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Meiosis

Volume 2

Cytological Methods

Edited by

Scott Keeney

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Preface

Each generation in a sexually reproducing organism such as a fly or a mouse passes through the bottleneck of meiosis, which is the specialized cell division that gives rise to haploid reproductive cells (sperm, eggs, spores, etc.). The principal function of meiosis is to reduce the genome complement by half, which is accomplished through sequential execution of one round of DNA replication followed by two rounds of chromosome segregation. Within the extended prophase between DNA replication and the first meiotic division in most organisms, homologous maternal and paternal chromosomes pair with one another and undergo homologous recombination, which establishes physical connections that link the homologous chromosomes until the time they are separated at anaphase I. Recombination also serves to increase genetic diversity from one generation to the next by breaking up linkage groups.

The unique chromosome dynamics of meiosis have fascinated scientists for well over a century, but in recent years there has been an explosion of new information about how meiotic chromosomes pair, recombine, and are segregated. Progress has been driven by advances in three main areas: (1) genetic identification of meiosis-defective mutants and cloning of the genes involved; (2) development of direct physical assays for DNA intermediates and products of recombination; and (3) increasingly sophisticated cytological methods that describe chromosome behaviors and the spatial and temporal patterns by which specific proteins associate with meiotic chromosomes. Often, the biggest insights have been obtained at the intersection between these historically separate approaches. New assays are being developed and classical methods are being applied in new ways, all in a diverse range of organisms from single-celled fungi, to plants, to animals both big and small.

These two volumes provide detailed protocols for genetic, molecular, and cytological methods for studying meiotic chromosome dynamics, in particular homologous recombination, higher-order chromosome structures, and chromosome segregation. Broad coverage is provided of many of the experimental organisms in which meiosis is often studied (e.g., the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the plant *Arabidopsis thaliana*, and the house mouse *Mus musculus*). Coverage is also provided of methods applicable to the study of meiosis in humans, as well as in other organisms which often offer distinct experimental advantages or unique mechanistic or evolutionary insights.

These books are aimed at scientists in (at least) three main categories: (1) Students of meiosis who want to “cross over” and apply basic techniques from other disciplines to the biological problems in which they are most interested. For example, cytologists who want to connect microscopic observations to the underlying DNA events will find guides to molecular methods for studying recombination. Likewise, geneticists who want to connect mutant phenotypes to the details of chromosome dynamics will find cytological methods that allow them to do so. (2) Students of meiosis in one organism

who wish to examine similar processes and conserved proteins in another organism, or who wish to gain a better understanding of both the possibilities and the limitations of methods for studying meiosis in other organisms. Ideally, the hope is that this book will play at least a small role in fostering crosstalk between investigators working in different experimental systems. (3) Students of basic chromosome biology in mitotically dividing cells who want to extend studies into meiosis, and, more generally, geneticists studying any biological process who find in hand a mutation that unexpectedly affects fertility and who need a handy primer on how to study this phenotype further. Indeed, this latter group has not been uncommon in the era of reverse genetic gene targeting in mouse.

The first volume, *Meiosis: Volume 1, Molecular and Genetic Methods*, is divided into two parts. The chapters in Part I of the first volume are devoted to genetic analyses, including methods for culturing and manipulating commonly used model organisms and methods for detecting and quantifying meiotic recombination or other aspects of chromosome dynamics. Part II of the first volume describes techniques for the direct study of meiotic recombination events through physical analysis of DNA or of protein-DNA interactions. Numerous approaches are described in budding and fission yeasts and in mouse and human.

The second volume, *Meiosis: Volume 2, Cytological Methods*, is subdivided for convenience by the general type of organism: fungi in Part I, plants and small animals (mostly invertebrates) in Part II, and mammals in Part III. Although there is some redundancy in certain aspects of the cytological methods, there are also many instances of species-specific differences—or even gender-specific differences within a species—that make it important to provide separate detailed protocols for different organisms. Cytology is a visual science, and the use of color and animation is often critical to the appropriate display of experimental results. As a consequence, Springer has graciously agreed to provide a companion CD for the second volume, on which can be found color versions of many of the figures that are reproduced in grayscale in the printed volume. The CD also contains a number of movies that illustrate results of real-time imaging of chromosome dynamics in yeasts, that show animations of three-dimensional reconstructions of meiotic nuclei, or that demonstrate particular experimental manipulations. A computer macro can also be found that provides analytical tools for evaluating the spatial distribution of cytological protein complexes on chromosomes. It is hoped that the contents of this CD will be a useful resource for readers of this volume.

I thank the many colleagues in the meiosis community for advice and suggestions on content, and the authors of chapters in these volumes for their hard work and willingness to share their expertise.

New York, NY
June 2008

Scott Keeney

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Chapter 1

Chromosome Spreading and Immunofluorescence Methods in *Saccharomyces cerevisiae*

Beth Rockmill

Abstract

Visualization of meiotic chromosomes in the model organism *S. cerevisiae* has become an integral part of the study of wild-type meiosis and the characterization of mutant phenotypes. This chapter describes a simple method for chromosome spreading, which is a variation on a protocol originally developed by Dresser and Giroux (1). This method uses osmotic pressure to spread the nuclear contents of spheroplasted meiotic cells over a glass slide enabling unobstructed inspection of the chromosomal morphology. Chromosomes from all meiotic stages can be analyzed using indirect immunofluorescence to visualize meiotic proteins involved in different processes of meiosis, including recombination, synapsis, sister chromatid cohesion, and chromosome disjunction.

Key words: Meiosis, chromosomes, yeast, chromosome spreads, immunofluorescence.

1. Introduction

Until the 1980s, the study of meiosis in yeast concentrated on the genetics of recombination and the biology of sporulation. Once spread meiotic chromosomes could be visualized, the structure, function, and development of yeast synaptonemal complex (SC) began to be dissected (2, 3). The SC has emerged as the focal point of much genetic and cytological experimentation, with direct parallels in other organisms (4, 5). The earliest demonstration of spread yeast chromosomes was in 1982, when Goetsch and Byers (6) spread protoplasts on a hypophase for the electron microscope. This approach spurred on two additional chromosome spreading methods. First, Dresser and Giroux published a method adapted from mice where spheroplasted cells were osmotically burst on a

glass slide (1). Subsequently, Loidl et al. (7) published a technique incorporating a detergent to aid bursting. The latter two methods are relatively simple, and versions of these are widely used today. This chapter will describe a simple variation of the Dresser method that fairly consistently produces the well-spread chromosomes necessary for some quantitative applications [e.g., quantification of foci and colocalization of two or more proteins (8, 9)]. This chapter is divided into three sections: sporulation, chromosome spreading, and immunofluorescence.

Diploids are efficiently induced to sporulate (undergo meiosis) using different protocols depending on the strain background. For optimal spreading, I have had the most success with “slow” sporulating strains, [i.e., BR1919-8B and derivatives (10)] and this background is considered for the basic method. Diploids (or other nonmating strains) are grown to late log phase/early stationary phase in rich medium and then directly introduced to sporulation medium. Full synapsis, i.e., pachytene, can be observed in a large proportion of the cells (up to 40%) from 12–18 h after induction, depending on the particular time course and conditions. For SK-1 (11), Y55 (12), and other “fast” sporulating strains, overnight cultures are diluted into presporulation medium 12 h prior to introduction to sporulation medium (*see Note 1*), where spreads at the pachytene stage can be observed after only ~4 h for SK-1 (13) and ~8 h for Y55 (Hoffmann, 2007, unpublished data).

Yeast cells at the appropriate time point after transfer to sporulation medium are harvested and enzymatically spheroplasted in an osmotically supportive medium. The gently pelleted spheroplasts are resuspended in low osmotic buffer and immediately fixed and spread on a glass slide. Later, most of the cell debris is washed off and the slide is left to air dry. A fraction of the burst nuclei adhere to the glass slide.

For antibody staining, a dilution of primary antibody(ies) in a blocking solution is added to the slide for 4–24 h, depending on the antibody and/or convenience. Several useful primary antibodies have been raised against meiotic yeast proteins [e.g., Red1, Zip1, and Dmc1 (14–16)]. However, greater flexibility can be achieved by tagging genes with epitopes such as c-MYC, HA, GFP, and GST (17, 18) (Rockmill and Hollingsworth, 2007, unpublished data). One caveat is that a tag may alter the stability and/or activity of the protein relative to the untagged protein. Although tagged genes may be phenotypically identical to wild type in some assays, defects may be observed in other assays and/or other strain backgrounds. One solution to this problem can be to construct diploids containing both a tagged and untagged copy of the gene [e.g., *ZIP3-GFP* in SK-1 (19)]. After incubation, the primary antibodies are washed off and replaced with secondary antibodies, which are usually incubated for a shorter time, and then washed again. Drained slides are prepared for the microscope by adding mount containing DAPI (to visualize chromatin).

2. Materials

2.1. Sporulation

1. YEPADU: 1% yeast extract, 2% dextrose, 2% bactopeptone, supplemented with adenine (10 mL of 50 mM adenine per liter) and uracil (10 mL of 20 mM uracil per liter). Autoclave to sterilize.
2. YPA (presporulation medium—optional, strain dependent): 1% yeast extract, 2% dextrose, 2% potassium acetate. Autoclave.
3. 2% KAc: 2% potassium acetate, pH 6.5 (adjusted with 1 N NaOH). Autoclave.

2.2. Chromosome Spreading

1. KAc-sorbitol: 1% potassium acetate and 1 M sorbitol (made from adding equal volumes of 2% potassium acetate and 2 M sorbitol). Store at room temperature.
2. Zymolyase stock: 10 mg/mL Zymolyase 100T in TE and 5% dextrose. For 5 mL Zymolyase stock: 50 mg Zymolyase 100T, 0.25 g dextrose in 5 mL of TE (10 mM Tris-Cl pH 7.5, 1 mM EDTA). Divide into 50 μ L aliquots and store at -20°C .
3. 1 M DTT stock: store 1 mL aliquots at -20°C .
4. Spheroplasting solution: per mL of KAc-Sorbitol, add 10 μ L of 1 M DTT and 20 μ L of Zymolyase stock.
5. 1% sarcosyl, kept at room temperature.
6. 2X MES: 0.2 M MES-NaOH (2-N-morpholino ethane sulfonic acid), pH 6.4, 2 mM EDTA, 1 mM MgCl₂. Filter sterilize and store at 4°C.
7. MES-sorbitol solution: 0.1 M MES-NaOH, pH 6.4, 1 mM EDTA, 0.5 mM MgCl₂, 1 M sorbitol. Prepare by mixing equal volumes of 2X MES and 2 M sorbitol. Store at 4°C.
8. 1X MES: Dilute 2X MES with dH₂O. Store at 4°C.
9. Borate buffer: 0.5 M boric acid adjusted to pH 9.2 with NaOH.
10. FIX: For 100 mL, add 4 g paraformaldehyde (kept at 4°, wear gloves and don't breathe powder or fumes) to 80 mL of ddH₂O in beaker with stir bar and cover. Warm to 50–60°C on hot plate. Add ~five drops of 1 N NaOH or until solution clears. Bring pH down below 8 with dilute HCl and then up to pH 8.2 with borate buffer and bring volume up to 100 mL with dH₂O. (Store at 4°C for up to a year, check pH before use—pH drifts down initially).
11. Photo-Flo: 0.4% v/v solution of Photo-Flo 200 solution (Kodak) in dH₂O. Adjust pH to ~8 with borate buffer.

2.3. Immunofluorescence

1. PBS: Phosphate-buffered saline (0.2 M phosphate, 1.5 M NaCl, pH 7.4) autoclaved.
2. PBS + 5% BSA: To PBS add 5% bovine serum albumen and store at 4°C.
3. PBS/FBS (Primary blocking solution): equal volumes PBS + 5% BSA and FBS (fetal bovine serum) kept in 1 mL aliquots at -20°C.
4. Mount + DAPI: Dissolve 100 mg p-phenylenediamine (an anti-fade agent, carcinogenic, wear gloves and use caution) in 10 mL PBS and adjust to pH 9 if needed. Stir at room temperature, covered with aluminum foil. Add 90 mL glycerol and mix. Store 1 mL aliquots frozen at -80°C. For working aliquot, add 1 µL of 1 mg/mL DAPI and store in aluminum foil at -20°C. Discard if aliquot becomes brown.

3. Methods

3.1. Sporulation

1. Grow an overnight culture. Innoculate a freshly grown strain (colony) into at least 2 mL of YEPADU and put on a rollerdrum at 30°C for ~24 h. When it is necessary to grow strains under selective conditions, *see Note 2*.
2. Sporulation: Pellet 1.5 mL of the saturated overnight culture by a 1 min centrifugation in a microfuge tube and remove the liquid. Resuspend the cell pellet in 10 mL of 2% KAc in a 125 mL Erlenmyer flask. This results in a cell concentration of $2-4 \times 10^7$ cells/mL. Shake the flask at ~250 rpm at 30°C.

3.2. Chromosome Spreading

1. Prepare a cocktail of spheroplasting solution: 0.5 mL for each 10 mL of sporulated culture.
2. Collect 10 mL of the sporulating culture from the flask at an appropriate time into a 14 mL polypropylene round-bottom tube (e.g., Falcon 352059) and spin down the cells in a tabletop centrifuge (~700g). Pour off the supernatant. Cells can be frozen for subsequent spreading (*see Note 3*).
3. Add 0.5 mL spheroplasting solution to each tube, resuspend pellet and return to roller drum at 30°C.
4. Prepare slides: Soak frosted glass slides in 70% EtOH for at least 5 min (or overnight) in a Coplin jar (a jar designed to hold several slides) and thoroughly rub the slides dry with a lint-free tissue (e.g., *kimwipe*). The slide should feel smooth and not give resistance as if there were a film on it (*see Note 4*). Label slides with an ethanol-resistant marker (e.g., VWR lab marker or pencil).

5. Monitor spheroplasting: After ~20 min of spheroplasting, pipette 3 µL of the cells onto a glass slide with 3 µL of 1% sarkosyl and place a coverslip on the slide for viewing using phase contrast microscopy. Evaluate the level of spheroplasting. Bluish and broken cells, and clumps of debris should fill most of the field. Unspheroplasted cells are bright and refractive and, ideally, these should be reduced to less than 20% within 20–30 min. If not, add more zymolyase and monitor after another 10 min. Strains vary in their sensitivity to zymolyase and rather than increase the incubation time, the zymolyase concentration should be optimized for a particular strain. Alternatively, if you suspect the cells are over-spheroplastic, look at cells under phase contrast, without sarkosyl, and determine if they are intact, as they should be. (Cells that have already burst will not make nice spreads.)
6. Wash cells with MES-Sorbitol: Add 2.5 mL of cold MES-Sorbitol to each tube and spin gently in a tabletop centrifuge (~400g) for 2 min. (If there are many tubes, the cell pellets may be left on ice.) (For SK-1 cells *see Note 5.*)
7. Place two (or more) coverslips (22 × 22 mm) on an elevated flat surface (e.g., a plastic Eppendorf tube rack).
8. Gently remove liquid from the cell pellet of one tube by inversion into a liquid waste container and then drain on an absorbent mat. Drain thoroughly, but take care not to allow pellet to slip down the tube. (Residual sorbitol will inhibit spreading).
9. Add 30 µL cold 1X MES (without sorbitol) onto the pellet and flick the tube several times to completely resuspend cells. Immediately add 60 µL FIX onto the cells with a pipettor, flick again and draw up the cells with the same pipette tip, and place onto coverslips. Split the suspension among two to three coverslips. *See Note 6* for optimizing spreading and alternative methods.
10. Place a labeled slide gently over the cell suspension and let the milky solution spread to the edges, minimizing any bubbles. Lay the slide “cover slip up” on a mat. Return to Step 8 for the next tube. Let the slides sit for at least 20 min.
11. Prepare fresh Photo-Flo solution. Pipette Photo-Flo onto the slides to help loosen the coverslip (or briefly submerge slides in a Coplin jar full of Photo-Flo). Dump the coverslip into waste as more Photo-Flo is gently streamed from a 5 mL pipette to encourage the coverslip to slip off. Nudging the coverslip with a finger or pipette tip may be necessary. Hold the slide at a slight angle from horizontal and gently pipette about one mL more Photo-Flo, or until slide is cleared of loose cellular debris. Drain the excess liquid and dry

horizontally (flat) on mat. Photo-Flo promotes uniform drying. *See Note 7* to increase the number of spreads adhering to the slide.

12. Assess spreading: Thaw mount + DAPI and add one drop to a new coverslip (22×40 mm) on an elevated level surface. Position a slide (with spreads side down) over the coverslip and allow mount to spread, minimizing air bubbles. Blot excess liquid by pressing the slide (coverslip down) on mat.
13. Paint edges of slides with clear nail polish and let dry.
14. Check slide for spreads under DAPI filter set on fluorescence microscope. Spreads may be unevenly distributed on the slide. Spreads in early prophase (before leptotene) tend to look round and cottony, whereas in later stages the chromatin is more condensed and irregular. DAPI and Zip3-GFP images of various stages of wild type are shown in **Fig. 1.1**. (Zip3-GFP is stable to fixing and can be viewed directly.) **Figure 1.2** contains DAPI images of three meiotic mutants.
15. DAPI-stained slides can be reused for immunofluorescence by removing the coverslip and soaking in a Coplin jar filled with PBS (two washes of 5 min each).
16. Freeze dry slides for storage in black plastic freezer boxes or use directly for immunofluorescence.

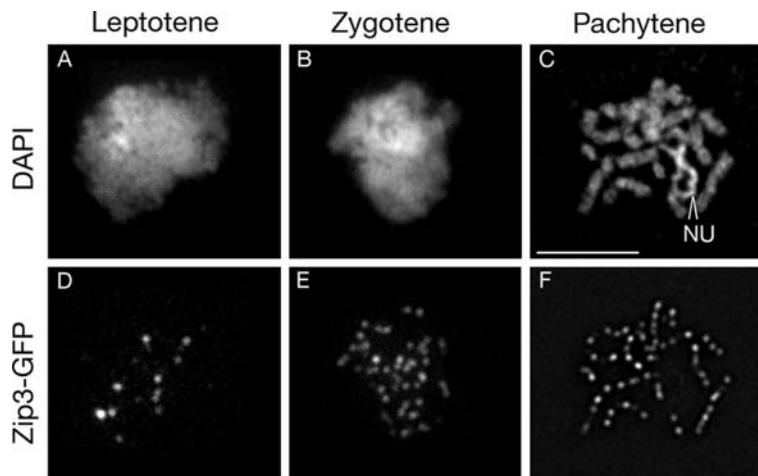


Fig. 1.1. Stages of prophase. Images of a wild-type diploid homozygous for *ZIP3-GFP* (20) are represented for leptotene (prior to SC formation, **A** and **D**), zygote (partial SC formation, **B** and **E**) and pachytene (full synapsis, **C** and **F**). **A–C** are DAPI images revealing the progressive condensation states of the chromatin. The caret points to the nucleolus, NU, which fails to form SC or have Zip3 foci. **D–F** reveals the increasing number and pattern of Zip3 foci, which are thought to mark future sites of crossovers (8, 21) as well as sites of initiation of SC (20) by direct fluorescence. Bar = 5 μ m.

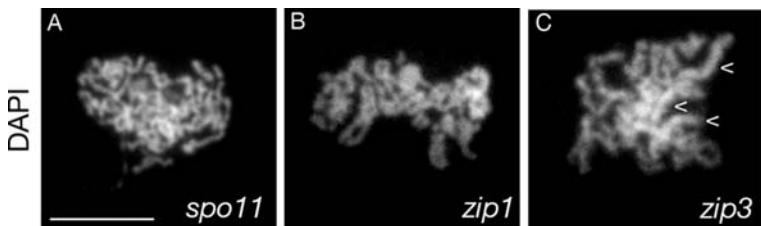


Fig. 1.2. Meiotic mutants. A sampling of DAPI images of mutants that fail to form wild-type SCs. **A.** *spo11* forms axes, but no axial associations. **B.** *zip1* forms axial associations but no SCs. **C.** *zip3* forms some SCs. Carets point to SCs. Bar = 5 μ m.

3.3. Immunofluorescence

1. Prepare humidity chamber, e.g., a plastic box with lid, fitted with old pipette tip holder inserts. Pour a shallow layer of water on the bottom.
2. Prepare antibody dilution in PBS/FBS. Typically, 1:100 dilution of primary antibody is used at a volume of 50 μ L per slide (*see Note 8*). Meiotic extract may be added to remove non-specific staining (*see Note 9*).
3. Place a coverslip (22 \times 40 mm) on a raised level platform and pipette 50 μ L of primary antibody solution onto the center of the coverslip. Gently place slide “spreads down” onto the coverslip, allowing solution to spread and minimizing air bubbles.
4. Incubate the slides in a humid chamber at room temperature for 4 h and/or overnight at 4°C.
5. Fill a Coplin jar with PBS. Remove the coverslips from the slides and put the slides in the jar. Rotate jar on an orbital shaker (optional) for 5 min. Repeat twice.
6. Prepare secondary antibody solution: Usually a 1:200 dilution of antibody(ies) (e.g., goat anti-rabbit FITC if a rabbit primary was used) in PBS + 5% BSA. Prepare 100 μ L per slide. *See Note 10* on specificity.
7. Drain the slides and place them in the humid chamber. Pipette 100 μ L of secondary antibody solution onto each slide and place a coverslip gently over, minimizing bubbles (or roll pre-cut pieces of parafilm over liquid, pushing air bubbles aside). Incubate 1.5 h or longer at room temperature. From this step on, keep in low light as much as possible.
8. Wash slides in three changes of PBS in a Coplin jar as in Step 5.
9. Thaw mount + DAPI and add one drop to a coverslip (22 \times 40 mm) on an elevated level surface. Position a well-drained slide (spreads side down) over the coverslip and allow the viscous mount to spread, minimizing air bubbles. Blot

excess liquid by pressing the slide (coverslip down) on absorbent mat. Make sure the thickness of the coverslip is optimal for your microscope's optics.

10. Paint edges of slides with clear nail polish and let dry.
11. View, or store slides in black boxes at -20°C for months with little deterioration. Immunofluorescence images in **Fig. 1.3** and Color Plate 1 illustrate the use of various antibodies directed against yeast proteins and representative tags.

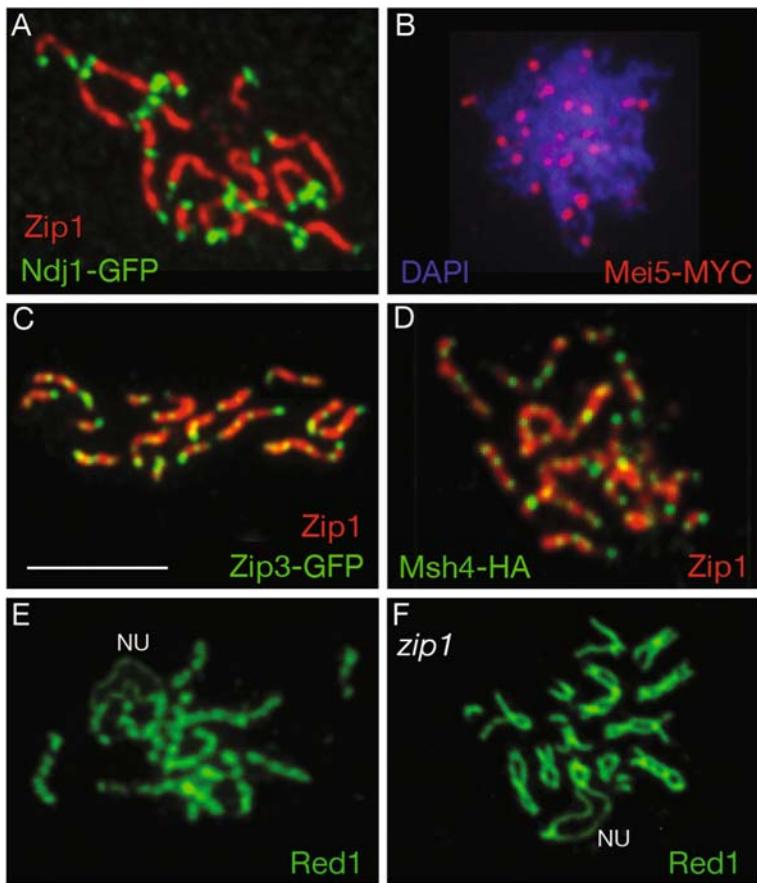


Fig. 1.3. Immunofluorescence of tagged and untagged meiotic proteins. **A–E** are wild type except for tagged proteins. For **A, C, and D**, rabbit anti-Zip1 antibodies (15) were used with anti-rabbit Texas Red secondary antibodies (Jackson ImmunoResearch). **A.** Telomeres were detected in a strain bearing an *NDJ1-GFP* high copy plasmid (22) with guinea pig anti-GFP antibodies (21) and an FITC conjugated secondary antibody (Jackson ImmunoResearch). **B.** Early meiotic recombination events are visualized via an integrated *MEI5-MYC* (23) using a mouse monoclonal (9E10, Covance) and FITC-conjugated secondary antibodies. **C** and **D**. Late recombination events, presumably future crossovers, are detected by either Zip3-GFP (20) or Msh4-HA (24). Commercially available mouse anti-HA antibodies (Covance) were used to detect the HA epitope. In wild type, **E**, Red1 staining is uneven. **F** is a *zip1Δ* spread nucleus, and is delayed at the pachytene stage, allowing the Red1 protein to accumulate on the axes (visualized with rabbit anti-Red1 antibodies (14)). Nucleoli are seen as unsynapsed axes and denoted "NU". Bar = 5 μm . (see Color Plate 1)

4. Notes



1. To prepare fast sporulators (Y55 and SK-1) for sporulation, freshly grown colonies (or freshly selected zygotes) are grown in YEPADU liquid for up to 24 h. SK-1 strains are constitutive sporulators and will sporulate on rich medium if left for several days, so be vigilant about their growth or select new zygotes before an experiment. Dilute cells into 10 mL YPA in 125 mL flasks at an OD₆₀₀ of 0.3. Vortex the cells immediately prior to reading OD, since SK-1 cells are flocculant. Grow until OD₆₀₀ of 1.2–1.4 is reached (about 13 h). Pellet the cells, wash with 2% KAc, and resuspend in 10 mL of 2% KAc (equivalent to $\sim 2 \times 10^7$ cells per mL).
2. Cells sporulate with reduced efficiency from synthetic medium, thus, a compromise between sporulation efficiency and plasmid retention is used. To maintain selection for an episomal plasmid, grow overnight cultures in synthetic medium, and then dilute with an equal volume of YEPADU 8 h prior to transfer into sporulation medium.
3. To freeze cells for subsequent spreading, resuspend pelleted cells in 50% glycerol (autoclaved) and transfer to a microfuge tube. Pellet the cells, pipette off the liquid and freeze at -80°C. To spread, thaw cells on ice and wash twice with KAc Sorbitol. Transfer to a 14 mL tube and proceed with spheroplasting (*see Section 3.2*).
4. Glass slides vary in their ability to adhere spreads. Be aware that different batches from the same company can give different results. The ethanol bath for the slides helps the spreads adhere.
5. SK-1 spheroplasts do not pellet well at this stage. Transfer the cells to a microfuge tube, add 800 μ L cold MES-Sorbitol and spin cells gently (6g for 30 s).
6. There is a balance between spreading and fixing the chromosomes. Addition of 1X MES to the pellet allows the cells and nuclei to osmotically burst, and the addition of FIX keeps the chromosomes in proximity. If predominantly intact nuclei are observed under DAPI fluorescence (*see Section 3.2*, Step 14), then allow more time after the addition of 1X MES and continue flicking the tube before addition of the FIX. The ratio of 1X MES to FIX can be increased (e.g., to 1:1). For an alternative method, resuspend the pellet in 100 μ L MES and 500 μ L FIX, and allow ~ 200 μ L per slide. Use a rectangular coverslip (e.g., 22 \times 40 mm).

7. To get more spreads to stick to the slides, allow some drying during the fixation process. The coverslip can be removed prior to the Photo-Flo wash and allowed to partially air dry. Alternatively, do not use a coverslip at all. In either case, do not let the slides completely dry. Since the slides dry unevenly, there will always be some part of the slide that dries first. If drying is prolonged, the washing will not remove the debris and the slides will be very “dirty.”
8. When determining the optimal concentration of primary antibody, remember that sometimes “less is more.” Occasionally a more dilute antibody will give a better signal.
9. Nonspecific staining may be reduced by adding meiotic extract made from the appropriate mutant strain to the primary antibodies prior to or at the time of incubation with the spreads. For immunofluorescence of a tag, a wild-type extract can be used, but keep in mind that this could interfere with staining other yeast proteins. To prepare extract, follow **Section 3.2**, Steps 1–9 but put the tube of fixed cells on ice for 30 min and then freeze in 0.5 mL aliquots at –80°C. Use as needed.
10. When using a primary or secondary antibody for the first time, do appropriate controls to ensure that the staining is specific. For example, use antibodies on slides containing spreads of strains deleted for the protein or lacking a tag against which the antibodies should react. In addition, to assure that there is no “bleed through” (fluorescence coming through more than one filter set), use antibodies individually and assess images on all channels. Pay close attention to the quality of the secondary antibodies when using more than one primary antibody so that your secondary antibodies are specific. The manufacturers list the animals against which the secondary antibodies have been pre-absorbed. Take into consideration the filter sets available on the microscope when choosing a fluorochrome.

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Chapter 2

Analysis of *Schizosaccharomyces pombe* Meiosis by Nuclear Spreading

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Abstract

The fission yeast, *Schizosaccharomyces pombe*, much like the budding yeast, is a particularly well-suited model organism for genetic research. However, the minuscule size of both yeasts' nuclei has hindered their success as research models for cytologists. A solution to this problem is provided by the spreading of nuclei, which increases their volume and allows for a better spatial resolution of nuclear contents. Here we describe nuclear spreading in fission yeast. Spreading of meiotic nuclei is particularly helpful in exposing the linear elements (LinEs), which are the fission yeasts' rudimentary version of the synaptonemal complex. Although the LinEs' role is still not fully understood, they serve as important meiotic hallmarks and their presence and morphology can be used in characterizing meiotic mutants. We first describe methods to induce meiosis in liquid cell cultures, then outline a method to break down cell and nuclear membranes by detergent treatment to release chromatin on cytological slides, and finally provide a set of protocols for analyzing these nuclei by immunostaining and fluorescence *in situ* hybridization (FISH), and by electron microscopy.

Key words: Linear element, meiosis, nuclear spreading, fluorescence *in situ* hybridization, immunocytochemistry, microscopy, Rad51, synaptonemal complex, chromosome, nucleus.

1. Introduction

Instead of a canonical synaptonemal complex (SC), linear elements (LinEs) are present during the stage when meiotic pairing and recombination take place in *S. pombe* (1). They are biochemically related to the axial elements of SCs and are likely evolutionarily derived from them (2, reviewed in Ref. 3). At the same time, this stage is characterized by the presence of elongated nuclei, the so-called horsetails which seem to be the fission yeasts' functional equivalent to the bouquet (4, 5). In these nuclei, the telomeres of all chromosomes are

assembled at the spindle pole body, which in turn is in contact with microtubules. They confer an oscillating movement to the nuclei during which they are stretched into the horsetail shape and which contributes to lining up the equal sized homologs inside the nucleus which promotes the initiation of homologous pairing (6, 7).

Horsetail nuclei are a good indicator of the pairing stage in a meiotic culture and can, in addition to flow cytometry (FACS analysis) of cell samples, be used to monitor the progress of meiosis, for example, for obtaining staged samples from a timecourse experiment for biochemical analyses. In addition to the staging of meiotic cultures, cytology is often performed in its own right for studies of individual cells. Several meiotic mutants are characterized by a lack of or by abnormal development of LinEs, and the appearance of foci of recombination proteins can also be used for the characterization of mutants.

SCs and LinEs were originally detected in ultrathin sections prepared and contrasted for electron microscopy (*see* Refs. 1, 8, 9). In conventionally fixed cells, SCs and LinEs were not visible by light microscopy. Only whole mount nuclear spreading methods, which liberate these structures from the surrounding chromatin made them amenable to detection after impregnation with silver. A variant of the spreading method using a detergent has found widespread application in animals and plants, as well as in the budding yeast (10). Later, it became possible to delineate LinEs by immunostaining of its various components (2), and while this may be feasible with conventionally prepared (unspread) nuclei in certain mutant backgrounds (11), some of the LinEs' characteristics can be analyzed conveniently only when nuclei are spread (12). The advantage of spreading is the unfolding of the densely packed nuclear contents, which results in a gain in spatial resolution. It is, however, achieved at the cost of destroying the integrity and spatial organization of nuclei.

Here we describe a detailed protocol for the spreading of meiotic *S. pombe* nuclei and the staining of LinEs and other nuclear structures in these spreads by immunostaining techniques. Moreover, we describe how these spreads can also be used for FISH and how spread nuclei can be transferred to the electron microscope for high-resolution analysis of silver-stained LinEs.

2. Materials

2.1. Cell Growth and Preparation

1. Rich medium, yeast extract medium (YE): 5 g/L yeast extract, 30 g/L glucose in distilled water, autoclaved. (For solid medium 18 g/L agar is added.)
2. Presporulation medium, *pombe* minimal medium (PM): 5 g/L NH₄Cl, 3 g/L potassium hydrogen phthalate, 1.8 g/L

Na_2HPO_4 , 20 g/L glucose, 20 mL/L 50 × salts stock (see Step 5), 1 mL/L 1,000 × vitamins stock (see Step 5), 0.1 mL/L 10,000 × minerals stock (see Step 5) in distilled water, autoclaved. (For solid medium, 18 g/L agar is added.)

3. Sporulation medium, *pombe* minimal medium without a nitrogen source (PM-N): 3 g/L potassium hydrogen phthalate, 1.8 g/L Na_2HPO_4 , 10 g/L glucose, 20 mL/L 50 × salts stock (see Step 5), 1 mL/L 1,000 × vitamins stock (see Step 5), 0.1 mL/L 10,000 × minerals stock (see Step 5) in distilled water, autoclaved.
4. Solid sporulation medium, malt extract agar medium (MEA): 30 g/L malt extract, 20 g/L agar in distilled water, pH adjusted to 5.4 with NaOH, autoclaved.
5. Stocks for minimal media (all stocks are stored at 4°C):
 - a) 50 × salts stock: 52.5 g/L (0.26 M) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 50 g/L (0.67 M) KCl, 2 g/L (14.1 mM) Na_2SO_4 , 0.735 g/L (4.99 mM) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water, autoclaved.
 - b) 1,000 × vitamins stock: 10 g/L nicotinic acid, 10 g/L inositol, 1 g/L pantothenic acid, 0.01 g/L biotin in distilled water, filter sterilized.
 - c) 10,000 × minerals stock: 10 g/L (47.6 mM) citric acid, 5 g/L (80.9 mM) boric acid, 4.5 g/L (23.7 mM) $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 4 g/L (13.9 mM) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g/L (7.4 mM) $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 1 g/L (6.02 mM) KI, 0.4 g/L (1.6 mM) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.36 g/L (2.47 mM) molybdenum(VI)oxide in distilled water, filter sterilized.
6. To ensure vigorous growth and sporulation of auxotrophic strains, the following supplements are added as required: adenine, uracil, lysine hydrochloride, leucine, histidine, and/or arginine monohydrochloride to a final concentration of 225 mg/L in YE as well as MEA, 75 mg/L in PM and 10 mg/L in PM-N (see Note 1).
7. Stocks for enzyme cocktail for digesting the yeast cell wall: Prepare stock solutions of 10 mg/mL Zymolyase 100T (Seikagaku Co., Tokyo, Japan) in distilled water, 100 mg/mL lysing enzyme from *Rhizoctonia solani* (L8757, Sigma, St Louis, MO, USA) and 250 mg/mL lysing enzyme from *Trichoderma harzianum* (L1412, Sigma) in 0.65 M KCl (see Note 2).
8. Spheroplasting solution: 0.65 M KCl with 10 mM dithiothreitol (prepared from a frozen 0.5 M stock). Add 20 μL Zymolyase, 20 μL L8757 and 60 μL L1412 stock solutions per mL.
9. Protease inhibitor: 1 M phenylmethylsulfonyl fluoride (PMSF) in dimethyl sulfoxide (DMSO).

10. Stop solution: 0.1 M 2-(*N*-morpholino)ethane sulfonic acid (MES), 1 mM EDTA, 0.5 mM MgCl₂, 1 M sorbitol in distilled water; adjust pH with NaOH to 6.4.
11. Detergent: Prepare a 1% solution of “Lipsol Detergent Concentrate” (now marketed by Barloworld Scientific, <http://www.barloworld-scientific.com/>) in distilled water. The working solution can be stored for several months in the refrigerator (*see Note 3*).
12. Fixative: 4% paraformaldehyde supplemented with 3.6% sucrose (*see Note 4*).
13. 10 × phosphate-buffered saline (PBS) stock: 80 g/L NaCl, 2 g/L KCl, 11.5 g/L Na₂HPO₄·7H₂O, 2 g/L anhydrous KH₂PO₄. Make up to 900 mL with distilled water, adjust pH to 7.5 using 1 N NaOH or 1 N HCl, add distilled water to 1,000 mL (*see Note 5*).

2.2. Immunostaining

1. 1 × PBS + Triton X-100 (PBS-T): Add 0.5 mL Triton X-100 to 1,000 mL 1 × PBS.
2. Humid chamber. Cover the bottom of an air-tight plastic box or glass container which will provide space for several horizontally placed slides with filter paper or some other water-absorbent material and moisten with distilled water. Put slides inside the box on a tray to avoid contact with the water. The humid chamber will prevent the drying out of preparations which are incubated with small amounts of liquid under the cover slip.
3. Primary antibodies. Antibodies against various components of LinEs, recombination proteins, nucleoli, spindle pole bodies, and other contents of meiotic nuclei are available upon request from noncommercial sources. Rabbit antibodies against Rec10 protein were created in the lab of Ramsay McFarlane (University of Wales at Bangor, UK) and a guinea pig antibody against Hop1 protein was made in the Loidl lab. A mouse monoclonal antibody against recombinant Rad51 protein (Clone 51RAD01, NeoMarkers, Fremont, CA, USA) works well with *S. pombe* Rad51. As an alternative to anti-Rec10 antibodies, an HA-tagged version of Rec10 constructed in the lab of Ramsay McFarlane can be used for the visualization of the LinEs. Strains expressing tagged versions of, for example, Rec7, Rec8, Rad32, Mek1, and Taz1 have been published (13–18). Most of these fusion proteins can be detected in spread nuclei (2, 19, and unpublished observations). The authors can provide details of sources of various antibodies and strains carrying tagged proteins which can be detected in nuclear spreads.
4. Antibodies against GFP-, Myc- and HA-tags, and secondary antibodies: Purchased from various vendors.

5. DAPI (4'6-diamidino-2-phenylindole) as a DNA-specific counterstain. Prepare a 1 mg/mL stock solution in distilled water which can be stored at -20°C. DAPI is applied at a final concentration of 1 µg/mL.
6. Antifade buffer to reduce bleaching: 245 mg diazabicyclo(2.2.2)octane + 200 µL 1 M NaHCO₃ (pH 8.0) + 800 µL distilled water + 9 mL glycerol. Alternatively, antifade buffers can be purchased under the tradenames Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) or Slow Fade (Molecular Probes Inc., Eugene, OR, USA) (*see Note 6*).
7. Rubber cement (e.g. Fixogum, Marabuwerke GmbH, Tamm, Germany) or nail varnish for sealing slides.

2.3. FISH

1. Cosmids. Clones from the desired chromosomal loci can be selected from the *Schizosaccharomyces pombe* Gene Database (<http://www.genedb.org/genedb/pombe/>) and obtained from John Woodward at the Sanger Centre, Hinxton, UK (http://www.sanger.ac.uk/Projects/S_pombe/cloneres.shtml) (*see Note 7*).
2. Probes produced by PCR. Fragments of ca. 5–10 kb sizes are produced by long-range PCR [suitable kits are available from various companies: e.g. Expand™ Long Template PCR System (Roche Diagnostics, Basel, Switzerland); TaKaRa Ex Taq (TaKaRa Shuzo Co., Ltd., Otsu, Japan)] with appropriate primers from the *Schizosaccharomyces pombe* Gene Database (*see Note 7*).
3. 10 × labeling buffer: 500 mM Tris-HCl, pH 8.0, 50 mM MgCl₂, 500 µg/mL BSA.
4. 1 mM dATP/dCTP/dGTP mixture.
5. Labeled nucleotides: e.g. Cy3-dUTP, Cy5-dUTP (Amersham Pharmacia Biotech, Uppsala, Sweden), fluorescein-dUTP, tetramethylrhodamin-dUTP, digoxigenin-dUTP, or biotin-dUTP (Roche Diagnostics, Basel, Switzerland).
6. 280 mM β-mercaptoethanol.
7. DNase I (Roche Diagnostics, Basel, Switzerland).
8. *E. coli* DNA polymerase I (10 U/µL, New England BioLabs, Beverly, MA, USA).
9. RNase, DNase free (Roche Diagnostics, Basel, Switzerland).
10. ST buffer: 4 × SSC (0.6 M NaCl, 60 mM trisodium citrate, pH 7.0), 0.1% Tween-20.
11. 20 × SSC: 3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0.
12. 3 M Na-acetate pH 5.5.
13. Hybridization mixture: 4 × SSC, 20 % dextran sulfate, 1 µg/µL sonicated salmon sperm DNA.

14. Rubber cement for sealing slides (as above).
15. Heating block or a thermocycler capable of holding slides (HYBAID Ltd., Ashford, England).
16. Blocking buffer: 3% BSA, 4 × SSC.
17. Detection buffer: 1% BSA, 4 × SSC, 0.1% Tween-20.
18. Detection reagents: e.g. Avidin-FITC conjugate, Extravidin®-FITC conjugate, Extravidin®-Cy3 conjugate; Biotin-conjugated anti-avidin monoclonal antibody (Sigma, Chemical Co., St. Louis, MO, USA); anti-digoxigenin-fluorescein, anti-digoxigenin-rhodamin, anti-digoxigenin-AMCA (Roche Diagnostics, Basel, Switzerland)
19. Anti-fading medium (as above).
20. DAPI (as above).

2.4. Silver Staining of Linear Elements

1. AgNO₃ solution: 5 g AgNO₃ in 10 mL ultrapure water (*see Note 8*). Caution: Corrosive; wear eye protection and gloves!
2. Polyamide cloth: e.g., Nybolt PA-100/31 (Swiss Silk Bolting Cloth Mfg. Co. Ltd., Zurich, Switzerland).

2.5. Spread Preparations for Electron Microscopy

1. N-hexane for removal of immersion oil.
2. 1% hydrofluoric acid. Prepared from 40% stock. Caution: Follow the safety regulations of your institution. Wear eye protection and gloves. Work under a chemical hood!
3. 1% solution of Formvar resin (polyvinyl formal) in chloroform.
4. EM grids (e.g. square 50 mesh, Agar Scientific Ltd., Stansted, England).
5. Benchkote paper (Schleicher & Schuell, Dassel, Germany).

3. Methods

For culturing, recombination testing and other methods which are relevant to the study of meiosis we refer the reader to Ref. (20) and the references given therein, including the web resource <http://www.pombe.net/> maintained by the Forsburg lab. All liquid cultures should be in flasks filled to only 1/10 of their volume and continuously agitated in a shaker.

3.1. Cell Growth and Sporulation

S. pombe is a haplont, i.e., when two starving haploid cells of opposite mating type (=sexes) mate to produce a diploid zygote, it will immediately undergo meiosis (=zygotic meiosis). However, the diploid state can be maintained in the lab if zygotes are quickly transferred to rich medium. The diploid state in fission yeast is

relatively unstable and cells will sporulate at a low frequency even on rich medium. The most common method of stabilizing *S. pombe* diploids is to use heterozygotes for the intragenetically complementing *ade6* alleles, *ade6-M210* and *ade6-M216*. While *ade6-M210* or *ade6-M216* haploid or aneuploid strains are *ade*⁻ and thus cannot grow on selective PM plates lacking adenine, the heterozygous diploid *ade6-M210/ade6-M216* is *ade*⁺ and able to grow on selective minimal plates (see Note 9). Azygotic meiosis in cultures of diploid cells can be induced more synchronously by transfer to nutrient-poor medium (PM-N) than the mating and zygotic meiosis by the mixing of haploid cultures. Therefore, if possible, it is recommended to work with diploid cells undergoing azygotic meiosis. *S. pombe* meiotic cytology is best done with cells which were sporulated in liquid medium but under certain circumstances cells can be scratched from sporulation plates. The following protocols describe a meiotic timecourse experiment of an *ade6-M210/ade6-M216* diploid (see Note 10).

3.1.1. Diploid ade6-M210/ade6-M216 Azygotic Timecourse (Meticulous Protocol)

1. Streak strain for single colonies on solid YE medium (see Note 11), and grow for 4 d at 30°C.
2. Inoculate three tubes of 10 mL liquid YE with a single colony each and incubate shaking for 23–24 h at 30°C.
3. Transfer 50–100 µL of each of the above precultures to 10 mL fresh liquid YE and incubate shaking for another 23–24 h at 30°C. In addition, drop 30–50 µL of each of the precultures onto an MEA plate and incubate at 30°C overnight.
4. Test the three cultures on MEA for sporulation by transferring some material to a drop of water on a slide and examining under the microscope. Continue the timecourse with the best-sporulating culture.
5. Dilute 2 and 4 mL of preculture into 200 mL presporulation medium (PM) each and incubate for 15–16 h (overnight) at 30°C (see Note 12).
6. Determine the cell density in the cultures; this should be between 1 and 2 × 10⁷ cells/mL (see Note 13).
7. Harvest the culture with the right cell density by centrifugation (700g, 4 min), wash once with 50 mL sterile distilled water, resuspend at 1 × 10⁷ cells/mL in PM-N and start the timecourse.
8. Take 1 mL samples every hour from t=0 to t=10 h (for some mutant backgrounds it may be necessary to extend this period) to assess the progression of meiosis. For this purpose, spin the sample shortly (~10 s) at ~12,000g, discard the supernatant and resuspend the cells in 70% ice-cold ethanol (samples can be kept at -20°C for several weeks). For microscopic evaluation collect the cells by centrifugation (~12,000g, 10 s) and

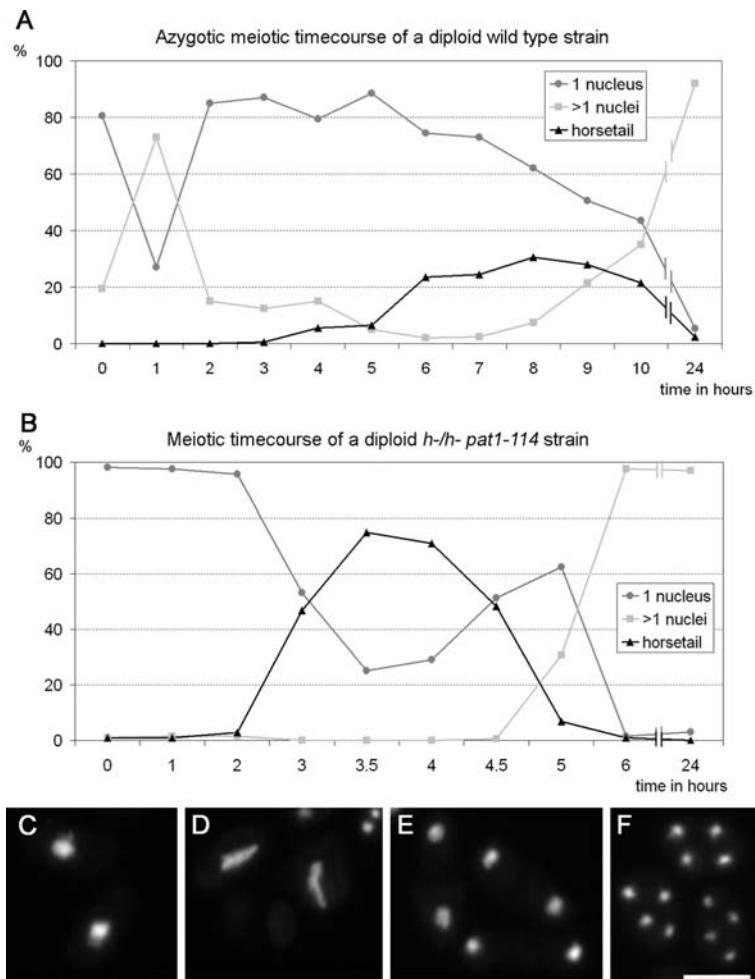


Fig. 2.1. (A, B) Frequencies of different morphological cell stages during typical meiotic timecourse experiments (at least 200 nuclei were inspected for each timepoint) and (C–F) examples of ethanol-fixed and DAPI-stained cells from a wild-type timecourse. (A) Azygotic wild-type timecourse as described in **Section 3.1.1**. Several criteria are used to determine synchronicity and proper meiotic progression: At timepoint $t=1$ h binucleate cells should be more abundant than uninucleate cells. Horsetail stage cells should peak between $t=5$ h and $t=8$ h. At $t=24$ h more than 85% of cells should have produced asci with four spores. (B) A diploid *pat1-114* timecourse as described in **Section 3.1.3**. Horsetail nuclei should not be frequent before $t=3$ h and normally peak at $t=3.5$ h or $t=4$ h. At $t=6$ h most of the cells should have undergone meiosis I. After 24 h >85% of the cells should have formed asci. (C) The “1 nucleus”-stage. (D) Horsetail nuclei. (E) Binucleate cells which represent post-mitotic stages at early timepoints and the stage between the first and the second meiotic division at later timepoints. (F) Groups of four nuclei, representing ascospores. Binucleate cells and asci are pooled into the class “>1 nucleus” in the diagrams. Bar in F represents 5 μ m.

resuspend in 20 µL of distilled water. Mix 5 µL of cell suspension with 5 µL DAPI in anti-fading medium on a slide and examine under the microscope for uni-, bi-, and tetranucleates as well as horsetail-stage cells (Fig. 2.1). There should be a high percentage of uninucleate cells (~80%) at t=0 and t=2 h, and >50% binucleate cells (representing the end of premeiotic mitosis) at t=1 h. In poorly synchronized cultures, these figures deviate; if good synchrony is crucial for the experiment, it should be terminated at this point (see Note 14). Alternatively or additionally, the progress of the timecourse can be monitored by determining the DNA content of the cells via flow cytometry (FACS) (see Ref. 20).

9. Take 5 mL samples every hour from t=3 to t=8 h (this may vary for mutants) for spreading (Section 3.2).

3.1.2. Diploid ade6-M210/ade6-M216 Azygotic Timecourse (Fast Track Protocol)

This protocol does not aim at a high sporulation frequency or good synchrony. It reduces the time from starting liquid cultures from a diploid strain on a plate to the harvest of meiotic cells from 4 to 2 d by dispensing with the selection of sporulation-competent colonies. This may produce a culture of which only a subset of cells will sporulate, but for most cytological applications (where meiosis is studied at the level of individual cells) it may be sufficient if meioses occur in a reasonable proportion of cells.

1. In the morning, choose three to five clearly visible white (*ade⁺*) colonies from YE plates which should not be older than one week and resuspend them in 10 mL YE liquid. Incubate the tube in the shaker at 30°C. The cells should reach a density of 0.5 to 1×10^7 cells/mL by the evening. (Doubling time is ~2.5 h.)
2. In the evening, inoculate PM with the pellet from the YE culture and incubate the PM culture in the shaker at 30°C. In PM, the generation time is about 3 h per doubling. Allowing for a lag time of 2–3 h (during which cells recover from handling and adjust to the new medium), a culture starting with 6×10^5 cells/mL should reach 1×10^7 cells/mL after 14–16 h (e.g., from 18:00 to 8:00).
3. In the morning, check the cell titer of the PM cultures with a counting chamber. It should be between 1 and 2×10^7 cells/mL (best is close to 1×10^7). If it is less than 1×10^7 , let the culture grow longer. If it is more than 2×10^7 , dilute to 0.5×10^7 cells/mL in fresh PM and allow growth to continue for 3 h.
4. Harvest the culture by centrifuging (700*g*, 4 min), wash once with 50 mL sterile, distilled water, and resuspend at 1×10^7 cells/mL in sporulation medium (PM-N) and start the timecourse.
5. Take samples; most LinEs will be found in the 5, 6, and 7 h timepoints.

3.1.3. Azygotic *pat1-114***Timecourse**

The *pat1-114* allele, which encodes for a temperature-sensitive version of the Ran1/Pat1 kinase, a key inhibitor of meiosis in *S. pombe* (see Ref. 5), is often used to induce meiosis in a highly synchronous fashion both in haploid as well as diploid cells. Meiosis in a *pat1-114* strain can be simply triggered by raising the temperature from the permissive 25°C to the restrictive 34°C. The cells will enter meiosis irrespective of their mating type, nutritional and cell cycle stage. It is essential that cells are well starved, otherwise a large proportion will enter meiosis from mitotic G₂ phase (21). In this case, chromosomes will have loaded mitotic cohesin and undergo meiotic missegregation. Also note that even diploid *pat1-114* meiosis deviates from wild-type meiosis in several respects (lower recombination rates, fewer Rad51-foci, aberrant LinE formation) (22, A. Baudrimont, J.L. and A.L., unpublished observations). Nevertheless, *pat1-114* strains can be useful for cytology, for example if a certain background is unstable as a diploid. The following protocol is modified after Ref. 23.

1. Inoculate 10 mL of liquid YE medium with a fresh *pat1-114* haploid or *ade6-M210/ade6-M216 pat1-114/pat1-114* diploid strain from a YE plate (see Note 11) and incubate at 25°C for ~20 h.
2. Inoculate 40 mL PM with 4 mL of preculture and let it grow for ~42 h at 25°C.
3. Determine cell density and resuspend in 200 mL PM at 2 × 10⁶ cells/mL. Let this culture grow for ~6 h at 25°C and determine the cell density (which should be around 4 × 10⁶ cells/mL). Centrifuge cells (700g, 4 min) and wash once with 50 mL of sterile distilled water. Resuspend cells in 200 mL PM-N at 4 × 10⁶ cells/mL and incubate at 25°C for 16–18 h.
4. Remove the culture from the incubator and raise temperature to 34°C. Check cell density; it should be between 0.8 and 2 × 10⁷ cells/mL (see Note 15). Add 2 mL of 50 g/L NH₄Cl and adenine, uracil, and lysine to a final concentration of 75 mg/L each. Add leucine, histidine, and/or arginine to a 75 mg/L end concentration depending on the auxotrophies of the strains. Put the culture back into the incubator and start the timecourse.
5. Draw test samples and samples for spreading as described in Section 3.1.1, Steps 8 and 9, respectively: 1 mL-samples for DAPI-staining are drawn hourly from t=0 to t=6 h and a final one after 24 h (see Note 14) (Fig. 2.1B). 5 mL-samples for spreading (see Section 3.2) are drawn every 30 min from t=3 h to t=5 h (may vary with mutant backgrounds) (see Note 16).

3.2. Nuclear Spreading

The spreading protocol described here is a modification by Lorenz et al. 2006 (19) of the method by Bähler et al. 1993 (12) (see Note 17). While in the original paper a mixture of Zymolyase

100T and Novozym 234 was used for spheroplasting, the modified method uses an enzyme cocktail (19) (see Sections 2.1.7 and 2.1.8) (see Note 18).

1. Take 5 mL of a cell suspension obtained according to one of the procedures under Section 3.1.
2. Spin and resuspend the pellet in 1 mL spheroplasting solution (see Section 2.1.8) and add 2 µL protease inhibitor (see Section 2.1.9).
3. Spheroplast cells for 25–30 min at 30°C on the shaker.
4. Add 9 mL of stop solution (see Section 2.1.10), harvest the spheroplasts by centrifugation (700g, 4 min) and resuspend them by carefully agitating with a pipette in 150–200 µL of stop solution (see Note 19). Add a further 1 µL of protease inhibitor.
5. Drop 20 µL of cell suspension onto a slide, add 40 µL fixative, 80 µL detergent, mix by slightly tilting the slide, wait for 30 s, then add another 80 µL fixative (see Notes 20, 21).
6. Spread out the mixture with a glass rod and put slides in a chemical hood overnight (see Note 22).
7. Continue with one of the Sections 3.3, 3.4.2, or 3.6, or freeze slides at –20°C for later use.

3.3. Immunostaining of LinEs and Other Structures in Spread Nuclei

Rec10 is the main protein of LinEs and Rec10 immunostaining is particularly useful for the detection of LinEs and the study of their development in the wild type and in mutants (Fig. 2.2A–E). Likewise, immunostaining of other LinE-associated proteins (such as Hop1 – Fig. 2.3A) and recombination proteins (such as Rad51 – Fig. 2.3B) can contribute to the characterization of meiotic mutants.

1. Wash slides that have been prepared according to Section 3.2 three times for 15 min in PBS-T (Section 2.2.1) and drain excess liquid.
2. Incubate slides with a drop of primary antibody (diluted in 1 × PBS; the appropriate dilution, usually 1:50 to 1:200, has to be tested empirically) under a coverslip in a humid chamber (Section 2.2.2) at room temperature overnight. Some of the background due to antibody cross-reaction can be removed by antibody preabsorption against total protein (acetone powder) extracted from an *S. pombe* strain that does not produce that specific protein (see Note 23).
3. Rinse coverslip away with 1 × PBS, wash slides three times for 15 min in PBS-T and drain excess liquid.
4. Incubate slides with a fluorochrome-conjugated secondary antibody (diluted in 1 × PBS according to the instructions of the provider) under a coverslip for 90 min at room temperature.
5. Rinse coverslip away with 1 × PBS, wash slides three times for 15 min in PBS-T and drain excess liquid.

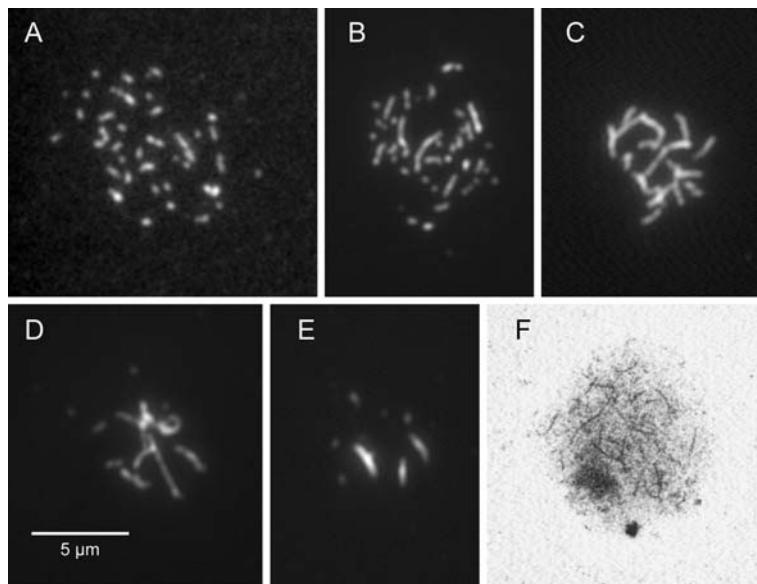


Fig. 2.2. Visualization of spread LinEs in the light microscope and in the electron microscope. (A–E) Rec10 immunostaining of LinEs at different stages of development. (F) LinEs visualized in the electron microscope by silver contrasting.

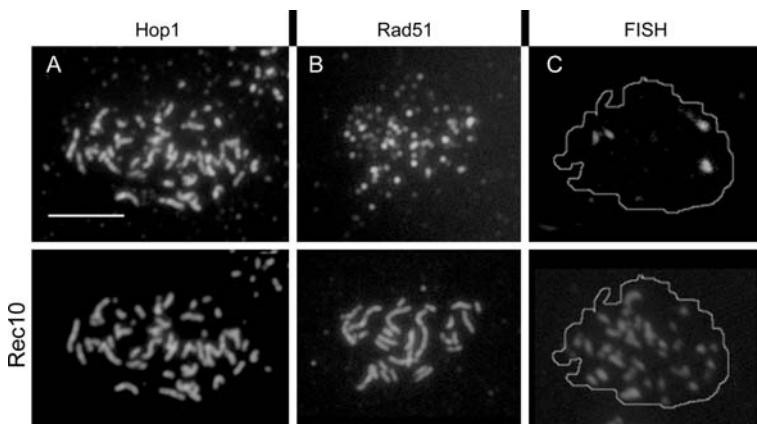


Fig. 2.3. Localization of LinE-associated proteins by double immunostaining and monitoring of homologous chromosome pairing by FISH in spread nuclei. (A) Double immunostaining of Hop1 (top) and Rec10 (bottom). Hop1 localizes to most of the length of the LinEs. (B) Double immunostaining of Rad51 (top) and Rec10 (bottom). The recombination protein Rad51 forms foci on LinEs. (C) Simultaneous FISH with two different probes (top), cosmids SPAC550 (the two spots to the left, originally delineated in red) and SPAC922 (to the right, originally delineated in green). The corresponding loci are unpaired. LinEs are immunostained for Rec10 (bottom). Contours of the nuclei as seen by DAPI counterstaining are indicated. Bar: 5 μ m. A color version of Fig. 2.3 is available on the companion CD for this volume.

6. Mount preparations in anti-fading medium supplemented with DAPI.
7. Seal with rubber cement if the preparations should be kept for more than a few days. The slides may be stored in the refrigerator for about one week. For longer periods they should be frozen at -20°C.

3.4. (Multicolor) FISH

FISH is useful for assessing homologous pairing (Fig. 2.3C) and segregation during meiosis similar to the *lacO*/LacI-GFP system. One obvious disadvantage is that FISH does not work with live cells to examine chromosome movements during a meiotic timecourse. On the other hand, it is comparably easy to highlight different loci without the need for constructing strains with appropriate *lacO* inserts.

3.4.1. Labeling of the Probes

The probes are labeled by the incorporation of Cy3-dUTP, Cy5-dUTP, fluorescein-dUTP, tetramethylrhodamin-dUTP (direct labeling) or digoxigenin-dUTP or biotin-dUTP (indirect labeling), using standard nick translation procedures (see Note 24). In the following, a typical nick translation labeling reaction is described:

1. Mix 2 µg probe DNA (see Note 25), 5 µL 10 × labeling buffer, 1.5 µL 280 mM β-mercaptoethanol, 5 µL 0.5 mM dATP/dCTP/dGTP mixture, 1.5 µL 1 mM labeled dUTP, 2 µL DNase I (~1:1,000 from a 1 mg/mL stock; the optimal dilution must be empirically determined), 1 µL *E. coli* DNA polymerase I (10 U/µL), and bring the reaction volume to 50 µL with dH₂O (see Note 26).
2. Incubate the reaction mixture at 15°C for 105–120 min.
3. Stop the reaction by adding 2.5 µL 0.5 M EDTA and incubate for 15 min at 65°C.
4. Precipitate DNA by adding 1/20 volume of 3 M Na-acetate (pH 5.5) and 2.5 volumes of ice-cold pure ethanol overnight at -20°C, centrifuge at 12,000*g* for 30 min at 4°C, discard the supernatant and add 300 µL 70% ethanol without disturbing the pellet, centrifuge again (12,000*g*, 15 min, 4°C), pour off the ethanol, air dry the pellet and resuspend in 30 µL 1 × TE or sterile distilled water.

3.4.2. Hybridization

For some applications, it is advantageous to inspect both immunostained structures and FISH signals in one and the same nucleus (e.g. if the spatial relationship of specific chromosomal loci to LinEs or recombination foci is of interest). While we did not succeed in immunostaining slides following the FISH procedure, FISH can be performed on immunostained slides. Some strong staining (such as by the anti-Rec10 antibody) is sometimes still weakly visible after subsequent FISH. If this is not the case, pictures have to be taken of immunostained nuclei and their coordinates on the slides recorded. Following FISH, the nuclei are looked up again (see Note 27).

1. When starting with spread preparations (**Section 3.2**) that were not subject to prior immunostaining, incubate the preparations in distilled water until the sucrose-layer has dissolved (~5–10 min). Continue with Step 3.
2. When starting with immunostained preparations (**Section 3.3**), remove immersion oil (if any) by slightly dabbing with filter paper and strip off the rubber sealing (if any). Rinse coverslip away with 1 × PBS, wash with distilled water (~5–10 min) to remove anti-fading medium. Continue with Step 3.
3. Drain the slide and let it dry (*see Note 28*).
4. Apply 50 µL DNase-free RNase (100 µg/mL in 2 × SSC) to each slide, cover with a coverslip, and incubate for 30 min at 37°C in a humid chamber (**Section 2.2.2**).
5. Incubate the slide in ST buffer (**Section 2.3.10**) at 37°C for 1–3 h.
6. To denature chromosomal DNA, place the slide in 70% formamide in 2 × SSC, pH 7.0 at 60°C for 2 min, then immediately immerse in ice-cold 70, 90, and 96% ethanol for 5 min each, and air dry.
7. Dry probe DNA in a vacuum concentrator. Use 1 µL (approx. 66 ng) per slide. If you apply two or three pooled probes to the same slide, mix 1 µL of each.
8. Resuspend the dried DNA in 3 µL 100% formamide by shaking vigorously for 30 min, add 3 µL of hybridization mix and shake for another 10–30 min.
9. Denature the probe DNA by heating to 95°C for 5 min and then chill on ice.
10. Apply 6 µL of denatured probe mix onto each slide, place a coverslip (12 × 12 mm) over the sample and seal with rubber cement.
11. Put slides on a thermocycler for slides and expose them for 10 min to 80°C (for co-denaturation of chromosomal DNA with probe mix) and then to 37°C for at least 36 h (for hybridization).
12. Peel off the rubber cement and gently rinse off the coverslip with 2 × SSC.
13. Wash slides for 5 min in each of the following buffers: 50% formamide in 2 × SSC (37°C), 2 × SSC (37°C), and 1 × SSC (room temperature).
14. After hybridization with directly labeled DNA, apply a drop of antifade medium with DAPI, and seal under a coverslip (*see Section 2.2*, agents 5, 6, and 7).
15. After hybridization with digoxigenin- or biotin-labeled DNA, go to **Section 3.4.3** (signal detection).

3.4.3. Detection of Digoxigenin- or Biotin-Labeled DNA

1. After washing the slides (**Section 3.4.2**, Step 13), put a large drop of blocking buffer (**Section 2.3.16**) under a coverslip and incubate slide for 1 h at 37°C in a humid chamber (**Section 2.2.2**).
2. To detect biotinylated probes, rinse coverslip away with detection buffer and incubate slides with 50 µL FITC- or Cy3-conjugated (Extra-) avidin diluted in detection buffer under a coverslip for 1 h at 37°. Continue with Step 4 or 9.
3. To detect digoxigenin-labeled probes, apply FITC-, rhodamin-, or AMCA-conjugated anti-digoxigenin antibody (*see Note 29*) and proceed as with applying secondary antibody in **Section 3.3** (immunostaining), Steps 3–6.
4. Weak signals of a biotin-labeled probe can be amplified as follows:
 5. Rinse coverslip away with ST buffer (**Section 2.3.10**), wash slides twice for 5 min in ST buffer and drain excess liquid.
 6. Incubate preparation with a biotin-conjugated anti-avidin monoclonal antibody for 1 h at 37°C as above.
 7. Rinse coverslip away with ST buffer, wash slides twice for 5 min in ST buffer and drain excess liquid.
 8. Incubate preparation with FITC- or Cy3-conjugated (Extra-) avidin as in Step 2.
 9. Rinse coverslip away with ST buffer, wash slides twice for 5 min in ST buffer and drain excess liquid.
 10. Mount preparation in anti-fading medium supplemented with DAPI.

3.5. Microscopic Evaluation of Fluorescent Signals

For immunostaining and FISH, an epifluorescence microscope equipped with a mercury lamp and appropriate filter sets for the excitation and emission of fluorescence spectra characteristic for the fluorochromes used, is necessary to visualize signals. The optimal combinations of excitation filter, beam splitter, and emission filter must be tested. Filter selection is a compromise between narrow band width (weaker signals) and wide spectrum (other fluorochrome may “leak through”) (10). Images are best recorded with a cooled CCD camera with high sensitivity to a wide spectrum of wavelengths, including far-red (as emitted by Cy5).

3.6. Silver Staining of Linear Elements

1. Incubate slide (from **Section 3.2**) in distilled water until the sucrose-layer has dissolved.
2. Drain the slide and let it dry for several hours.
3. Apply several drops of AgNO₃ solution to the slide and cover with a piece of polyamide cloth, trimmed to the size of a coverslip (*see Note 30*).
4. Incubate slides in a humid chamber (**Section 2.2.2**) at 60°C for 40 min.

5. Rinse away the cloth with distilled water and allow the preparation to dry.
6. Inspect the slide (*see Note 31*) without embedding under a coverslip, as most microscopical mounting media will bleach or dissolve the silver deposit. For prolonged storage, immersion oil can be removed from the slide by 1–3 min incubation in hexane.

**3.7. Transfer
of Light-Microscope
Preparations to the
Electron Microscope
(EM)**

Silver-impregnation produces highly contrasted structures which are suitable for inspection in the electron microscope (**Fig. 2.2F**). For this purpose, preparations on glass slides can be transferred to electron microscopy grids.

1. If required, remove immersion oil by incubating the slide for 1–3 min in hexane.
2. Dip slide in Formvar solution and retract slowly such that a thin supporting film will deposit on the slide (*see Note 32*).
3. Identify at low magnification (no immersion oil!) regions of interest and mark them with a water-resistant marker pen.
4. Scratch the coating with a diamond glass-writer around the area with the markings. Put small drops of 1% hydrofluoric acid onto the slide; it will dissolve the glass surface and help to detach the Formvar film together with the cells from the slide. Caution: hydrofluoric acid and its vapors are corrosive and toxic. Follow the safety regulations of your institution. Work in a chemical hood and wear eye protection and gloves.
5. Add water until the plastic film floats. Submerge the slide in a large bowl with water such that the coating will come off and float.
6. Place EM grids on the marked regions of interest.
7. Push the plastic film together with the grids under the surface with the edge of a piece of Benchkote paper such that the plastic film will attach to the smooth side of the paper and the EM grids will become sandwiched between the plastic film and the paper (*see Note 33*).
8. After the film has dried, pick off the EM grids together with the adhering plastic film with fine tweezers; they are now ready for inspection in the EM.

4. Notes



1. Supplements can be directly added in powder form to the YE and MEA before autoclaving; for use with PM and PM-N, it is more practical to prepare mixed supplement stocks of appropriate concentrations (e.g. 7.5 g/L) in distilled water and filter sterilize. Adenine and uracil tend to precipitate, so warm up the stocks before adding to the medium.

2. The stocks can be stored at -20°C for several months and repeatedly frozen and thawed. In the Zymolyase stock, the powder does not dissolve completely; therefore, after thawing a pellet is present which must be stirred up before use. Alternatively, the stock can be prepared in 10% (w/v) glucose instead of water.
3. Lipsol is a laboratory cleaning agent, a mixture of nonionic and anionic detergents plus a chelating agent and builders (information from the manufacturer). Several standard laboratory detergents (Nonidet, Triton X-100, sodium dodecyl sulfate, *N*-lauroyl sarcosine) were tested as alternatives but gave unsatisfactory results in our hands.
4. 4 g Paraformaldehyde is heated in 90 mL distilled water on a magnetic stirrer to 80°C (Caution: formaldehyde vapors! Work under a chemical hood!). After 20–30 min the solution should become clear. If it stays opaque, add 1 M NaOH until it becomes clear. Add 3.4 g sucrose to the solution after cooling. If NaOH has been added, the solution has to be titrated back to pH 8.5 with HCl. Make up the volume to 100 mL. If the fixative is not completely clear, it can be filtered. It can be stored for several months in the refrigerator.
5. There are almost as many recipes for PBS as there are labs. A simpler alternative for 10 × PBS is: 90 g/L NaCl, 11.5 g/L anhydrous Na₂HPO₄, 2.3 g/L anhydrous NaH₂PO₄, make up to 1,000 mL with distilled water. Both worked in our hands.
6. It is convenient to prepare antifade medium containing 1 µg/mL DAPI so that embedding and DAPI staining can be performed in a single step.
7. FISH probes should be carefully selected and their sequence checked against the database to avoid the inclusion of repetitive genomic elements (e.g. transposons), which would result in unspecific speckled background staining.
8. Working solution can be stored for several months in the refrigerator. If a black or brown deposit is formed, the solution should be filtered. Old AgNO₃ or used AgNO₃ solution should not be released into the environment. Check with a recycling service; it can be collected together with photographic fixer for the recycling of silver.
9. If kept on PM for extended periods, diploid strains will sporulate. Therefore, they should be maintained on YE plates with no adenine supplement. While some haploid *ade*⁻ colonies will establish themselves on these plates, they can be easily discerned from the diploids because they are smaller and colored red (*ade6-M210*) or pink (*ade6-M216*) (20, 24). Slowly growing strains can be kept on casein plates (1.7 g/L

yeast nitrogen base without ammonium sulfate and amino acids, 3.7 g/L sodium glutamate, 20 g/L glucose, 10 g/L casamino acids, 0.1 g/L uracil, 20 g/L agar).

10. If possible, the strain should not carry auxotrophic markers other than *ade6*, since they impair synchronous sporulation of the strain.
11. Media in this experiment must not contain adenine supplement to avoid the untimely emergence of haploid cells.
12. Two cultures with different cell densities are started to improve the chance that one of them will have the right density the next morning.
13. If the cell density is much higher than 2×10^7 cells/mL, the culture should be discarded and the experiment started anew, since subsequent sporulation will be asynchronous.
14. While the careful monitoring of meiotic progression may not be crucial for all experiments, it is advisable to take a sample after ~ 24 h to assess the sporulation efficiency of the culture.
15. If the cell density is much higher you may prefer to abort the experiment since the frequency of cells entering meiosis will drop.
16. After 4 h, the temperature should be decreased to 30°C, since the cells are already committed to meiosis and it is unnecessary to stress them unreasonably.
17. This spreading protocol was originally developed for diploid strains. Spreading of haploid (e.g. *pat1-114*) strains seems to work better if the cells are killed by adding Na-azide (final concentration 0.1% from a 10% stock in water; ATTENTION: toxic!) to the cell suspension in **Section 3.2, Step 1**.
18. Novozym 234 is not produced any more and the alternatively used lysing enzyme L2265 (Sigma) (25) is no longer available either.
19. This suspension can be stored on ice for several hours.
20. The presence of sucrose in the fixative has the additional advantage that the mixture is hygroscopic and does not dry out completely. Therefore, this kind of preparation can be used for immunostaining even after storage for several days in the refrigerator or several months in the freezer.
21. Fixative is added to the slide before and after the detergent. A small amount of fixative present during detergent spreading prevents the disruption of spheroplasts but does not interfere too much with spreading. The relative amounts and order of application of nuclear suspension, detergent, and fixative should be optimized by testing since the optimal spreading depends on the density of nuclei in the suspension, the degree

of spheroplasting and the age of solutions. The process of spreading can be watched in the phase contrast microscope at low magnification without a coverslip. Spheroplasts should swell slowly, and turn continuously from white to black and then to gray. They should not “explode” instantly!

22. Spheroplasts readily stick to the slides. It is, therefore, not necessary to coat slides with polylysine, but it may be a good idea to clean them with ethanol before use.
23. To prepare acetone powder (26), collect 100 mL of a meiotically induced cell culture by centrifugation, wash once with a sterile 0.9% NaCl-solution and finally resuspend the cells in the same solution (1 mL of saline per 1 g of cells), keep on ice for 5 min. Add 4 mL acetone (-20°C) per 1 mL cell suspension and mix vigorously, incubate on ice for 30 min (mix every 5 min). Harvest by centrifugation ($10,000g$, 10 min), discard the supernatant and resuspend precipitate in 100% acetone (-20°C) by mixing vigorously and keep on ice for another 10 min. Collect precipitate again ($10,000g$, 10 min), discard supernatant and spread the precipitate onto a clean sheet of filter paper, let the pellet air-dry at room temperature while dispersing it on the filter paper (avoid formation of large lumps). The dry acetone powder is moved to an airtight tube and can be stored at 4°C . For preabsorption, incubate diluted antiserum for 30 min at 4°C with the acetone powder at a final concentration of approximately 1% (w/v) in an Eppendorf tube, centrifuge for 10 min at $\sim 12,000g$ and transfer the supernatant to a fresh Eppendorf tube.
24. Since nucleotides tagged with fluorochromes (Cy3-dUTP, Cy5-dUTP, fluorescein-dUTP) have become commercially available, direct probe labeling is becoming more and more common, and it is sufficient for most applications. However, if numerous different fluorochromes are needed for multi-color FISH or if fluorescence intensity of directly labeled probes is insufficient, it may be useful to work with indirectly labeled probes. Direct labeling of the DNA probe with the fluorescent tags Cy3, Cy5, or FITC usually produces a sufficiently strong signal to be readily detected. For small probes, indirect labeling is recommended.
25. We isolate cosmid DNA with the help of the QIAGEN Plasmid Midi Kit (Qiagen Inc.), following the instructions from the manufacturer.
26. Labeling reactions should be optimized to give labeled products of 100–500 bp in length, by adjusting the DNase I concentration. The size of the product should be monitored on an agarose gel.

27. Finding nuclei is facilitated if low-magnification overviews of DAPI-stained groups of nuclei are also photographed. These patterns are easily recognized in the microscope at low magnification. We have a success rate of close to 90% in re-locating nuclei.
28. We noticed that slides which were left at this point to season for a couple of days worked better for FISH than fresh slides.
29. AMCA is a blue fluorescent dye. It cannot be used in combination with DAPI as a DNA counterstain. Use propidium iodide (red) instead of DAPI.
30. Polyamide cloth produces a homogeneous staining all over the coated area, whereas by using a coverslip instead, regions near the edges are stronger stained than the interior. A possible explanation for the enhancing effect, based on the chemical interaction of the polyamide with the staining reaction, is given by Herickhoff et al. (27).
31. If the silver staining turns out too pale, modify the protocol by transferring slides to sodium tetraborate buffer (pH 9.2; Merck, Darmstadt, Germany) for 30 s after Step 1. Also, using different brands or batches of nylon cloth may influence staining intensity. Increasing the time or temperature of the incubation with silver nitrate solution is not recommended as it tends to enhance unspecific precipitation of silver.
32. Work in a dry environment. Moist slides may cause holes in the plastic coating.
33. Parafilm can be used instead of Benchkote.

Acknowledgments

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Chapter 3

Assaying Chromosome Pairing by FISH Analysis of Spread *Saccharomyces cerevisiae* Nuclei

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Abstract

Fluorescent in situ hybridization (FISH) provides a powerful tool to study the localization of DNA sequences in relationship to one another. FISH has the advantage over other methods, notably use of GFP-tagged repressor/operator arrays, that an almost unlimited number of probes can be utilized without having to make new strains for each new locus one wants to study. Also, the number of sites that can be visualized at the same time is limited only by the number of fluorophores that are available and can be distinguished by the available microscope. Described here is a method for FISH analysis and its application to analysis of chromosome pairing during meiosis in *S. cerevisiae*.

Key words: *S. cerevisiae*, chromosome pairing, meiosis, fluorescent in situ hybridization, FISH, nuclear spreads.

1. Introduction

One of the most fundamental aspects of meiosis is the formation of connections between homologous chromosomes. These connections are required for the faithful segregation of chromosomes leading to viable spores. We have shown (1–3) using fluorescent in situ hybridization (FISH) that alignment of homologous chromosomal loci in *S. cerevisiae* occurs prior to meiotic DNA replication, in fact even in mitosis. In both meiosis and mitosis, pairing is transiently lost during replication and is re-established after replication. Significant juxtaposition of homologous loci occurs in the absence of either double-strand breaks or visible cytological structures, a process we refer to as “recombination-independent pairing”. FISH can also be used to analyze the entire process of

meiotic homolog juxtaposition which, by analogy with other organisms (e.g., 4) includes progressive DSB-mediated juxtaposition to a “presynaptic alignment” distance and, finally, formation of synaptonemal complex (SC). FISH allows one to study the position of DNA loci without adding the complication of inserting extraneous non-native sequences. Yeast provides an attractive system for such studies because of the potential for genetic manipulation (1, 3). Proper set up of cultures for analysis, methods for both slide and probe preparation and for hybridization as well as approaches to data analysis will be described in this chapter. We also discuss important considerations and caveats to the interpretation of such data. FISH can also be used for analysis of whole chromosomal domains [“chromosome painting” (5)]. The methods described in this chapter are generally applicable to any DNA sequence and cellular system.

2. Materials

2.1. Meiotic Time

Courses – SK1 Strains

(see Notes 1 and 2)

1. YEPG plates: 3% v/v glycerol, 2% w/v bactopeptone, 1% w/v yeast extract, 2% w/v bactoagar. Autoclave on liquid cycle.
2. YEPD liquid and plates: 2% w/v bactopeptone, 1% w/v yeast extract. Add 2% w/v bactoagar for plates. Make a 40% w/v stock solution of glucose. Autoclave on liquid cycle. Add glucose to medium to a final of 2% (see Note 3).
3. YEPA; 1% w/v potassium acetate, 2% w/v bactopeptone, 1% w/v yeast extract, two drops per liter antifoam (Sigma). Autoclave on liquid cycle.
4. SPM: 0.3% w/v potassium acetate, 0.02% w/v raffinose, two drops per liter antifoam (Sigma). Autoclave on liquid cycle.
5. 40% ethanol, 0.1 M sorbitol.
6. Low-speed centrifuge such as Beckman Coulter J6B or the Sorvall SuperT21.

2.2. Fluorescent In Situ Hybridization

2.2.1. Spreading of Nuclei

1. Microscope slides: 25 × 75 mm, frosted. Use of frosted slides provides both a convenient labeling surface and a way to feel which side is on top in low light.
2. Liquid dish detergent: Dawn and Joy are two common brands that work.
3. Histostik (Accurate Chemicals), diluted six drops per 200 mL water. Can be stored at 4°C. Mix well before using.
4. 1 M Tris-HCl pH 7.5.
5. Tris buffer: 0.2 M Tris-HCl pH 7.5.

6. 1 M DTT (dithiothreitol): Store 1 mL aliquots at -20°C.
7. ZK buffer: 50 mM Tris-HCl pH 7.5, 0.5 M KCl.
8. Zymolyase solution: 3 mg Zymolyase 100T, 7.5 µL of 40% w/v glucose, 7.5 µL 1 M Tris-HCl pH 7.5, 150 µL sterile water. Make up 1 h before use, solubilize on ice.
9. MES wash: 1 M sorbital, 0.1 M MES(2-(N-morpholino)ethanesulfonic acid)-NaOH, 1 mM EDTA, 0.5 mM MgCl₂, pH 6.5.
10. MES lysis: 0.1 M MES-NaOH, 1 mM EDTA, 0.5 mM MgCl₂, pH 6.5.
11. 1% Paraformaldehyde: Mix paraformaldehyde in 60–70°C water for 10 min then cool. With NaOH (1 N) adjust the pH to 7. Use pH paper to measure the pH (*see Note 4*).
12. 0.4% v/v Photoflo (Eastman Kodak, Rochester, NY).

2.2.2. Probe Preparation

1. 10x Nick translation Buffer: 0.5 M Tris-HCl pH 7.2, 0.1 M MgSO₄, 1 mM DTT, 500 µg/mL BSA (bovine serum albumin Pentax fraction V).
2. DNA polymerase I, *E. coli*.
3. DNase I: 1 mg/mL stock in 50% v/v glycerol. Store at -20°C. Dilute to 1 µg/mL with ice cold water immediately before use.
4. dNTP mix: 0.5 mM dATP, dGTP, dCTP.
5. Labeled dUTP. Many different nucleotides and labeling methods are available. *See Note 5* for some suggestions.
6. 0.5 M EDTA.
7. 10% SDS.
8. Nucleotide purification spin column such as ProbeQuant G-50 Microcolumns (GE Healthcare, Piscataway, NJ).

2.2.3. Hybridization and Washing

1. 20x SSC: 0.3 M sodium citrate, 3 M sodium chloride pH 7.0.
2. 100 µg/mL preboiled RNase A in 2x SSC.
3. 2 mg/mL salmon sperm DNA, phenol:chloroform extracted then ethanol precipitated and dissolved in water.
4. 5 mg/mL tRNA from *E. coli*.
5. Formamide: molecular biology grade (Sigma, St. Louis, MO).
6. Coverslips: 40 × 20 mm.
7. Hybridization Buffer: 4x SSC, 200 mM sodium phosphate pH 7.2, 40% dextran sulfate
8. PCR In Situ block on a PT-200 thermocycler (MJ Research). Any block which will heat to 80°C will work or a water bath may be used.

9. Rubber cement.
10. Blocking solution: 3% w/v BSA, 4x SSC.
11. Buffer A: 1% w/v BSA, 4x SSC, 0.1% v/v Tween20.
12. Wash A: 4x SSC, 0.1% Tween20.
13. Wash B: 4x SSC, 0.1% Triton X-100.
14. 2 mg/mL DAPI (4',6-diamidino-2-phenylindole).
15. Wash C: 4x SSC, 1 µg/mL DAPI.
16. Slow Fade (Invitrogen, Carlsbad, CA). Any brand antifade should work.

2.3. Visualization

1. Microscope: 100x objective, high-resolution/high-sensitivity camera, appropriate filters for the fluorescent probes used.
2. Software for measuring xy positions in an image.

3. Methods

3.1. Meiotic Time Courses

In order to properly evaluate chromosome localization at different stages of meiosis, it is critical to work with cultures that are highly synchronous. The procedures required to obtain such cultures, and then to perform the time course experiment itself, will take in total ~7 days. Many of these steps can be adjusted slightly but best synchrony is most reliably obtained by following the suggested timing closely (6). The protocol described below, referred to as the “YPA” protocol, is one of several variations used in our laboratory. For another version, the “SPS” protocol, see Chapter 6 in this volume.

1. Day 1 – In the evening, patch cells from -80°C glycerol stock onto YEPG plates. This step ensures that cells emerging into growth are respiration-proficient, critical for entry into meiosis.
2. Day 2 – The next morning, streak for single colonies on YEPD plates. Make sure colonies are sparse so that they will grow large and healthy (*see Note 6*).
3. Day 4 – In the evening, pick a whole colony and put into 5 mL YEPD liquid in a 10 × 13 mm culture tube. Vortex and then grow overnight at 30°C on a roller drum. Typically we set up at least two cultures for each strain to be analyzed (*see Note 7*).
4. Day 5 – Grow YEPD cultures for 24–30 h, vortexing tubes a couple of times during the day. Then add the cells to a large volume of YEPA, to a cell density of 1.8×10^6 cells per mL. This typically involves a 1/100 to 1/150 dilution. Note that surface-to-volume ratios in the culture flasks at this stage are important. Use 100 mL YEPA in a 1 L flask, or no more than

250 mL in a 2 l flask. It is easiest to prepare a range of dilutions and then decide at Step 6 which will give the best results. This also helps if you are not going to hit precisely the 13.5 h time point described in the next step. Typically we set up two to four flasks for each strain.

5. Grow YEPA cultures at 30°C, shaking at 300–350 rpm, making the culture swirl up the edges of the flask to create the largest surface area possible.
6. Day 6 – YEPA cultures should be grown for a total of 13.5 h. Remove samples from cultures and assess synchrony (*see Note 8*).
7. Once culture(s) for analysis are selected, harvest cells by centrifugation at 1,400g for 3 min at room temperature.
8. Wash cells in an equal volume of sterile distilled water (pre-warmed to 30°C) and resuspend in an equal volume of pre-warmed SPM. Try to get the cells into the SPM within 45 min of the first centrifugation (Step 7); faster is better.
9. Take zero time point and place culture in incubator at 30°C, shaking as before (*see Note 9*).
10. Harvest samples at desired intervals (usually 20–60 min). Place a 10 mL sample in a plastic tube on ice for further processing (*see Section 3.2.1, Step 2*).
11. Take 1 mL samples at hourly intervals to assess meiotic progression (MI and MII divisions) by DAPI staining. Pellet samples at 13,000g for 10 s then resuspend in 40% ethanol, 0.1 M sorbitol (*see Note 10*).

3.2. Fluorescent In Situ Hybridization of Spread Chromosomes

A critical variable in assessment of relative locus positions is the overall diameter of the spread nucleus that, in turn, depends critically on the conditions for spreading. The method below yields nuclei of approximately 5–8 µm in diameter. The spreading protocol described here (adapted from Refs. 6 and 7) appears to optimize spatial separation while minimizing unwanted disruption (*see Note 11*).

3.2.1. Spreading of Nuclear Contents

1. Prepare slides by washing with liquid dish detergent, rinsing well with distilled water, then rinsing in 95% ethanol. After the slides are dry, dip them in Histostik. 0.1% Polylysine also works. Let them dry again. Store at room temperature.
2. Take 10 mL of meiotic culture, pellet at 1,400 g for 2 min.
3. Resuspend in 2.5 mL 0.2 M Tris-HCl pH 7.5.
4. Add 50 µL 1 M DTT.
5. Let stand 2 min at room temperature.

6. Pellet as above and remove supernatant.
7. Resuspend in 2.5 mL ZK buffer. Use only plastic from this point: spheroplasted cells stick to glass.
8. Add 20 μ L zymolyase solution.
9. Incubate on roller drum for 25–30 min at 30°C, inverting tube a few times during incubation (*see Note 12*).
10. Pellet cells at 1,600 g for 3 min.
11. Resuspend in 2.5 mL ice-cold MES wash. Pellet cells at 1,600 g for 3 min.
12. Lyse in MES lysis buffer. Solution should be slightly cloudy. Usually 10 mL of starting cell culture requires the use of 0.5–1 mL of lysis buffer. Let lyse 5 min at room temperature.
13. Add equal volume 1% paraformaldehyde.
14. Place aliquots of cells on coated slides. Make as many slides as possible, using the entire preparation. 10 mL of original cell culture yields ~2 mL of fixed cells, which is enough for ~ four slides with about 0.5 mL per slide. If you need more than four slides, start with a larger aliquot of cells and scale up the above protocol. Let slides sit 10 min at room temperature. The above protocol should give \leq 1 cell per field, which is a good number. Larger numbers of nuclei require too much probe; small numbers of nuclei are too sparse for easy identification under the microscope.
15. Tilt off excess liquid. Discard paraformaldehyde as hazardous waste. With a pipette rinse the slide with 1.5 mL 0.4% Photo-flo. Let dry.
16. Slides can be stored at –20°C in a slide box. Slides remain analyzable for at least one year and possibly longer.

3.2.2. Probe Preparation

An almost unlimited number of different tagged nucleotides are currently available. The number needed in a given experiment will depend on the subject of interest. Accurate assessment of homolog pairing requires at least two different probes whose fluorescence can be distinguished from each other and the DAPI stained total DNA (below).

1. Combine 10 μ g probe DNA, 10 μ L 10x nick translation buffer, 10 μ L dNTP mix, 1 mM labeled dUTP, 10 μ L DNase I, 2 μ L DNA Polymerase I. Add sterile water to 100 μ L (*see Note 13*).
2. Incubate at 15°C for 2 h (*see Note 14*).
3. Stop reaction by addition of 2.5 μ L 0.5 MEDTA plus 1 μ L 10% SDS.
4. Purify probe using nucleotide purification spin column.

Fluorescently labeled probes should be exposed only to indirect light. This can be accomplished by turning off overhead lights and otherwise keeping the samples covered.

3.2.3. Hybridization

1. Heat slides for 3 h at 65°C in a dry oven.
2. Add 100 µL RNase A solution then a coverslip and incubate for 1 h at 37°C
3. Dehydrate in three successive steps by dipping the slides in 70, 95, and 100% ethanol for 5 min each.
4. Mix the DNA probe with 5 µg salmon sperm DNA and 20 µg tRNA. Approximately 100 ng of labeled probe is needed. The longer the probe, the more DNA is required. Do an initial titration to determine the amount required for your probe.
5. Precipitate DNA with ethanol. Resuspend probe in 5 µL 100% formamide. Heat at 42°C to dissolve, then add an equal volume of hybridization buffer.
6. Add 10 µL hybridization solution to each slide.
7. Cover with 40 × 20 mm coverslip.
8. Denature 3 min at 80°C on a heated block (e.g. PCR In Situ block; MJ Research, *see Note 15*). Cool to 4°C.
9. Seal coverslip to slide with rubber cement around the edges.
10. Place slide in dark, humidified chamber. A plastic or metal box containing wet paper towels works well.
11. Hybridize at 37°C over two nights.
12. Remove coverslip.
13. Rinse 30 min in 50% formamide (doesn't have to be Sigma), 2X SSC at 37–42°C in a Coplin jar. Discard formamide as hazardous waste.
14. Rinse 30 min in 2X SSC at 37–42°C.
15. Rinse 30 min in 1X SSC at room temperature.
16. The following steps vary according to the nature of the probe. If using a fluorescently labeled probe go to step 21. Otherwise add 200 µL Blocking solution to each slide, place coverslip, and incubate 30 min to 3 h at 37°C in a moist chamber. The longer times are necessary if high background is a problem.
17. Drain, add 100 µL primary antibody diluted according to manufacturer's directions in Buffer A and incubate 1 h at 37°C in a moist chamber.
18. Rinse 15 min with Wash A at 37–42°C.
19. If primary antibody is fluorescently labeled go to Step 21. Otherwise add 100 µL secondary antibody diluted 1:250 in Buffer A and again incubate at 1 h at 37°C in a moist chamber.
20. Rinse 10 min in Wash A at 37–42°C.

21. Rinse 10 min in 4x SSC at room temperature.
22. Rinse 10 min in Wash B at room temperature.
23. Rinse 10 min. in Wash C at room temperature.
24. Drain slides, add one drop SlowFade or other antifade, then cover with a 22 × 60 mm coverslip.
25. Seal edges with clear nail polish. Slides can be stored at -20°C or -80°C (*see Note 16*)

3.3. Visualization and Analysis

Nuclei are observed under appropriate excitation and emission wavelengths for the particular fluorophores being used. Because of the small size of FISH signals, a 100X objective is required. Especially with smaller probes, the best optics and a highly sensitive camera are essential. In addition, a computer is needed for acquiring images and subsequent analysis.

1. Acquire 50–100 images of nuclei. Nuclei can be located on the slide based on their DAPI staining. Take pictures of the DAPI-stained nucleus and the spots resulting from the hybridization (Fig. 3.1A).
2. For each nucleus record the XY coordinates of every spot. (Because these are spreads there should be only one focal plane).

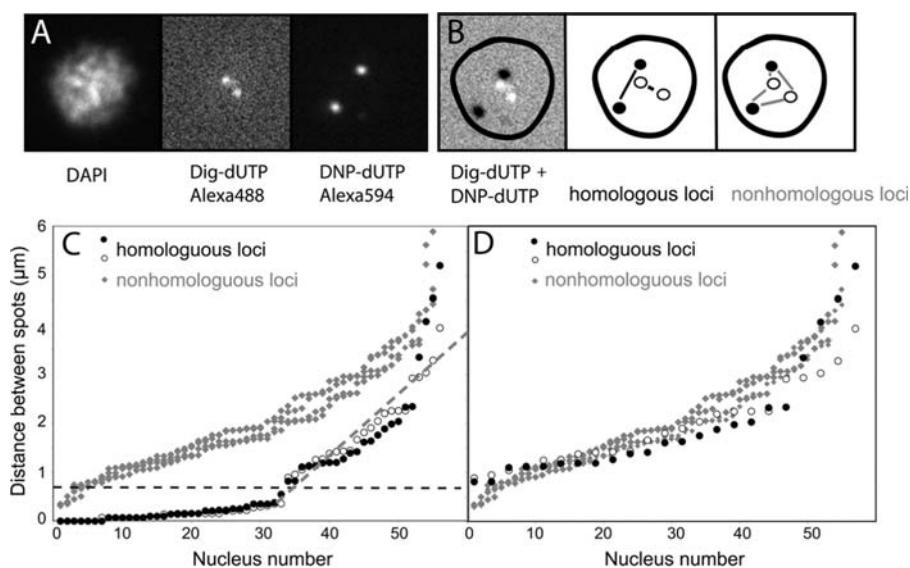


Fig. 3.1. Example of data acquisition and analysis. **(A)** Epifluorescent micrographs showing a spread nucleus with DAPI staining of the chromatin and the *in situ* hybridization signal from probes to loci on two different chromosomes. **(B)** The combined image of the *in situ* hybridization signal. The cartoon on the right shows the measurements taken between the two sets of homologous loci and the four sets of nonhomologous loci. **(C)** Measurements as shown in **(B)** are calculated for at least 50 nuclei. Distances within each set are ordered according to increasing distance and plotted as a function of the position in its own array. Homologous distances are shown in black for the two different loci. The four nonhomologous measurements are indistinguishable from each other and are all shown in gray. **(D)** The distances from homologous loci delineated by the gray dashed line in **(C)** are compared to the nonhomologous measurements.

3. Calculate the distance between each pair of spots. For two probes there will be six possible pairs. Two measurements are between homologous chromosomes and represent possible interactions. Four measurements are from spots on nonhomologous chromosomes and represent the chance that two chromosomal loci fall randomly together (**Fig. 3.1B**). Some microscopy programs will convert pixels to microns automatically. Otherwise the conversion can be done at the end. As a practical matter, it is not required to convert since in the end one compares the % of homologous interactions to the % of nonhomologous “accidents.”
4. Rank-order each set of distances from smallest to largest and graph in ascending rank order (**Fig. 3.1C**).
5. The distance between two homologous spots defined as “paired” is dependent on level of spreading. It can be empirically defined using strains known to show high levels, such as *ndt80* where most chromosomes exhibit full-length SC at t~7 h (*1*). Alternatively, one can use the shape of the curves to arrive at a numerical definition of pairing. In this case, the maximum distance where two loci are considered to be paired is the point at which the curve for homologous distances changes slope from being “relatively flat” to a slope which, if it comprised the entire population, would match the curve for nonhomologous distances (**Fig. 3.1D**). The percentage of loci paired in a particular sample is the difference between the percentage homologous loci at the pairing distance or closer and the percentage of nonhomologous loci whose distances also fall into this range. Importantly: if nuclei are not optimally spread, the “background” from nonhomologous loci is very large, and subtraction of this value leads to an underestimate of the actual level of interaction for homologous loci (*see Note 17*).

4. Notes



1. If strain is a *trp1* or *ura3* auxotroph, bring plates and liquid YEPA to 40 µM tryptophan and/or 20 µM uracil after autoclaving.
2. We have tried many different waters and results seem random. House distilled seems to work as well or better than MilliQ filtered. Time courses during summer seem especially finicky. Some people swear by bottled water such as Poland Springs as being more reproducible year round.
3. The sugar and amino acids in media will be chemically modified if autoclaved together (Maillard reaction). Autoclave the glucose as a separate 40% stock solution at 110°C. The rest of

the media components can be combined then autoclaved at 121°C. After autoclaving add the glucose to the media to a final concentration of 2%.

4. Paraformaldehyde decomposes with high heat and time. Store one week at most. Discard as hazardous waste.
5. Probes can either incorporate a fluorescent-labeled dUTP or use primary and even secondary antibodies to visualize a non-fluorescent tag. The use of antibodies increases the sensitivity of the assay allowing shorter lengths of DNA sequences but also increases the potential level of nonspecific background. Ideally one wants to use the fewest steps necessary to get a positive signal. This will depend not only on the choice of label but also the quality of the microscope and sensitivity of the camera used to collect the images. There are a large number of sources for labeling and visualizing the probe. The ARES labeling system (Invitrogen, Carlsbad, CA) incorporates an allyl-dUTP then chemically attaches a fluorophore. In addition the company sells a line called "Chromatides" which are fluorescently labeled dUTP nucleotides. Vector Laboratories (Burlingame, CA) also sells kits for chemically labeling DNA with either fluorophores or haptens. Hapten conjugated dUTP nucleotides (biotin, DNP, and digoxigenin) are available from Invitrogen and Roche Applied Sciences (Indianapolis, IN). These can then be hybridized either with fluorescent-labeled primary antibodies or further amplified using fluorescently labeled secondary antibodies (Jackson Immunoresearch, West Grove, PA and Evident Technologies, Troy, NY are two additional distributors). Biotin-dUTP can also be labeled using streptavidin conjugated to fluorophores (Vector Laboratories and Invitrogen). This is just a small sample. Many other companies sell similar products.
6. For *S. cerevisiae* strain SK1 one can readily distinguish diploid and haploid: diploids produce smaller, smoother, shinier colonies with a distinct "nipple"; haploids are broader, flatter and duller with wrinkled edges. Because SK1 haploids tend to "clump," they also sink much more quickly in liquid culture.
7. For mutants that have a mitotic growth defect (e.g. *rad51* and *rad52*) each growth step can be extended to allow for sufficient density of cells at the start of the time course. Also it is harder to distinguish haploid and diploids so more YPD cultures are started to insure an adequate selection. Often one must judge haploid and diploid under the microscope with haploids being smaller and budding apically while diploids are larger and bud from alternate poles.
8. There are three criteria for judging the synchrony of a culture:
 - a. Optical Density: On our spectrophotometer we take between 1.3 and 1.45 OD₆₀₀ measured on the undiluted

culture (i.e. not a true OD because we do not dilute the cells into the range that the spectrophotometer can measure in a linear fashion). Each spectrophotometer will give a different reading according to light path length. This OD corresponds to $\sim 2.1 \times 10^7$ cells per mL at $OD_{600}=1.35$. The lower densities seem to be consistently more synchronous and are best for cytological studies. We have found that if cultures are too dense, diluting with SPM improves the synchrony of the subsequent time course.

- b. Percentage of G1/G0 Cells: Stain cells with DAPI by centrifuging a 500 μ L sample for 10 s at 13,000*g*. Resuspend in 40% ethanol, 0.1 M sorbitol. On a microscope slide add 2 μ L of ethanol-permeabilized cell suspension to 2 μ L of 1 μ g/mL DAPI. Assess the percentage of cells in vegetative S-phase (small buds without DAPI bodies) and mitosis (large buds with DNA at, or stretched across, the neck). A good culture will have $\leq 10\%$ replicating and dividing cells. The absence of mitotic figures *may* indicate that the culture has prematurely entered meiosis.
- c. Cell Morphology: Strings of three to five cells with daughter and mother cells of about the same size seem to correlate with synchronous cultures.
9. At temperatures $>30^\circ\text{C}$, SK1 meiosis is sub-optimal; at $\geq 34^\circ\text{C}$ meiosis is inhibited. Check the temperature of your incubator carefully – it may fluctuate throughout the day. Lower temperatures (23–30°C) may be less problematic but you will have to be consistent. Meiotic DNA replication is particularly sensitive to high temperature. If it is necessary to perform a time course at higher temperature it is preferable to shift from 30°C after initiation of DNA replication (~ 2 h).
10. DAPI staining: On a microscope slide add 2 μ L of ethanol-permeabilized cell suspension to 2 μ L of 1 μ g/mL DAPI. Cells that have done anaphase I or anaphase II will have 2 or 4 DAPI staining bodies, respectively.
11. Methods that give less spreading (e.g., Ref. 8) provide less spatial resolution and, in the case of chromosome pairing assays, increase the percentage of nonhomologous loci that fall close to each other. Methods that give more spreading would lower the nonhomologous background but risk more extensive destruction of important connections.
12. Cells will get clumpy then unclump. Check progress by placing a small amount of cell suspension in a drop of water and observing them osmotically lyse under the microscope.
13. Probes can be chosen from any sequence in the genome. For the purpose of studying chromosome pairing of typical loci, one should choose a single copy sequence. Existing cloned

DNA can be used or specific sequences can be isolated by PCR and cloned for subsequent use. The minimum sequence length depends on the final brightness of the probe and the sensitivity of the microscope camera. Unfortunately the only real way to know the minimum length for one's system is to test different size probes. We have found that one can use probes as small as 500 bp for bacteria; however, detection of the same sequence when present in yeast requires a probe of at least 4 kb in length. This may reflect the fact that yeast has more highly ordered chromatin (e.g. nucleosomes) which interferes with probe access for hybridization. When probing short regions, careful probe preparation is critical. We recommend: (a) use of plasmid DNA for labeling and (b) preparation of labeled probe by nick translation. The rationale for (a) is that, when the probe includes sequences other than the region of interest, these sequences form "networks" by hybridization to one another, thereby concomitantly recruiting more than one copy of probe DNA for a particular locus (9). Nick translation is the labeling method of choice because it amplifies the total amount of DNA, gives high efficiency incorporation of labeled nucleotide, and provides random fragments of the required size (*see Note 14*). In contrast, when probing a larger region, other probe preparations are possible. For example, large probes (e.g. cosmids of 30–45 kb) can be used at lesser concentrations, and with lower efficiency labeling, and still give a clear signal.

14. Lower backgrounds are achieved when both the probe DNA and the carrier salmon sperm DNA are cleaved into fragments predominately between 300 and 500 bp (9). Salmon sperm DNA is incubated with DNase I and the size is monitored by agarose gel electrophoresis. The probe fragment length is achieved by titrating the ratio of DNase I and DNA polymerase I in the nick translation reaction or adjusting the incubation time. Size can be checked on an agarose gel if the probe is fluorescent. If the probe is not fluorescent then the gel can be blotted to nylon membrane and the DNA visualized by hybridization with appropriate antibodies (e.g. anti-DNP alkaline phosphatase followed by suitable substrate). Fragmentation of the probes also means the stochastic distribution of label to all loci obviating the worry that hybridization occurred at only a subset of loci.
15. If a PCR machine capable of handling microscope slides or an 80°C block are not available then a protocol utilizing a water bath is as follows: In this version the slides and probe are denatured separately. Preheat a Coplin jar containing 70% formamide, 2x SSC to 70–80°C in a water bath. Add slides and incubate for 2 min. At the same time resuspend probe in

100% formamide and heat to 70–80°C for 5 min then add an equal volume of hybridization buffer and immediately put 10 µL on each slide. Proceed to step 10 of the hybridization protocol (**Section 3.2.3**).

16. We have looked at slides a year later and been able to observe signals similar to when they were first prepared.
17. It is critical to note that the reliable detection of pairing requires the comparison of homologous and nonhomologous distances; in the absence of information regarding nonhomologous distances, the only information available is the percentage of nuclei exhibiting one spot, versus two spots, i.e. so-called “one-spot/two-spot analysis.” This value is highly variable according to spreading conditions and, in any case, formally, the relationships between homologous signals cannot be inferred to reflect pairing in the absence of a control.

In this regard, an important issue concerns the possible contributions of nonspecific interchromosomal interactions (e.g. telomere or centromere clustering; see Refs. 1, 2, 10). In principle, spreading should (and in our protocol does) disrupt these interactions. Nonetheless, in an ideal case, nonhomologous probes should be localized equi-distant from their respective centromeres and telomeres to control for the effects that these interactions have on global chromosome localization (11).

Some FISH studies of spread yeast chromosomes have failed to detect “recombination-independent” pairing, e.g. in yeast *spo11* mutants (12). Lack of extensive spreading may account for this discrepancy. Some FISH studies of whole cells have also failed to identify recombination-independent pairing (13). This may be attributable to the fact that the signal (distances between loci) expected for whole cells is extremely small. As discussed in detail elsewhere (2), the yeast nucleus is small, the chromosomes are highly flexible and very mobile, and pairing contacts are very far apart. Given these factors, the relative local concentrations of homologous sequences are normally very small, only ~two-fold higher than that of comparable nonhomologous loci. We note, however, that, despite the limitations of “one-spot/two-spot” analysis, studies of fluorescent repressor/operator arrays also provide evidence for pairing of homologs at t = 0 of meiosis in intact whole cells (14).

An important additional caveat of FISH analysis is that the actual level of pairing signal depends not only on the frequency of pairing contacts but also on the level of chromosome stiffness. This is because a pairing contact at one position will influence pairing levels at nearby positions to a greater extent if the chromosome is stiff than if it is highly flexible. Thus, if the probed locus is far away from a point of

contact, the level of pairing will be higher if the chromosome is stiffer than if it is more flexible. This consideration is not so important for recombination-independent pairing, which tends to occur in situations where axial chromosome organization is minimal. However, it can be very important for analysis of “homolog juxtaposition” (pairing in the generic sense) during meiotic prophase, because chromosome stiffness increases progressively during this period. Thus, for example, when FISH studies of spread chromosomes identify defects or delays in “pairing” of homologs compared to wild type, it must be kept in mind that the identified defects could be entirely, or in part, due to defects in development of axial stiffness.

Finally, one needs to keep in mind that, after meiotic DNA replication there are four copies of each locus present in the nucleus. There are many proteins involved in sister chromatid cohesion. While wild type maintains its sister chromatids in close contact (1) many mutant strains may have up to four distinguishable spots (15). In these cases it is very difficult to determine whether homologous sisters still interact.

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Chapter 4

Live-Cell Fluorescence Imaging of Meiotic Chromosome Dynamics in *Schizosaccharomyces pombe*

Haruhiko Asakawa and Yasushi Hiraoka

Abstract

The fission yeast *Schizosaccharomyces pombe* has provided a useful experimental system to study nuclear structures during meiosis. Unlike many higher animals in which meiosis takes place only in specialized tissues deep inside their bodies, *S. pombe* is a unicellular eukaryote and its meiosis can be induced simply by depleting nitrogen sources from the culture medium. The entire process of meiosis is completed within several hours, and thus can be followed in individual living cells. These features provide ease of microscopic observation. A more trivial merit is its rod-like cell shape, which aids microscopic observation, as the long axis of cells is kept in the microscope image plane. Here we describe methods for induction of meiosis and fluorescence microscopy observation in living cells of *S. pombe*.

Key words: Fission yeast, meiosis, chromosome, live-cell imaging.

1. Introduction

S. pombe cells are maintained in the haploid state during the vegetative growth stage of their life cycle. Upon nitrogen starvation, haploid cells of opposite mating types (h^+ and h^-) conjugate to form a diploid nucleus through karyogamy. Following cell conjugation, the process of meiosis normally begins immediately without intervening mitotic cycles (zygotic meiosis). On the other hand, under the selective pressure of some auxotrophic markers, cells can also be maintained in the diploid state; nitrogen starvation induces meiosis in a diploid cell (azygotic meiosis). Upon entering meiosis, zygotic or azygotic, the nucleus elongates and migrates between the cell poles, showing the “horsetail” nucleus, and

undergoes two rounds of nuclear division to form four haploid spores (Fig. 4.1). These processes of meiosis can be followed in individual living cells using a fluorescence microscope. To visualize chromosome dynamics, various *S. pombe* strains expressing GFP fused to chromatin protein (e.g. histones) are available. Living cells of *S. pombe* are observed by either of two methods: sandwiching the cells between coverslips or by mounting the cells on a glass-bottom culture dish. Time-lapse images are obtained using a computer-controlled microscope system equipped with temperature-control devices.

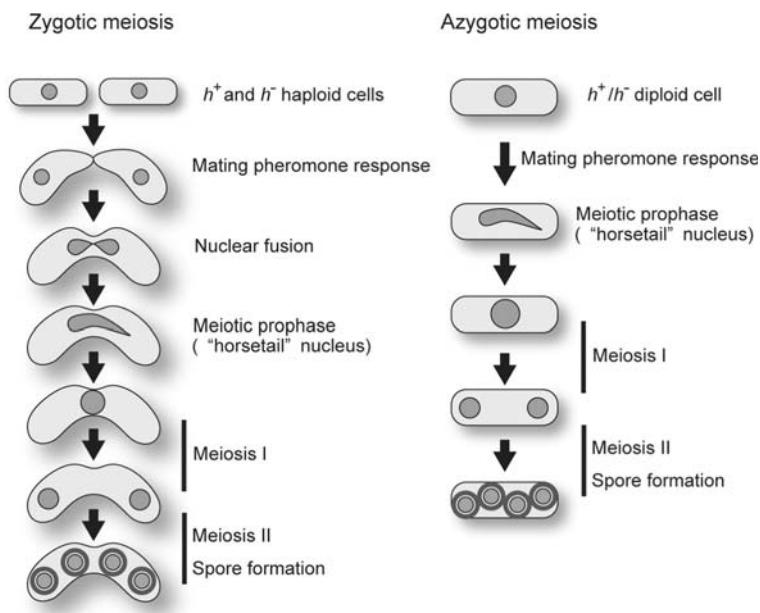


Fig. 4.1. Zygotic and azygotic meiosis in *S. pombe*. (Left) Zygotic meiosis. When starved of nitrogen sources, haploid cells of the h^+ and h^- mating type are arrested in G1, and secrete mating pheromone to the opposite mating type cells. Upon sensing the pheromone, the cell elongates toward the opposite mating type cell and the cells fuse with each other (conjugation), and this is followed by fusion of the haploid nuclei. The fused diploid nucleus then elongates (known as the "horsetail" nucleus), and moves back and forth between the cell ends (meiotic prophase). Meiotic DNA replication occurs at the beginning of this period, yielding a nucleus with 4C DNA content. After movement ceases, the horsetail nucleus becomes rounded at the center of the cell, and proceeds with the first and second meiotic divisions to create four 1C nuclei. (Right) Azygotic meiosis. When starved of nitrogen sources, diploid cells harboring both mating type genes (h^+ and h^-) are arrested in G1 with 2C DNA content, and enter the meiotic process without undergoing conjugation. The mating pheromone response induces expression of mating type genes, and promotes meiosis initiation. Horsetail nuclear movement occurs in azygotic meiosis as in zygotic meiosis, following meiotic DNA replication, which yields a nucleus with 4C DNA content. Two rounds of nuclear divisions results in four 1C nuclei.

2. Materials

2.1. Strains

- Cells that express GFP-fusion of chromatin protein such as histone H3 (*see Note 1*).
- For zygotic meiosis, haploid strains are used (**Table 4.1**; *see Note 2*).
- For azygotic meiosis, diploid strains are used (**Table 4.1**; *see Note 3*).
- For synchronous induction of meiosis, strains harboring *patl-114* temperature-sensitive mutation are used (**Table 4.1**; *see Note 4*).

Table 4.1
S. pombe strains

Strain name	Genotype
Zygotic meiosis	
AY160-14D	<i>b</i> ⁹⁰ <i>ade6-M216 leu1-32 lys1-131 ura4-D18</i>
YY231-6C	<i>b</i> ⁺ <i>ade6-M210 leu1-32 ura4-D18 his2 hbt2</i> ⁺ - GFP::ura4 ⁺
YY231-5C	<i>b</i> ⁻ <i>ade6-M216 leu1-32 ura4-D18 hbt2</i> ⁺ -GFP::ura4 ⁺
Azygotic meiosis	
YN487	<i>b</i> ⁺ / <i>b</i> ⁻ <i>ade6-M210/ade6-M216</i>
Synchronous meiosis	
CRL2412	<i>b</i> ⁻ <i>patl-114 lys1</i> ⁺ :: <i>mat1-Pc</i>
AY1395	<i>b</i> ⁺ <i>ade6-210 leu1-32 ura4-D18 patl-114</i> <i>lys1</i> ⁺ :: <i>mat1-Mc</i>
AY2463d	<i>b</i> ⁻ / <i>b</i> ⁻ <i>leu1-32/leu1</i> ⁺ <i>ura4</i> ⁺ / <i>ura4-D18 patl-114/</i> <i>patl-114 lys1</i> ⁺ :: <i>mat1-Pc/lys1</i> ⁺ :: <i>lacOp his7</i> ⁺ / <i>his7</i> ⁺ ::GFP-lacI

All these strains listed are available from Yeast Genetic Resource Center (YGRCC), Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html).

2.2. Culture Media

- YES medium for routine culture of *S. pombe* cells: 5 g/L yeast extract (DIFCO), 30 g/L glucose, and 0.225 g/L of supplements (adenine sulfate, L-leucine, uracil, L-histidine hydrochloride monohydrate, and L(+)-lysine hydrochloride). For agar plate medium, add 15 g/L agar.

2. EMM2 medium for culture of specific auxotroph strains:
20 g/L glucose, 5 g/L NH₄Cl, 2.2 g/L Na₂HPO₄ (or
5.5 g/L Na₂HPO₄·12H₂O), 3 g/L potassium hydrogen
phthalate, 20 mL/L salt solution (50× stock), 1 mL/L
trace element solution (1,000× stock), 1 mL/L vitamin
solution (1,000× stock), and if necessary, 0.225 g/mL of
each supplement. For agar plate medium, add 15 g/L agar.
3. Salt solution (50× stock): 52.5 g/L MgCl₂·6H₂O, 0.735 mg/
L CaCl₂·6H₂O, 50 g/L KCl, 2 g/L Na₂SO₄. Store at 4°C.
4. Trace element solution (1,000× stock): 0.5 g/L boric acid
(H₃BO₃), 0.4 g/L MnSO₄, 0.4 g/L ZnSO₄·7H₂O, 0.2 g/L
FeCl₂·6H₂O, 0.04 g/L molybdic acid (H₂MoO₄), 0.1 g/L
KI, 0.04 g/L CuSO₄·5H₂O, 1 g/L citric acid. Store at 4°C.

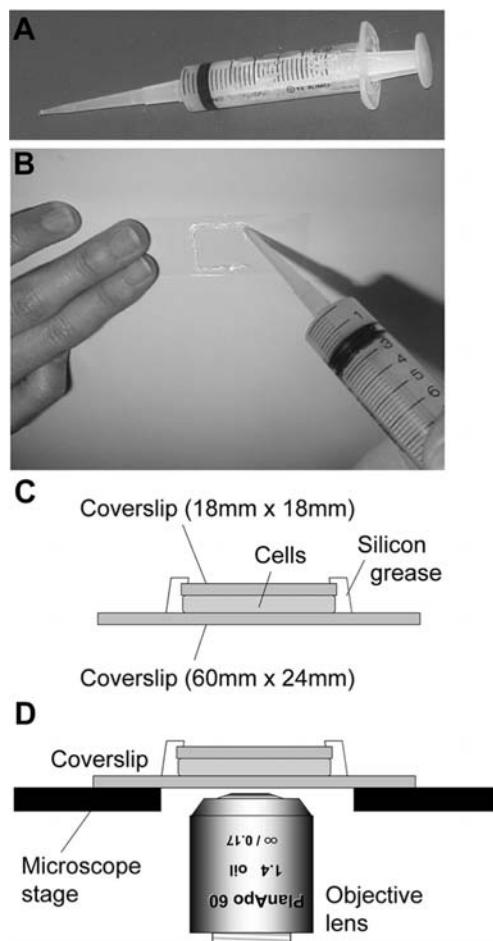


Fig. 4.2. Mounting cells between coverslips for microscopic observation. (A) Syringe packed with silicon grease and capped with a micropipette tip. (B) Sealing the edges of a coverslip with silicon grease. (C) Cells mounted between two coverslips. (D) Microscope observation.

5. Vitamin solution ($1,000 \times$ stock): 1 g/L pantothenic acid, 10 g/L nicotinic acid, 10 g/L myo-inositol, 10 mg/L biotin. Store as 50 mL aliquots in a freezer.
6. ME medium for induction of meiosis: 30 g/L malt extract (Oriental Yeast Co., Japan), 0.225 g/L of each supplement (see YES medium above). For agar plate medium, adjust to pH 5.5 and add 15 g/L agar.
7. EMM2-N medium for induction of meiosis using the *patl-114* mutant strain: same as EMM2 with the exception that NH₄Cl (the nitrogen source) is omitted. EMM2-N is also used for washing cells before induction of meiosis.

2.3. Microscope Sample Preparation

1. Coverslips: 60 × 24 mm and 18 × 18 mm.
2. Silicon grease packed in the syringe: Squeeze a tube of silicon grease into a syringe. Cap the syringe with a micropipette tip that is trimmed at its tip to make an appropriate sized opening (Fig. 4.2A).
3. Glass-bottom culture dish (commercially available, e.g., from MatTek, Ashland, MA) (Fig. 4.3A): Coat a glass-bottom culture dish with concanavalin A or lectin shortly before use. Spread 50 μL of 2 mg/mL concanavalin A or 0.2 mg/mL soybean lectin on the glass bottom of the dish, remove excess solution and dry.

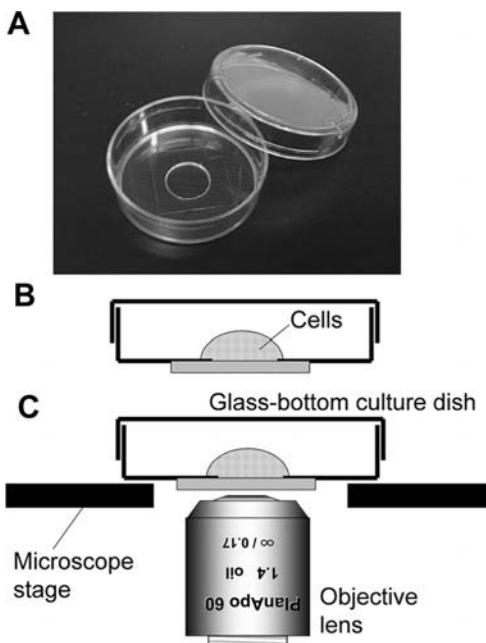


Fig. 4.3. Mounting cells in a glass-bottom culture dish for microscopic observation. (A) A glass-bottom culture dish. (B) Cells mounted in a glass-bottom culture dish. (C) Microscope observation.

3. Methods

3.1. Zygotic and Azygotic Meiosis

3.1.1. Vegetative Growth (Preculture)

1. Isolate single colonies by streaking the desired strain on a YES plate. Use an EMM2 plate when auxotrophic selection is applied. It is recommended that long-term culture on EMM2 plate should be avoided, as it induces cells to enter mating and meiosis.
2. Incubate at 26–33°C for 1–4 d.
3. Pick a single colony and suspend in 5–10 mL YES liquid medium.
4. Incubate at 26–33°C with vigorous shaking until the culture reaches $0.2\text{--}5 \times 10^7$ cells/mL.
5. The generation times of *S. pombe* haploid cells are shown in **Table 4.2**.

Table 4.2
Generation times for haploid strains of *S. pombe*

Medium	Temperature (°C)	Generation time (h)
YES	20	~6
	25	3
	29	2.5
	32	2.2
	35.5	2
EMM2	20	~8
	25	4
	29	3
	32	2.5
	35.5	2.3

Partly adapted from Ref. 13.

3.1.2. Mating and Zygotic Meiosis

1. Use b^{90} cells, or mix b^+ and b^- cells at Step 7 below (see **Table 4.1**).
2. Prepare a preculture of the cells as described in **Section 3.1.1**.
3. Harvest the cells by centrifugation for 5 min at 1,200*g* (or 3,000 rpm in a benchtop centrifuge), and discard the supernatant.
4. Resuspend the pellet in EMM2-N.

5. Harvest the cells by centrifugation for 5 min at 1,200*g* (or 3,000 rpm in a benchtop centrifuge), and discard the supernatant.
6. Repeat Steps 4 and 5.
7. Resuspend the pellet in 0.2–0.5 mL EMM2-N. When *b*⁺ and *b*[−] cells are prepared for zygotic meiosis, combine them in one tube with EMM2-N at this step.
8. Drop the cell suspension onto an ME agar plate and leave for a few minutes until the EMM2-N liquid medium is absorbed by the plate.
9. Incubate the plate overnight at 20–26 °C.

3.1.3. Azygotic Meiosis

1. Use *b*⁺/*b*[−] diploid cells (see Table 4.1).
2. Prepare a preculture of the cells as described in Section 3.1.1.
3. Harvest the cells by centrifugation for 5 min at 1,200*g* (or 3,000 rpm in a benchtop centrifuge), and discard the supernatant.
4. Resuspend the pellet in EMM2-N.
5. Harvest the cells by centrifugation for 5 min at 1,200*g* (or 3,000 rpm in a benchtop centrifuge), and discard the supernatant.
6. Repeat Steps 4 and 5.
7. Resuspend the pellet in 0.2–0.5 mL EMM2-N.
8. Drop the cell suspension onto an ME agar plate. Leave for a few minutes until EMM2-N liquid medium is absorbed by the plate.
9. Incubate the plate overnight at 20–26°C.

3.2. Synchronous Induction of Meiosis

1. Isolate a single colony by streaking the appropriate strain on a YES plate. Use an EMM2 plate when auxotrophic selection is applied. It is recommended that long-term culture on EMM2 plate should be avoided.
2. Incubate below 26°C for 2–4 d.
3. Pick a single colony and suspend in 5–10 mL YES liquid medium.
4. Incubate at 26°C with vigorous shaking until the culture reaches 2–5 × 10⁶ cells/mL.
5. Harvest the cells by centrifugation for 5 min at 1,200*g* (or 3,000 rpm in a benchtop centrifuge), and discard the supernatant.
6. Resuspend the cell pellet in EMM2-N liquid medium.
7. Harvest the cells by centrifugation for 5 min at 1,200*g* (or 3,000 rpm in a benchtop centrifuge), and discard the supernatant.
8. Repeat Steps 6 and 7.

9. Resuspend the cells in EMM2-N at a density of 2×10^6 cells/mL.
10. Incubate the cells with vigorous shaking at 26°C for 15 h to induce G1 arrest (*see Note 5*).
11. Mount the cells on a glass-bottom culture dish (*see Section 3.4 below*).
12. Shift up the culture temperature to 34°C on a microscope stage.

3.3. Sample**Preparation for Short-Term Observation
(Sandwiching Between Coverslips)**

1. Suspend cells in EMM2-N liquid medium (*see Note 6*).
2. For short-term observation (1–2 h or less), cells can be mounted between coverslips. Place 2.5 µL of cells in EMM2 liquid medium on a large coverslip (60 × 24 mm), and cover with a small coverslip (18 × 18 mm).
3. Gently remove any excess fluid with a piece of lens paper or paper towel (fluorescence-free).
4. Seal the four edges of the smaller coverslip with silicon grease (**Fig. 4.2B, C**) (*see Note 7*).
5. Place the specimen on a microscope stage for observation (**Fig. 4.2D**).

3.4. Sample**Preparation for Long-Term Observation
(Glass-Bottom Culture Dish)**

1. For long-term observation (more than 2 h), cells must be mounted in a glass-bottom culture dish because the availability of nutrition and oxygen is limited when mounted between coverslips.
2. Prepare a glass-bottom culture dish as described in **Section 2.3**.
3. Suspend cells in EMM2-N (*see Note 6*).
4. Place 50 µL of cells in EMM2-N medium on the glass bottom of the culture dish (**Fig. 4.3B**).
5. Place a few drops of water or a few pieces of moist paper in the culture dish. Put a lid on the dish and seal with Parafilm.
6. Place the specimen on a microscope stage for observation (**Fig. 4.3C**).

3.5. Live-Cell Observation

1. Start up a computer-controlled fluorescence microscope system (*see Note 8*).
2. Adjust the temperature of the microscope atmosphere to an appropriate setting (*see Note 9*).
3. Place the specimen on the microscope stage and leave the specimen for 30 min before image data acquisition to allow the specimen to settle down and to reach the observation temperature.
4. Run image acquisition software to obtain time-lapse image data (**Fig. 4.4** and *Supplemental movie available on the companion CD for this volume*).

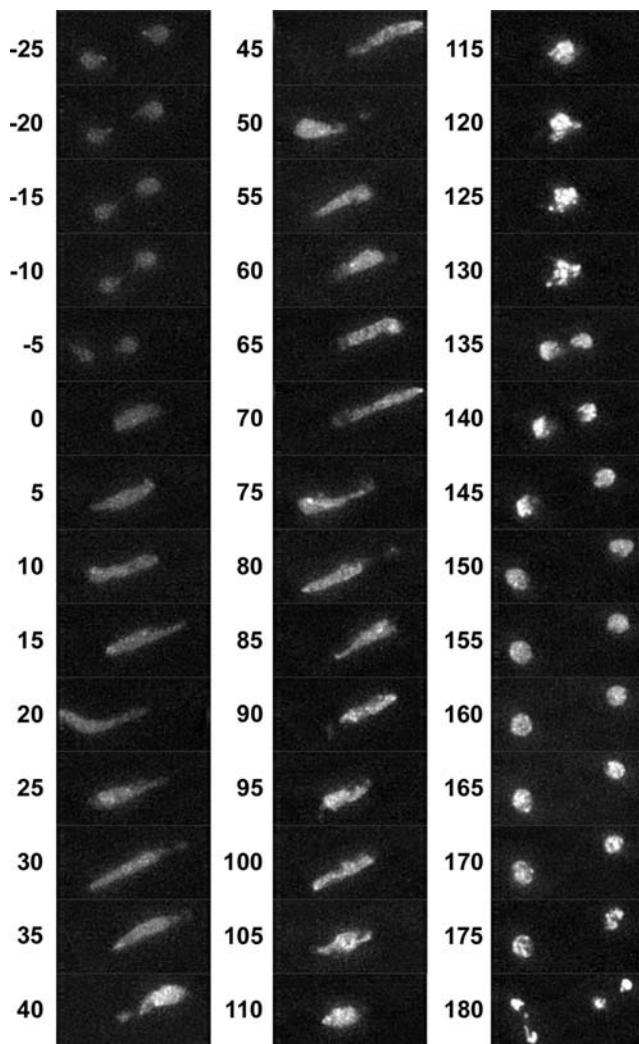


Fig. 4.4. Live-cell imaging of chromosomes during meiosis. Chromosomes were observed during a process of zygotic meiosis using *S. pombe* cells expressing GFP-fused histone H3. Haploid cells of the opposite mating types (YY231-5C and YY231-6C) were mixed for mating as described in **Section 3.1.2**. A single zygote was observed at 26°C on a DeltaVision microscope system. Time-lapse images were obtained at 5 min intervals. Numbers on the left indicate the time in minutes: time 0 represents the time of nuclear fusion. Adapted from Ref. 8. A movie of chromosome dynamics is available on the companion CD for this volume.

4. Notes



1. To visualize chromosomes, GFP fusions of histone proteins are widely used (1, 2). Alternatively, a DNA-specific fluorescent dye, Hoechst 33342, can be used to doubly stain with other GFP fusion proteins (3, 4). Libraries of GFP- or

YFP-fusion constructs are also available in *S. pombe* (5, 6). The LacO/LacI-GFP system is useful to follow specific chromosomal loci in living cells (7, 8).

2. The most convenient way is to use *h*⁹⁰ mating type strains. Strains harboring the *h*⁹⁰ mating type can switch their mating type between *h*⁺ and *h*⁻. Thus, they are a mixture of *h*⁺ and *h*⁻ mating type cells. *h*⁹⁰ strains are therefore homothallic for mating, even if they grow from a single colony. A combination of *h*⁺ and *h*⁻ strains can also be used to induce zygotic meiosis. Standard strains are available from strain stock centers.
3. Diploid cells are distinguishable from haploid cells by microscopic observation, because diploid cells are longer and wider than haploid cells, and their nuclei are also larger than haploid nuclei. Some standard diploid strains are available from strain stock centers. Alternatively, diploid strains can be made by crossing haploid *h*⁺ and *h*⁻ mating type strains of specific genetic backgrounds (e.g. YY231-6C and YY231-5C, in Table 4.1). In brief, mix *h*⁺ and *h*⁻ mating type strains on sporulation medium, incubate overnight, and spread the mixture of cells onto a selective medium. To maintain the cell ploidy during a course of experiments, most diploid strains harbor *ade6-M210/ade6-M216* (often described as *ade6-210* and *ade6-216*), alleles of the *ade6*⁺ gene. The *ade6-M210* and *ade6-M216* alleles intragenetically complement each other, and thus diploid cells harboring *ade6-M210* and *ade6-M216* can be isolated by incubating on a selective medium that is adenine deficient.
4. Cells harboring the *pat1-114* mutation start meiosis upon temperature-shift to the restrictive temperature (Pat1 is a negative regulator of meiosis) (9, 10). It should be noted that meiosis induced using the *pat1-114* mutation shows aberrant chromosome segregation in meiosis I. This aberrant segregation is complemented by expressing an opposite mating type gene that is integrated into an ectopic chromosomal locus (i.e. *mat1-Pc* for *h*⁻, and *mat1-Mc* for *h*⁺ mating type strains) (7, 11; reviewed in Ref. 12). Thus, the introduction of an opposite mating type gene is recommended for leading to the normal process of chromosome dynamics in the *pat1-114* background. The addition of a mating type gene is also effective for achieving the best possible synchrony. The time course of meiotic events in synchronous culture using *pat1-114* mutation is shown in Table 4.3.
5. Cells of *pat1-114* can enter meiosis irrespective of their ploidy and cell cycle phase. Thus, cells must be arrested in G1 before the initiation of synchronous meiosis. To arrest cells in G1 phase, cells are cultured overnight in nitrogen-free medium (EMM2-N, see Materials). Diploid cells harboring *h*^{+/h⁻ mating type genes enter meiosis at this preculture step, and}

Table 4.3
Time course of meiotic events in synchronous culture using cells with the *pat1-114* mutation

Mating types	Genetic backgrounds	Times after induction of meiosis			References
		DNA replication	Meiosis-I	Meiosis-II	
<i>b</i> ⁺	<i>pat1-114</i>	3 h	7 h	8 h	(7)
<i>b</i> ⁻ / <i>b</i> ⁻	<i>pat1-114/pat1-114</i>	2–3 h	6–7 h	7–8 h	(7), (11)
<i>b</i> ⁻	<i>pat1-114 mat1-Pc</i>	1–2 h	5 h	6	(2), (7)

therefore *b*⁻/*b*⁻ diploid cells are used for diploid *pat1-114* meiosis. Diploid cells homozygous for mating type genes (*b*⁺/*b*⁺ or *b*⁻/*b*⁻) are generated by a protoplast fusion method (14).

6. For microscopic observation, use an optically clear and colorless medium such as EMM2-N. Use of a turbid medium disturbs microscopic observation.
7. Do not use nail enamel to seal a coverslip as organic solvents in the nail enamel can be toxic to the cells.
8. Several fluorescence microscope systems capable of live-cell imaging are commercially available: for example, DeltaVision (Applied Precision, Inc., Washington, Seattle) or Leica AF6000.
9. For temperature control, our microscope system is kept in a temperature-controlled room, and the microscope is controlled remotely from outside the room (4). Alternatively, various types of temperature-control equipment are commercially available. Choice of such equipment requires careful consideration to achieve accurate temperature control during observation (15).

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Chapter 5

Time-Lapse Fluorescence Microscopy of *Saccharomyces cerevisiae* in Meiosis

Michael E. Dresser

Abstract

Movements are implicit in the chromosome behaviors of bouquet formation, pairing and synapsis during meiotic prophase. In *S. cerevisiae*, the positions of chromosomes, specific structures, and individual chromosomal loci marked by fluorescent fusion proteins are easily visualized in living cells. Time-lapse analyses have revealed rapid and varied chromosome movements throughout meiotic prophase. To facilitate the analysis of these movements, we have developed a simple, inexpensive, and efficient method to prepare sporulating cells for fluorescence microscopy. This method produces a monolayer of cells that progress from meiosis through spore formation, allows visualization of hundreds of cells in a single high-resolution frame and is suitable for most methods of fluorescence microscopy.

Key words: Meiosis, fluorescence microscopy, live cell, time-lapse analysis, yeast, *S. cerevisiae*.

1. Introduction

Fluorescence microscopy is on course to rival electron microscopy in providing biologists with capabilities more limited by sample preparation than by the inherent resolution of the instruments themselves. Under the right conditions, a single fluorescent molecule or point source, adequately sampled, can be localized to well within 10 nm in the XY (focal) plane (1) and to within 30 nm along the Z (focusing) axis using currently available equipment [R. Ober, personal communication, using multi-focal plane imaging (2)]. “Adequate” sampling generally means collecting relatively large numbers of photons, which can be time-consuming and inappropriate for time-lapse acquisitions but with sufficient care makes it possible to determine the numbers of proteins in

individual structures (3). However, improved detector sensitivities and geometries, faster digital data flow, better-regulated illumination, higher duty cycle and wavelength-controlled fluorochromes, improved algorithms for processing and analyzing images and faster, higher precision mechanical components all are developing quickly and are being packaged in ever more clever combinations that steadily are improving the resolution gained from time-lapse analyses.

Paralleling the technological achievements are improvements in the cells themselves. This is nowhere more prominent than in the yeast *S. cerevisiae*, where fluorescent fusion proteins routinely are used in place of wild-type proteins and cell characteristics are tailored to support an amazing range of experimental designs. The flexibility of approach provided by yeast continues to draw those interested in the most durable questions of cell biology, a class that includes meiotic chromosome behavior.

Chromosomes behave as they do in first meiotic prophase in order to haploidize the genome; this has been evident for more than a hundred years. However, it has been difficult to address the details of meiotic chromosome behavior which lead to appropriate chromosome orientation in the first division, i.e., the molecular mechanisms, structures and activities which generate and regulate bouquet formation, pairing, synapsis, and recombination. This is true particularly for behaviors in early prophase, before chromosomes have formed the distinct axes that have helped microscopists to sort out later events.

Recent results from yeast indicate that crossover regulation occurs prior to or coincident with synapsis (4–6), underscoring the importance of understanding early meiotic prophase chromosome behavior. Providing enormous help in this regard is Belmont's method of tagging specific chromosome loci with fluorescent spots (7) which quickly was shown by the Murray lab to enable analysis of chromosome behaviors that previously had not been accessible (8). Functional fluorescent markers of telomeres (9), centromeres (10), chromosome axes (11), synaptonemal complexes (12, 13), recombination complexes (14), spindle pole bodies (15), nuclear pores (16), microtubules (17), actin filaments (18) and more, provide context and internal controls for the behavior of the individual chromosome loci and for one another. Given a specific biological question that can be addressed using some combination of fluorescent markers, the questions become how best to carry out image capture, processing, and analysis.

The method described here for sample preparation is based on a method developed for *Dictyostelium discoideum* (19) and is designed to support time-lapse microscopy of sporulating *S. cerevisiae* using simple materials and standard upright fluorescence microscopes (although it should be a simple matter to adapt this method for use on inverted microscopes). With a little

practice, the method produces a monolayer of nonflattened cells adjacent to the coverslip, where spherical aberration is minimized when using oil-immersion optics to visualize cells in an aqueous medium. This method also can be used to prepare samples of fluorescent protein-tagged whole cells for high-resolution microscopy, where multiple focal planes are acquired using confocal microscopy or wide-field microscopy followed by deconvolution; these samples typically are briefly fixed in formaldehyde at a final concentration of 2% and rinsed quickly to minimize loss of fluorescence while still preventing intracellular movements during imaging. The advantage of the monolayer is that large numbers of cells can be captured in a single frame without interfering signal from overlapping cells (**Fig. 5.1**).

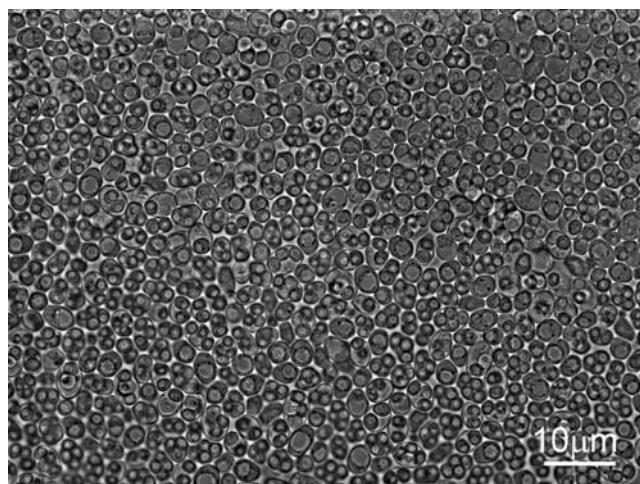


Fig. 5.1. Bright-field image of a field of cells that finished sporulation under the agarose pad following fluorescence time-lapse imaging during meiotic prophase.

2. Materials

1. Coverslips. Treat coverslips (24 × 50 mm, #1.5 thickness) just before use with polyethylenimine (PEI) to make the surface sticky (*see Note 1*). Make a stock of 0.5% PEI by diluting 50% polyethylenimine 1/100 in water; this stock can be kept in a brown bottle at room temperature for several weeks. Spread approximately 5 µL of 0.5% PEI across each coverslip using the tip of the pipette; if the PEI does not spread evenly, discard the coverslip. Rinse treated coverslips immediately with a stream of distilled water and allow to air dry.
2. Gaskets. Reusable perfusion chambers (Electron Microscopy Sciences, 70326-26) are thick-sided, rectangular silicone

gaskets with flat tops and bottoms that form a tight seal when pressed between a coverslip and a clean slide. Wipe the gaskets to clean between uses and re-use multiple times. The relatively large volume of air trapped in the chamber is important to allow completion of sporulation.

3. Agarose pads. Prepare thin strips from the end of a #1.5 coverslip by first drawing a line with a diamond-tipped marker (it helps to use a ruler), then snapping the strip free. Place a strip at each end of a clean glass microscope slide and glue in place using clear nail polish. These strips act as spacers to set the thickness of the final agarose pads. Prepare a solution of 1% agarose (Fisher, Low EEO Electrophoresis Grade, BP160) in pH 7.4 phosphate-buffered saline (PBS) and heat to dissolve the agarose. Place 1 mL of the agarose solution between the spacers on the slide and press a second clean microscope slide over the first, so that when cooled there is a thin, flat layer of 1% of agarose between the slides. Trim the agarose layer along the sides with a clean razor blade, separate the slides, and cut the agarose pad into ~3 mm² pads by chopping with the razor blade. Store the pads in a Petri dish in PBS at 4°C for up to a month, barring contamination.
4. Strains. Most fluorescent marker proteins are produced by stable gene replacements and require little maintenance. However, the long DNA concatemers that are the basis for marking specific chromosomal loci (7) are susceptible to recombination events that can reduce the number of repeats. Although expression of lacI-GFP from a meiosis-specific promoter generally is adequate to visualize spots on chromosomes in meiotic prophase, it can be helpful additionally to express lacI-GFP using a promoter that is active during vegetative growth so that several cell isolates can be checked for spot brightness (related to number of repeats) prior to sporulation and to identify appropriate haploid cells generated in crosses. Alternatively, meiosis-specific expression alone allows one to ignore nonmeiotic cells. Where this is desired, it is a good idea to use several isolates in a single experiment.

3. Methods

3.1. Sporulation Conditions

Prepare sporulating cells using standard conditions, for example in vigorously aerated cultures at 30°C, at a concentration of 1×10^8 cells/mL in 1% potassium acetate supplemented as required by auxotrophs. Proper growth and handling of cells prior to the

shift into sporulation medium is critical for fast, efficient sporulation and varies with strain background (20). Detailed methods can be found in Chapters 9, 10, 14, 17, and 18 in this volume.

3.2. Preparation of Cell Monolayer

1. Sample cultures just prior to imaging, to keep the kinetics of sporulation as close to standard as possible. Place 0.5 mL aliquots of culture in plastic microfuge tubes and centrifuge for 5–8 s, as gently as possible, to make a soft pellet. Remove supernatant to a separate tube and resuspend the cells by tapping the tube (*see Note 2*).
2. Remove an agarose pad from the stock by picking it up on the tip of a thin spatula (a second spatula is helpful in manipulating the pads). Replace the PBS with sporulation medium by adding a drop of the saved culture medium to the pad and then draining by touching a corner of the pad to a laboratory tissue (Kimwipe); repeat the add/drain two more times. Alternatively, soak pads briefly in pre-conditioned sporulation medium (*see Note 3*).
3. Place 5 μ L of cell suspension on the coverslip in the area treated with PEI and spread the suspension slightly with the pipette tip. Place the agarose pad over the top of the cell suspension. Remove excess suspension by blotting two or three times, gently, with a tissue. This part takes practice; too much medium and the pad and/or cells drift; too little medium and the cells become flattened. Three to six preparations can be made on a single coverslip and, if spaced and blotted appropriately, will remain separate throughout the imaging. Place a gasket on a clean slide, add a drop of sporulation medium to the chamber (*see Note 4*), invert the coverslip with pads and cells over the chamber and press gently along the edges to seal the chamber (**Fig. 5.2**).

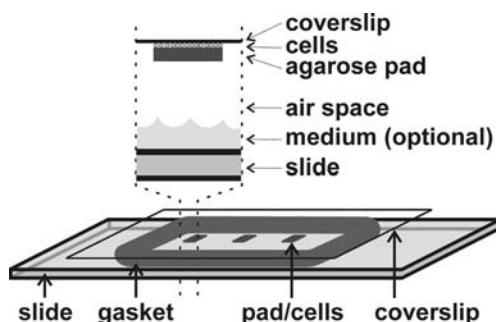


Fig. 5.2. Diagram of assembled chamber with cells trapped between cover slip and agarose pad.

3.3. Image Acquisition

1. *Temperature and cell behavior.* The effects of temperature are more subtle than a simple shortening of the duration of meiotic prophase at higher temperatures [see (6)], so it is important to control temperature carefully. Microscope-based temperature control may be required if it is necessary to carry out the imaging at specific temperatures, for example, to assess the effects of temperature-sensitive alleles. Heating (e.g., to 34°C) is easier than cooling (e.g., to 17°C), and controlled enclosures generally offer the best microscope and temperature stability. Absent of such a requirement, the simplest approach is to find a temperature from 25 to 30°C at which the microscope room can be held, despite seasonal changes and after factoring in heat from the microscope equipment and microscopists. If the microscope room is small enough, an inexpensive space heater can supply sufficient heat to hold the pre-determined “standard” temperature.
2. *Illumination and cell viability.* *S. cerevisiae* is relatively susceptible to damage by the illumination used in fluorescence microscopy, so it is important to minimize illumination intensity and the numbers and lengths of exposures. Shorter exposures improve the resolution of moving objects and conventional wisdom in the field is that the cells themselves may be less sensitive to higher intensity/shorter exposures than to lower intensity/longer exposures. A standard claim is that if the cells continue as expected after image acquisition, for example by finishing sporulation, then the imaging captured “normal” behavior. A better indicator for the absence of phototoxic effects is that movements are the same whether captured early or late in a time-lapse acquisition. Another reason for reducing exposures as much as possible is to minimize fading of the fluorescence signal. Fading can be corrected to some extent (or ignored), unlike changes in illumination intensity during acquisition of a time-lapse series or of an image stack. Illumination changes due, e.g., to lamp flicker, are difficult to eliminate entirely but must be minimized as they cause problems in down-stream image processing.
3. *Spatial resolution in the plane of focus.* In digital imaging-based microscopy, spatial resolution in the XY plane (the “plane of focus”) is determined mainly by the numerical aperture of the objective and by the spatial frequency at which the object is sampled. The latter is set, for example, by the point-to-point distance for scanning microscopes or by the optical magnification and pixel spacing on camera chips. Theoretical considerations indicate that the optimal pixel-to-pixel spacing should represent ~0.09 µm in the sample in raw

images made using a microscope objective with a numerical aperture of 1.4 (the current standard for good oil-immersion 63X or 100X objectives) and a digital camera with square pixels (21); practical considerations indicate that sampling should be at least three times finer than the details to be resolved and thus, for best resolution, closer to $\sim 0.06\text{ }\mu\text{m}$. Intermediate optics, between the objective and camera, can be used to change the final magnification to match this optimum, usually at the cost of some of the signal. Undersampling, usually the result of using a camera with large pixels, reduces resolution but can reduce the effects of noise inherent in single molecule detection. Oversampling, where the pixel spacing represents less than $0.06\text{--}0.09\text{ }\mu\text{m}$, often produces images with a more pleasing, less pixilated appearance; this is particularly noticeable where images are enlarged without interpolation for presentation or publication (*see Note 5*), and only interferes with downstream processing if the signal per pixel is too low (*see Note 6*). Oversampling also reduces the size of the field, which can limit the numbers of cells that can be analyzed.

4. *Spatial resolution along the focusing, Z, axis.* This is a complicated subject but involves similar trade-offs—finer spatial sampling improves resolution but exposes the cells to more illumination. Generally, there are two issues. The first issue is that out-of-focus signal blurs a single-plane image. Blur reduces contrast, which makes identification of individual objects more difficult, particularly for automated systems. To generate a crisper, higher contrast image, confocal microscopes most commonly use one or more pin-holes to reject the out-of-focus signal, which “wastes” photons that contain information about the distribution of signal in the sample. Wide-field (standard) microscopes capture these photons but, in order to produce images with similar contrast, require the acquisition of a series of images along the focusing axis to produce an image stack, or volume, which then is deconvolved to reduce the blur. The second issue is that objects of interest generally are spread out in all three physical dimensions, including along the focusing axis. Whether the specific goal is to detect all the fluorescent objects in the cell, to watch an object as it moves across planes during a time-lapse series or to quantify distances moved or between objects, it generally is necessary to acquire an image stack, whether using confocal or wide-field microscopy. The critical question then becomes how far to change the focus between images in the stack. In time-lapse experiments, capturing a complete image volume before there is movement that interferes with analysis generally sets the limit for possible resolution in Z. For fluorescent objects in yeast cells visualized using a

wide-field microscope, a starting Z spacing of 0.4–0.7 μm between each of the 7–10 focal planes per volume is a reasonable starting point for attempts to follow single objects through time. For best resolution, and for 3D images that appear well-resolved even when rotated for viewing information spread along the Z-axis, using a Z spacing as close as 0.1 μm in a volume composed of 50–60 images may be required, followed by deconvolution even of confocal images. This level of resolution is difficult for live cell microscopy using current equipment (but see discussion below).

5. *Temporal resolution.* The rate at which successive images must be acquired in order to measure the velocity of an object often depends on factors that cannot be known in advance – how fast, how far, how continuously and along what path the object moves. Even given a straight path, movement can be smooth or saltatory, directed or frequently reversing, and so forth. A related issue is the need to follow individuals in a field of similar objects, where too much movement between frames confuses identification. Clearly, the more rapidly successive frames are acquired, the more detail is possible in the analysis—and, unfortunately, the lower the signal/noise ratio for a given level of illumination. As with many experimental designs, a good starting place is to assess the limits then pick standard experimental parameters. At this point it helps to conceive of the microscope as a means of detecting rather than of photographing, i.e., to acquire images at the lowest *reasonable* signal/noise ratio, physical resolution, frame size, and number in order to capture images as rapidly and long as possible before fading or cell damage intrude (*see Note 7*). These images are likely to be visually unappealing even with processing but will indicate movements that can be detected and will guide the questions that can be asked. It is important to keep in mind that, as with physical location, some temporal information likely lies below the limits of resolution. Intermittent activities can be particularly problematic if they involve relatively fast movements that occur in a background of slower movements that are being visualized by long time-courses with long between-acquisition times. As automated acquisition becomes more sophisticated, routines using machine vision to recognize when to change acquisition rates will help to resolve the details of movements that occur during infrequent bursts of activity.
6. *Confocal microscopy.* The blur from out-of-focus objects obscures details of in-plane movements or the individuality of separate objects. Laser-scanning confocal microscopes reject out-of-focus light mechanically to reduce this problem. Where the diameter of the pinhole that rejects the light can be

varied, single images capture fluorescent signal from a focal slab that gets thinner as the pinhole diameter is reduced. The limitation, that objects disappear when they move out of focus, can be circumvented in a single-focus time series simply by limiting measurements to movements that occur in the focal plane/slab, i.e., orthogonal to the direction of focus (22, 23). Nevertheless, collection at multiple focal planes/slabs at each acquisition in a time-lapse experiment, i.e., 4D microscopy, has become the standard approach (24). Spinning disk confocal microscopy, which uses a CCD camera instead of a photomultiplier tube to capture signal, similarly rejects out-of-focus signal mechanically and is a user-friendly alternative to using scanning confocal to visualize live cells (for example, *see* (25); Fig. 5.3). Newer, more sensitive detectors, more rapid scanning devices and clever approaches to increase resolution by decreasing the effective illumination spot size [for example, *see* (26)] combine to continually reset the limits of confocal microscopy. Coupled with the ability to analyze small volumes of the sample individually [via fluorescence correlation spectroscopy; for example, *see* (27)], these advances insure that confocal microscopy will continue to contribute to live-cell imaging.

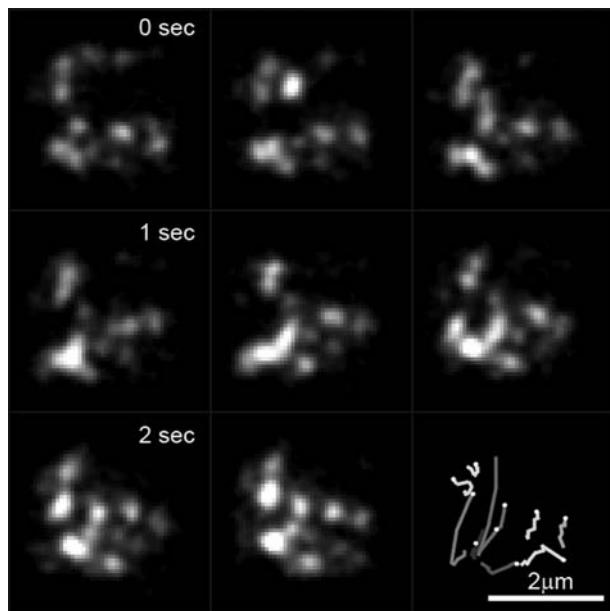


Fig. 5.3. Time-lapse analysis of telomere movements during meiotic prophase using spinning disk confocal microscopy. Telomeres tagged by Mps3-GFP and Rap1-GFP were visualized at the “top” surface of a single nucleus in images acquired at 3 frames per second. Traces of individual spots (each of which represent multiple telomeres) are shown at lower right.

7. *Wide-field microscopy.* Because no photons are purposely rejected, wide-field microscopy is inherently more sensitive than confocal at capturing live-cell fluorescence (which generally is limited by the signal-to-noise ratio). More laboratories have regular access to nonconfocal instruments which, given efficient light transmission from the cell to the camera, can be outfitted as needed for live-cell imaging. Typical upgrades include a sensitive camera, a computer designed to handle large amounts of image data quickly, flexible user-friendly acquisition software, automated focusing and shuttering, and filter sets (or equivalents) to select illumination and fluorescence emission wavelengths. The choice of equipment must be tailored to the particular experimental question.

3.4. To begin

Preliminary results gained from sub-optimal equipment can guide experimental design (*see Note 8*) and indicate the sort of equipment needed for improved results.

1. *Where objects can be modeled as point sources.* A fully implemented system can produce remarkably detailed results, particularly if the objects being visualized can be modeled as point sources (for example, as provided by a concatemer of DNA binding sites or a spindle pole body) and the analytical approach is sophisticated (28). Fluorescence speckle microscopy (29), where a small fraction of the subunits of a large macromolecular structure are fluorescently labeled and decorate the structure with local peaks of fluorescence, can provide detailed information about movements and flow in larger structures such as spindles and actin networks by providing localized, point source-like detail (30). In the extreme, images can be constructed solely from signals from single fluorescent molecules with the result that relatively large structures can be resolved on the nanometer scale (31) and movements of single molecules within multiple ensembles can be tracked in time-lapse experiments (32).
2. *Narrowing the focal slab.* Total internal reflection microscopy (TIRF) reduces out of focus blur by illuminating only those parts of the cell which are within 50–80 nm of the coverslip. TIRF provides greater contrast for fluorescence speckle microscopy as well as for more standard visualizations in yeast (33) but obviously is limited to events occurring near the cell wall.
3. *Widening the focal slab.* It is possible to capture the fluorescent signal from all focal levels nearly simultaneously by exposing the camera while moving the sample through focus, i.e., along the Z-axis, at rates of up to 1 $\mu\text{m}/\text{ms}$

(using a standard Zeiss motorized stand), then to deblur the resulting single image by deconvolution (34). Termed “thru-focus” (34) or “optical axis integration” [Applied Precision, Issaquah, WA; for example, see (35)] microscopy, this approach works well with the method for preparing samples described above. Using this method, 4D (XYZT) information is reduced to 3D (XYT), i.e., information about position along the Z-axis is lost (although it can be recovered by making pairs of angled focusing movements). Fluorescent signals must be sufficiently strong and/or movements sufficiently slow to produce images with little blur but, where applicable, this method provides a particularly efficient means of acquiring large numbers of observations [*see Supplemental Movie 1*, reproduced with permission from (36)].

4. *Simultaneous detection at different focal planes.* Where movements occur in a limited distribution along the Z-axis, it is possible to place two or more cameras at different levels of focus in order to capture different focal planes simultaneously. Without changing focus during the time-lapse acquisition, this arrangement makes it possible to visualize and, for approximately point-source objects, to localize objects as they move in all three dimensions (37).
5. *Rapid 4D microscopy.* A standard approach is to collect individual images at a series of focal levels for each frame of a time-lapse dataset, and to deconvolve the image stack to remove blur (38). Acquisition of a stack of images rapidly enough to avoid movement-induced artifacts in the final deconvolved 3D dataset, but at high enough resolution to warrant using this approach, requires careful attention to a large number of details (39). However, improvements in equipment, as outlined in the introduction, make this approach increasingly accessible.

3.5. Post-Acquisition Image Handling

1. *Image storage.* Digital storage capabilities always seem to be one step behind the ability of laboratories to acquire large numbers of huge time-lapse image datasets. Organizing and archiving raw image data is as essential as it is difficult. The Open Microscopy Environment (<http://www.openmicroscopy.org/>) is a collaborative, freely available effort to address these issues as well as several of those outlined below.
2. *Image processing.* Numerous software packages, free and commercial, are available to process images to emphasize information and suppress noise. It is particularly important to understand how the manipulations affect the information so that the images can be processed appropriately; the textbook by Russ is a good place to start (40).

3. *Image visualization.* Fewer software packages are available that support facile visualization of 3D, much less of 4D, image datasets. Microscope manufacturers sell proprietary software which can be quite good, as are the several packages of more broadly aimed commercial and free software [for examples, *see* (41)]. A critical and often overlooked feature is the ability to import and export images in different file formats, as proprietary file formats can defeat the best of plans.
4. *Image analysis.* Even fewer software packages are available that can automate analysis of the huge amounts of data that most live-cell experiments generate. Commercial products tend to be expensive but, if they satisfactorily address the biological question at hand, probably are worth the expense. Much of the work currently being performed is carried out in collaboration with computer scientists who devise and implement purpose-driven algorithms [*see* (42)]. Given the range of methods and questions in combination, this is likely to continue to be the case for some time.

4. Notes



1. The lectin Concanavalin A, applied at ~1 mg/mL in water then dried, has been used similarly to stick yeast cells to coverslips or to the bottoms of flow chambers. Note that if it is required to add chemicals, e.g., the actin depolymerizing drug latrunculin, while imaging, a flow chamber may be simpler to use. With the agarose pad overlay method, such agents must be added during sample preparation. Alternative methods for trapping, treating, and visualizing cells, for example using microfluidics, are now commercially available (for example, *see* <http://www.cellasic.com/>).
2. The final concentration of cells is critical to get a monolayer of cells at the end. Because subsequent steps are susceptible to variation between individual researchers, this requires some practice.
3. An old trick is to use conditioned sporulation medium to improve a subsequent sporulation. Following completed sporulation in liquid medium, cells are pelleted and the supernatant saved at 4°C for up to a week. Note that preparing the agarose pad initially in sporulation medium does NOT work, as heating the medium is deleterious.
4. The purpose of the sporulation medium is to prevent drying of the preparations. Too much medium can cause the gasket to separate from the slide.

5. The use only of noninterpolating image processing is a rule for many journals. A good rule of thumb for enlarging digital images is to increase image size only by integral amounts and to use the “nearest neighbor” algorithm, a noninterpolating means of generating intensity values for the newly created image pixels.
6. The definition of “too low” is that the signal/noise ratio is below what is required for the experiment. More sensitive cameras help but, a note of caution: digital cameras designed to be particularly light-sensitive frequently have pixels that are so large as to require more than $100 \times$ magnification of the sample to produce adequate final resolution and thus require additional optics that may steal signal.
7. Shorter exposures will reduce signal/noise ratio; binning or using camera chips with large pixels without added magnification, to increase signal/noise ratio, will reduce physical resolution; using a small region of interest instead of the whole frame will increase the rate at which each frame is completely transferred from camera to computer but will reduce the numbers of cells captured.
8. Quantification is best but you have to start somewhere.

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Chapter 6

Real-Time Imaging of Meiotic Chromosomes in *Saccharomyces cerevisiae*

Romain Koszul, Sei Kameoka, and Beth M. Weiner

Abstract

Important information on cellular physiology can be obtained by directly observing living cells. The nucleus, and the chromatin within, is of particular interest to many researchers. Monitoring the behavior of specific DNA loci in the living cell is now commonly achieved through the insertion of binding sites for fluorescently tagged proteins at the sequence of interest (e.g. Ref 1). However, visualizing the behavior of full length chromosomes can only be achieved when they constitute discrete, relatively well individualized units. During meiotic mid-prophase, chromosomes of budding yeast are well-organized structures that present such characteristics, making them remarkably suited for visualization. Here we describe the optimized protocols and techniques that allow monitoring of chromosome behavior during meiotic prophase in budding yeast.

Key words: *S. cerevisiae*, chromosome dynamics, *in vivo*, meiosis, prophase.

1. Introduction

A fundamental step of meiosis consists of the juxtaposition and interaction of homologous chromosomes all along their lengths. In the classical meiotic program, as happens in budding yeast, juxtaposition culminates with formation of the synaptonemal complex (SC) between homologous chromosomes (the zygotene stage). At the pachytene stage, SC appears complete all along the axes, with regular arrays of chromatin loops emanating outward from both sides. Furthermore, telomeres of pachytene chromosomes are anchored to the nuclear envelope (NE) through telomere/NE complexes. Recent studies from our laboratory and others have exposed the dynamic behavior of mid-prophase

chromosomes in *S. cerevisiae* (2–4). This chapter provides a detailed protocol for optimal conditions to monitor chromosomes in real time during prophase.

2. Materials

2.1. Strains and Chromosome Labeling

To visualize the chromatin, strains expressing fluorescently tagged protein(s) are constructed using standard techniques for transformation of *S. cerevisiae*.

2.2. Meiotic Time Courses – SK1 Strains (see Notes 1 and 2)

1. YEPG plates: 3% v/v glycerol, 2% w/v bactopeptone, 1% w/v yeast extract, 2% w/v bactoagar. Autoclave on liquid cycle
2. YEPD liquid and plates: 2% w/v bactopeptone, 1% w/v yeast extract. Add 2% w/v bactoagar for plates. Make a 40% w/v stock solution of glucose. Autoclave on liquid cycle. Add glucose to media to a final of 2% (see Note 3).
3. SPS: 1% w/v potassium acetate, 1% w/v bactopeptone, 0.5% w/v yeast extract, 0.17% w/v yeast nitrogen base with ammonium sulfate and without amino acids, 0.5% w/v ammonium sulfate, 0.05 M potassium biptalate, two drops of antifoam (Sigma) per liter. Adjust pH to 5.5 with 10 N KOH. Autoclave on liquid cycle
4. SPM: 1% w/v potassium acetate, 0.02% w/v raffinose, two drops of antifoam (Sigma) per liter. Autoclave on liquid cycle.
5. 40% ethanol, 0.1 M sorbitol.

2.3. Cell Preparation

1. DAPI (4',6-diamidino-2-phenylindole): 2 mg/mL stock solution in water.
2. 25 × 75 mm microscope slides.
3. 22 × 22 mm coverslips.

2.4. Epifluorescence Microscopy

Microscope: 100x objective, high-resolution/high-sensitivity camera, appropriate filters for the fluorescent probes used, and appropriate software for image acquisition (see Note 4).

3. Methods

3.1. Chromosome Labeling

In vivo visualization of individual prophase chromosomes can be achieved by fluorescently labeling proteins involved in structural organization. Proteins that compose the chromosomal axes and the SC are targets of choice and, in principle, should allow

illumination of individual chromatids and homologous pairs, respectively. It is important that the tagged protein provides a distinguishable signal without affecting meiotic progression as confirmed by FACS analysis, kinetics of divisions/sporulation, and spore viability. Visualization of the chromosomal axis has been achieved by fusing a GFP tag to the meiosis-specific cohesin, Rec8 (4). The SC can be illuminated using a GFP-tagged Zip1 protein (2, 3). In the latter case, although homozygous strains exhibit a brighter signal, good resolution can nonetheless be achieved using a heterozygous allele. Telomeres can be illuminated using a GFP-tagged Rap1 protein. In this case a heterozygous allele is faint though still visible.

Besides monitoring chromosomes directly labeled with fluorescent tags, a live cell DAPI-labeling method can be used to reveal the dynamic nature of chromosomes during prophase. This technique presents the great advantage, beside its simplicity, that one can monitor chromosomes in any cell undergoing meiosis without having to modify the genome to introduce a tagged protein into each new strain of interest. Best results are achieved for the pachytene stage, where chromosomes are well individualized. Movement also can be observed at zygotene though it is difficult to clearly distinguish individual chromosomes. Furthermore, it is easy to combine imaging of DAPI-labeled chromosomes with one's favorite fluorescently tagged protein located on the chromosomes or elsewhere (e.g. Ref. 3).

3.2. Meiotic Time Courses

Proper synchronization of a meiotic culture is critical for virtually all studies. In order to analyze a population of cells at specific stages or, conversely, to monitor certain events as a function of stage, most of the cells must enter into the different prophase steps at approximately the same time. It is possible to observe cells undergoing meiosis using techniques such as immobilization onto agarose patches and use of flow cell observation chambers. These approaches result in slower progression through meiosis, probably largely due to aeration problems, which also dramatically affect chromosome dynamics (R.K., unpublished observations; Ref. 5). Thus far, only sporulation in liquid medium allows optimal synchrony of the population. The synchrony of meiotic progression can be evaluated by monitoring meiotic replication by FACS analysis (*see Note 5*) and the two meiotic divisions by DAPI staining (*see Note 6*). The following is one of several available protocols, referred to here as the "SPS" protocol.

1. Day 1 – In the evening, patch cells from -80°C glycerol stock onto YEPG plates. This step ensures that cells emerging into growth will be respiration-proficient, which readily becomes defective in SK1 strains.

2. Day 2 – The next morning, streak for single colonies on YEPD plates. Make sure colonies are sparse so that they will grow large and healthy (*see Note 7*).
3. Day 4 – In the afternoon, pick a whole colony and put it into 5 mL YEPD liquid in a 10 × 13 mm culture tube. Vortex and then grow overnight at 30°C on a roller drum.
4. Day 5 – Grow YEPD cultures for 24 h, vortexing tubes a couple of times during the day. Set up at least two SPS cultures by diluting the YEPD cultures 1/500 and 1/1,000 in SPS. For slow-growing strains, include a 1/250 dilution. Note that surface-to-volume ratios in the culture flasks at this stage are important. Use 50 or 100 mL SPS in a 1 l flask, or 250 mL in a 2 L flask.
5. Grow SPS cultures at 30°C, shaking at 325–350 rpm, making the culture swirl up the edges of the flask to create the largest surface area possible.
6. Day 6 – SPS cultures should be grown for a total of 18 h. Remove samples from cultures and assess synchrony (*see Note 8*).
7. Once culture(s) for analysis are selected, harvest cells by centrifugation at 3,000*g* for 3 min at room temperature.
8. Wash cells in an equal volume of 1% SPM (prewarmed to 30°C) and resuspend in two-times the original volume of prewarmed SPM.
9. Take zero time point and place culture in incubator at 30°C, shaking as before (*see Note 9*).
10. Take 1 mL samples at hourly intervals to assess meiotic progression (MI and MII divisions) by DAPI staining. Pellet samples at 13,000*g* for 10 s then resuspend in 40% ethanol, 0.1 M sorbitol (*see Note 10*).

3.3. Cell Preparation

1. Take aliquots of cells to be analyzed from the sporulation medium culture at appropriate time points after initiation of meiosis. Because full aeration is essential to full chromosome dynamics during prophase, special care has to be taken at all steps of the protocol described below.
2. Harvest 500 µL samples. If possible, proceed immediately to image acquisition. If one wants more concentrated samples, let sit on ice for 20 min then remove supernatant.
3. For DAPI staining, add DAPI directly into two aliquots of the culture to a final concentration of 10 and 20 µg/mL and incubate overnight in ice in a 4°C room (*see Note 11*).
4. Once the sample is ready for visualization, either immediately for visualization of GFP-labeled proteins or later for DAPI visualization, vortex for 7 s to reoxygenate the solution and then deposit 3 µL of suspension onto the slide (*see Note 12*).

5. Add the coverslip (*see Note 13*). A few air bubbles must remain stuck under the coverslip: the cells located in their vicinity correspond to cells where aeration, and thus dynamic movements, last the longest. Therefore it is better to deposit the sample at three or four positions, located within the total surface covered by a coverslip, in order to maximize the chance of trapping an air bubble once the coverslip is added. Repeat as needed, discarding slides with no trapped air bubbles until a correct one is obtained.
6. Proceed quickly to imaging. One slide can be used for ~7–8 min once ready before motion deteriorates.

3.4. Image Acquisition

The microscope apparatus listed below (*see Note 1*) proved well-suited for the monitoring of GFP labeled as well as DAPI-stained chromosomes. The combination of a highly sensitive CCD camera with the piezo Z-motor allows short exposure times in rapid succession as needed to help maintain cell viability.

3.4.1. Two-Dimensional Time-Lapse Recording

Because mid-prophase chromosomes in wild-type budding yeast show dramatic movements, it is convenient to monitor them within a single focal plane. Useful measurements can be recovered from such 2D timelapse analysis (2, 3).

1. Nuclei are observed under appropriate excitation and emission wavelengths for the particular fluorophores being used.
2. View slides under phase contrast microscopy to locate the cells. For each time point, record images of cells located in the vicinity of a trapped air bubble (ideally up to five cell diameters away from the air/water interface).
3. Conditions for capturing 2D time-lapse series: for both GFP- and DAPI-labeled chromosomes, 90–120 images can usually be recorded before movement diminishes (perhaps as a result of photodamage) and/or photobleaching impedes satisfactory detection. Exposure of 800 ms is usually sufficient to achieve good quality pictures, although in homozygous Zip1-GFP strains it can be reduced down to ~350–500 ms. A series of exposures taken every one to three seconds present a good quality and are sufficient to reveal details of chromosome dynamics (*see Fig. 6.1*). Whereas smaller time intervals outline details of chromosome movements, larger ones underline more global trends such as large transitions and nucleus displacement. Eventually, longer time intervals can be used to achieve longer periods of observation (*see Note 14*).

3.4.2. Three-Dimensional Time-Lapse Recording

Three-dimensional analysis of chromosomes is achievable. However, little information about zygotene/pachytene chromosome disposition is directly extractable from such recordings in a wild-type background due to the highly dynamic nature of the nucleus.

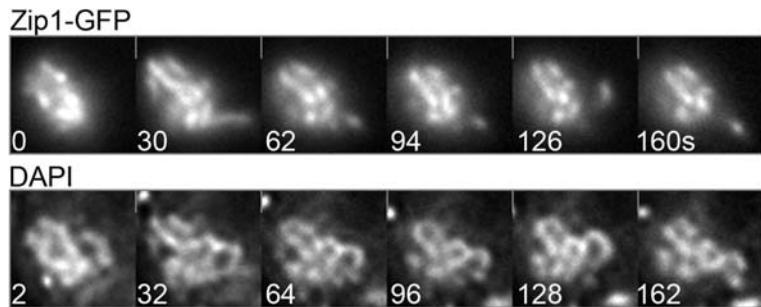


Fig. 6.1. Two-dimensional time lapse of *S. cerevisiae* zygote/pachytene nuclei with chromosomes co-labeled with Zip1-GFP and DAPI, 5 hrs after transfer to sporulation medium. Upper panel: series of images showing the Zip1-GFP chromosomes, with 800 ms exposure and at 2 s intervals. Lower panel: DAPI images of the same nuclei taken 2 s after the Zip1-GFP picture shown in the above panel (800 ms exposure). At some locations, DAPI signal reveals regions where chromosomes are not yet entirely synapsed and therefore where no GFP signal is already visible. For other examples, see (2, 3). A supplementary movie showing chromosome movements is available on the companion CD for this volume.

However, mutations or conditions that disrupt motion can be analyzed using 3D time lapse. We recommend conditions as follows: $16 \times 0.25 \mu\text{m}$ or $10 \times 0.4 \mu\text{m}$ z-sections taken every 30–60 s, with 400 ms exposures.

4. Notes



1. If strain is a *trp1* or *ura3* auxotroph, bring plates and liquid YEPA to 40 μM tryptophan and/or 20 μM uracil after autoclaving.
2. We have tried many different sources of water and results seem random. House distilled seems to work as well or better than MilliQ filtered. Time courses during summer seem especially finicky. Some people swear by bottled water such as Poland Springs as being more reproducible year round.
3. The sugar and amino acids in media will be chemically modified if autoclaved together (Maillard reaction). Autoclave the glucose as a separate 40% stock solution at 110°C. The rest of the media components can be combined then autoclaved at 121°C. After autoclaving add the glucose to the media to a final concentration of 2%.
4. Any good epifluorescent microscope can be used. Important features are: high resolution and sensitivity of the camera to minimize exposure times, fast frame rates to allow minimal times between acquisition of images, a z-stepper capable of

rapid, small steps, and an image acquisition program that can drive all hardware. We use an Axioplan 2 IE motorized microscope (Zeiss, Gottingen, Germany) with a 100x objective (NA=1.45 Ph3 Plan-Apochromat or alpha-PlanFluar NA=1.4) and filter sets appropriate to the fluorophores being imaged (GFP or DAPI in our case). Our EM-CCD camera is a Photometrics Cascade 512B (Roper Scientific, Tuscan, AZ). For three-dimensional analysis we use either the computerized z-motor of the Axioplan microscope's stage, or a piezoelectric translation z-stepper driven by the amplifier E662 LVPTZ (Physik-Instrumente, Karlsruhe, Germany) mounted on the microscope objective. We use Metamorph software (MDS Analytical Technologies, Mississauga, ONT, Canada) for data acquisition.

5. If the population is correctly synchronized, replication should be achieved within 3 h and pachytene reached by 5 h after transfer into sporulation medium.
6. Meiotic progression can be monitored by DAPI staining: every hour after inoculation into SPM to initiate meiosis, spin down 1 mL of culture and resuspend in 40% ethanol plus 0.1 M sorbitol. On a microscope slide, add 2 μ L of ethanol-permeabilized cell suspension to 2 μ L of 1 μ g/mL DAPI. Cells that have passed through anaphase I or anaphase II will contain two or four DAPI staining bodies, respectively.
7. For mutants that have a mitotic growth defect (e.g. *rad51* and *rad52*) each growth step can be extended to allow for sufficient density of cells at the start of the time course. Also it is harder to distinguish haploids and diploids so more YPD cultures are started to insure an adequate selection. Often one must judge haploid and diploid under the microscope with haploids being smaller and budding apically while diploids are larger and bud from alternate poles.
8. There are three criteria for judging the synchrony of a culture:
 - a. Optical Density: On our spectrophotometer we take between 1.3 and 1.45 OD₆₀₀ measured on the undiluted culture (i.e., not a true OD because we do not dilute the cells into the range the spectrophotometer can measure in a linear fashion). Each spectrophotometer will give a different reading according to light path length. This OD corresponds to $\sim 2.1 \times 10^7$ cells per mL at OD₆₀₀=1.35. The lower densities seem to be consistently more synchronous and are best for cytological studies. We have found that if cultures are too dense, diluting with SPM improves the synchrony of the subsequent time course.
 - b. Percentage of G1/G0 Cells: Stain cells with DAPI by centrifuging a 500 μ L sample for 10 s at 13,000*g*.

Resuspend in 40% ethanol, 0.1 M sorbitol. On a microscope slide add 2 μ L of ethanol-permeabilized cell suspension to 2 μ L of 1 μ g/mL DAPI. Assess the percentage of cells in vegetative S-phase (small buds without DAPI bodies) and mitosis (large buds with DNA at, or stretched across the neck). A good culture will have \leq 10% replicating and dividing cells. The absence mitotic figures *may* indicate that the culture has prematurely entered meiosis.

- c. Cell Morphology: The presence of daughter and mother cells of about the same size is a good indication that ensuing meiosis will be synchronous. Such cells often occur in strings of three to five cells.
9. At temperatures $>30^{\circ}\text{C}$, SK1 meiosis is sub-optimal; at $\geq 34^{\circ}\text{C}$ meiosis is inhibited. Check the temperature of your incubator carefully – it may fluctuate throughout the day. Lower temperatures (23–30 $^{\circ}\text{C}$) may be less problematic but you will have to be consistent. Meiotic DNA replication is particularly sensitive to high temperature. If it is necessary to perform a time course at higher temperature it is preferable to shift from 30 $^{\circ}\text{C}$ after initiation of DNA replication (~2 h).
10. DAPI staining: On a microscope slide add 2 μ L of ethanol-permeabilized cell suspension to 2 μ L of 1 μ g/mL DAPI. Cells that have done anaphase I or anaphase II will have two or four DAPI staining bodies, respectively.
11. Live-cell DAPI staining: it is better to prepare duplicate cell aliquots because of sample to sample variations. In general, up to 40% of the cells treated with DAPI are dead and exhibit very strong DAPI signal with little movement. Some others show very little staining and should be discarded as well. However, between 10 and 30% of all the cells show a weak but distinguishable signal, which corresponds to the live cells with stained chromosomes. Note that mitochondrial DNA is labeled as well with this protocol, corresponding to the tubular structures and dots observed at the cell poles and equator, respectively.
12. In order to increase the cell concentration and the number of cells in the vicinity of trapped air bubbles, it is recommended to remove the supernatant of the tube (after cells have settled to the bottom overnight) before the 7 s vortex. Avoid spinning. Deposit the 3 μ L at three to four places on the slide, so that the chance to trap an air bubble when adding the coverslip is maximized.
13. Do not press on the coverslip. Let it fall down gently on the sample and wait ~1 min before positioning it under the objective. However, over longer periods of time and in the set-up described here, access to oxygen diminishes and motion may be attenuated.

14. Using longer time intervals allows one to monitor cells over longer periods of time, but may induce complications due to insufficient aeration, and/or stage drifting. However, significant results have nonetheless been achieved (5 s time interval over 44 min, Ref. 2).

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Chapter 7

Observing Meiosis in Filamentous Fungi: *Sordaria* and *Neurospora*

Denise Zickler

Abstract

The filamentous fungi *Neurospora crassa* and *Sordaria macrospora* are materials of choice for recombination studies because each of the DNA strands involved in meiosis can be visually analyzed using spore-color mutants. Well-advanced molecular genetic methodologies have been developed for each of these fungi, and several mutants defective in recombination and/or pairing are available. Moreover, the complete genome sequence of *N. crassa* has made it possible to clone virtually any gene involved in their life cycle. Both fungi provide also a particularly attractive experimental system for cytological analysis of meiosis: stages can be determined independently of chromosomal morphology and their seven chromosomes are easily identified. The techniques for light, immunofluorescence and electron microscopy presented here have been used, with success, for monitoring of chromosome behavior during both meiotic and sporulation processes. They have also proved useful for the analysis of mitochondria and peroxisomes as well as cytoskeleton and spindle pole-body components. Moreover, all techniques of this chapter can be easily applied to other filamentous ascomycetes, including other *Sordaria* and *Neurospora* species as well as *Podospora*, *Ascobolus*, *Ascophanus*, *Fusarium*, *Neotiella*, and *Aspergillus* species.

Key words: Meiosis, homologous pairing, methods for light and electron microscopy, immunofluorescence, synaptonemal complex, fungi, *Neurospora*, *Sordaria*.

1. Introduction

Mycelial fungi like *Sordaria macrospora* and *Neurospora crassa* are particularly well suited to meiosis and genetic studies. First, both have a brief life cycle during which several hundred meiocytes (asci) and the resulting gametes (ascospores) can be analyzed: a Petri dish contains over 1,000 fruiting bodies, each of which contains 150–200 ascii. Also, samples can be removed (e.g. for drug treatment) at different times from the culture, in which fruiting bodies develop synchronously, without any apparent

effect on development of the remaining fruiting bodies. Second, the four products of a single meiosis are held together in a large cell (ascus). Moreover, the four resulting haploid nuclei are arranged in a linear order, which reflects the preceding nuclear/spindle position: this allows easy detection of pre- or post-segregation of allelic pairs (Fig. 7.1A). Third, meiosis is followed by a post-meiotic mitosis giving a linear series of eight haploid nuclei that allow determination of the genetic constitution of each of the DNA strands involved in meiosis by visual analysis of the 8-spored ascus (including post-meiotic segregations, e.g. 1, 2). The ability to discern the genetic information of each DNA strand at a particular locus has provided considerable insight into recombination mechanisms (reviewed in 1–3). Moreover, the eight ripe ascospores remain mostly associated when ejected from fruiting bodies on agar plates. Ascospore-color mutations can be used to recognize recombination events (Fig. 7.1A) and to score (and map) the different types of chromosome rearrangements (e.g., 4–6 for respectively *Neurospora* and *Sordaria*). Fourth, all linkage groups are mapped genetically and assigned to cytologically distinguished chromosomes in both species (5–7). Finally, since mutations that

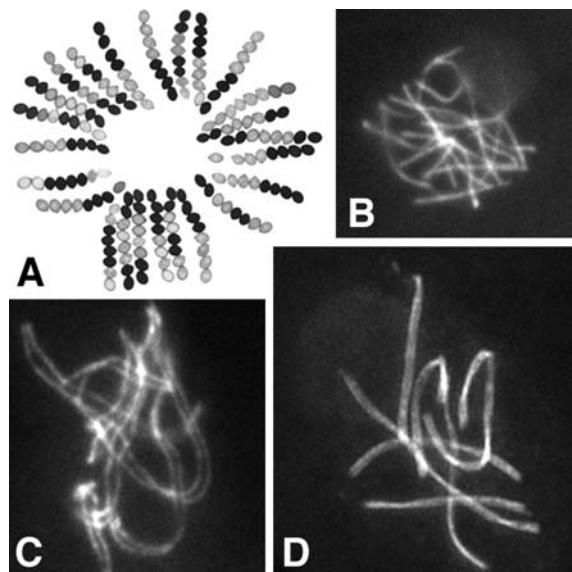


Fig. 7.1. Recombination and meiosis of *Sordaria*. (A) Rosette of 8-spored asci showing the 4:4 segregation of black (B) and white (W) ascospores. Asci with 4B and 4 W ascospores imply segregation at first meiotic division without a crossover having occurred between the centromere and the spore-color marker. Crossover between the white gene locus and the centromere results in 2 W:2B:2 W:2B or 2 W:4B:2 W spored-asci (and reciprocal segregations), depending on how homologs segregated to the poles of the spindles during the second meiotic division. (B–D) Meiotic prophase: chromosome axes are stained with the cohesin-associated protein Spo76/Pds5 tagged with GFP. At early leptotene (B) chromosomes are not paired while they are all aligned at late leptotene (C), and finally synapsed (D).

affect meiosis lead to defects in sporulation due to aneuploid meiotic products (e.g. 8), the presence of abnormal ascospores (generally white when compared to the black spores in wild type) or absence of 8-spored ascii on the Petri-dish lid provides an easy screen for potential meiotic mutants (after mutagenizing strains or protoplasts and inducing them to fruit).

S. macrospora is self-fertile (homothallic) and thus able, by self-mating, to form a diploid nucleus homozygous for any induced mutation. Homothallism not only guarantees isogenic genomes but allows one to generate and screen directly for recessive (and dominant) mutants affecting meiosis and to isolate molecularly transformed or tagged genes: the homokaryotic mycelium established from a single haploid nucleus has the potentiality to progress through the dikaryotic stage, meiosis and ascospore formation. For easy genetic analyses, selfing can be prevented by the use of nonallelic self-sterile but cross-fertile mutants (e.g. 6–8). *N. crassa*, in contrast, is self-incompatible (heterothallic): mating between homokaryotic mycelia of mating-type *a* and mating-type *A* is required to complete the sexual cycle (4, 5, 9).

Also, *Neurospora* has developed several ways of controlling the integrity of its genome (e.g., inactivation of duplications created by transformation) during both premeiosis [RIP for Repeat-Induced point Mutation (reviewed in 9)] and meiotic prophase [Silencing by Unpaired DNA or MSUD (10, 11)]. *Sordaria*, in contrast, does not exhibit such inactivation, allowing easy introduction of modified or GFP-tagged sequences (8, 12). On the other hand, as RIP extensively mutates endogenous genes with G-C to A-T transitions when a second copy of the gene is introduced to the *Neurospora* genome and the duplication strain is subsequently crossed, RIP provides an interesting tool for in vivo mutagenesis of specific genes of interest, available from related organisms (reviewed in 9). Finally, the genome of *N. crassa* (estimated size of 41 Mb) is completely sequenced (www.genome.wi.mit.edu). The genome of *S. macrospora* is not, but the two species being very close, availability of the *Neurospora* genome allows easy primer design to clone *Sordaria* genes of interest (e.g. 8, 12).

Both fungi also provide several particularly attractive features for examination of meiotic pairing (**Fig. 7.1B and C**) and synapsis (**Fig. 7.1D**). First, their chromosome number is low ($n=7$) and chromosomes can be recognized by length and centromere position in electron microscopy (EM) (6, 7, 12, 13, 18 for *Sordaria*; 14–17, 19 for *Neurospora*). Second, the progression of nuclei through the various stages of meiosis can be monitored independently of chromosome status, by progressive increase in ascus size (from 10 μm just after karyogamy to 60 μm at zygotene, 100 μm at mid-pachytene and 150 μm at diplotene), thus permitting a clear establishment of event time lines in mutant situations in comparison to wild-type meiosis (e.g., 8, 12, 18). Third, chromosome axes seen by cohesins (e.g. tagged with GFP, **Fig. 7.1B–D**) or

synaptonemal complex (SC) axial elements emerge concomitant with DNA replication and occur all along the lengths of the chromosomes at early prophase, just before appearance of foci of the RecA homolog Rad51, thus very soon after double-strand break formation, which initiates meiotic recombination. This allows an accurate analysis of the alignment and chromosome location and movements during the recognition and juxtaposition processes (see 7, 12, 13, 18, 20 for *S. macrospora* and 14, 15, 16, 17, 19 for *N. crassa*). Fourth, the volume of the nucleus increases steadily from leptotene to late pachytene; consequently the seven bivalents are widely spaced within the nucleus, allowing easy observation of alignment and synapsis (Fig. 7.1B–D). Fifth, contrary to most eukaryotes, the two sets of homologous chromosomes are in separated nuclei before meiosis starts: progression of homologue juxtaposition can therefore be conveniently described with respect to two major landmarks: karyogamy and SC formation. These advantages have, for example, permitted elucidation of the mechanism of presynaptic coalignment (12). Finally, *Sordaria* and *Neurospora* meiotic mutants do not arrest when either recombination or synapsis is defective: although defective for segregation at anaphase I and/or II, they form ascospores around nuclei whatever the number of chromosomes present in the nucleus (e.g. six or only two, instead of the seven expected, as seen in the *spo11* mutant of *Sordaria*, Fig. 7.2B).

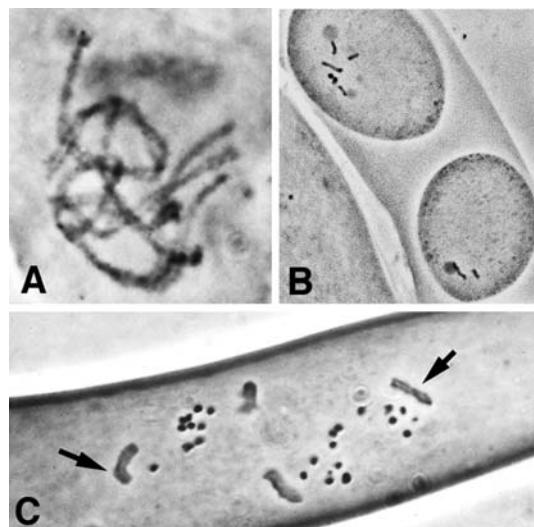


Fig. 7.2. Hematoxylin staining (*Sordaria*). (A) Pachytene nucleus with bright chromosome axes. (B) Post-meiotic mitosis in two ascospores of the *spo11* mutant (no recombination is initiated) with respectively six (upper) and two chromosomes (lower spore). Hematoxylin stains both prometaphase condensed chromosomes and nucleolus (round dark ball attached to a chromosome), indicating that both spores contain a nucleolar organizer chromosome. (C) Anaphase of second meiotic division: chromosomes segregate along the spindles indicated by the spindle-pole bodies (arrows) that are also stained by this procedure.

2. Materials

2.1. Strains and Culture Media

1. *N. crassa* and *S. macrospora* laboratory strains: Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, KS). Strains can also be collected from nature (see Note 1).
2. *Sordaria* growth and fruiting medium: To 1 L of distilled water add successively: 10 g glucose, 5 mL TKP (TKP: 5 g KH₂PO₄, 6 g K₂HPO₄ in 100 mL H₂O), 5 mL 5% MgSO₄, 5 mL 10% urea, 5 mL of 12 mg/mL biotin, 0.1 mL mineral concentrate (5 g ascorbic acid·1H₂O, 5 g ZnSO₄·7H₂O, 1 g Fe(NH₄)₂(SO₄)₂·6H₂O, 0.25 g CuSO₄·5H₂O, 0.05 g MnSO₄·1H₂O, 0.05 g H₃BO₄, 0.05 g Na₂MoO₄·2H₂O for 100 mL). Store concentrate at room temperature. Solidify with 15 g agar. Autoclave 20 min at 120°C. For ascospore germination, replace urea with 0.44% ammonium acetate in minimal medium.
3. *Neurospora* synthetic cross medium: To 1 L of distilled water add 3 g KNO₃, 1.4 g K₂HPO₄, 1 g KH₂PO₄, 1 g MgSO₄·7H₂O, 0.2 g NaCl, 0.2 g CaCl₂·2H₂O, 0.1 mL biotin (5 mg in 100 mL of 50% ethanol, stored at -20°C), 0.2 mL mineral concentrate (same as for *Sordaria*) and 1% sucrose. Solidify with 2% agar. (21, and see Note 2).

2.2. Strain Storage Medium

1. *Sordaria*: 216 g saccharose in 1 L of minimal medium. Store agar strips with mycelium in 1.5 mL aliquots at -80°C.
2. *Neurospora*: 5 mg biotin in 100 mL of 50% ethanol. Store in 2.5 mL aliquots at -20°C. Conidia are kept on minimal media in slants at -20°C or on anhydrous silica gel (21).

2.3. Iron-Hematoxylin Staining

1. Iron mordant: prepare stock solution with 10 g ferric acetate in 100 mL of 50% aqueous propionic acid (glass bottle). Working solution: 0.5–1 mL in 50 mL 50% aqueous propionic acid. Keep in dark at room temperature.
2. Hematoxylin: 2% (Gurr, London or Sigma) in 50% aqueous propionic acid. Keep in dark at room temperature. Better when 1–2 months old.
3. Fixative for *Neurospora*: 9 vol of 95% ethanol, 6 vol of propionic acid, and 2 vol of 10% aqueous chromic acid.
4. Fixative for *Sordaria* and *Podospora*: 9 vol of butanol, 6 vol of glacial acetic acid, and 2 vol of 10% aqueous chromic acid.
5. Hydrolysis solution for *Neurospora* 1:1 (v/v) 12 N HCl and 95% ethanol.
6. Hydrolysis solution for *Sordaria* and *Podospora*: 1 N HCl.
7. Glycerin: 1 vol glycerin plus 1 vol of 45% acetic acid.

2.4. Acriflavine Staining

1. Hydrolysis solution: 4 N HCl.
2. Acriflavine (Sigma): 100–200 µg/mL in K₂S₂O₅ (5 mg/mL in 0.1 N HCl).
3. Washing solution: 2 vol HCl in 98 vol 70% ethanol.

2.5. Immuno-fluorescence: Antibodies and GFP/RFP

1. Siliconized slides. Wash slides in 50% ethanol and dry. Under fume hood, dip each slide quickly into dimethyldichlorosilane (2%, Merk 103014.0500) and dry on pipette. Store in slide box to avoid dust. Both slides and dimethyldichlorosilane (filter if necessary) can be used two to three times.
2. Polylysine (Sigma): add 24.875 mL distilled water and 125 µL of 10% Triton to a 25 g polylysine tube (fractioning is difficult). Filter and distribute as 500 µL samples in Eppendorf tubes. Store at –20°C. Do not refreeze more than twice.
3. Polylysine-coated coverslips. Clean coverslips with 95% ethanol, let dry and place them into a large Petri dish (14 cm diameter, allows easy transfer) on parafilm. With a pipette tip, cover each coverslip with 10 µL polylysine. Let dry for 10 min (cover the dish with lid to avoid dust) and wash coverslips one by one in distilled water (hold edge with thin tweezers). Air dry (dab the last drop of liquid with Kimwipes paper) and place coverslips with polylysine side up into a second large Petri dish also covered with parafilm. Prepare coverslips one night before (room temperature) or at least 4 h before staining (dry at 37°C) and keep in dark.
4. PEM buffer: In 600 µL distilled water add 9 mL 100 mM Pipes, 200 µL 500 mM EGTA, 200 µL 500 mM MgCl₂. Store PEM, as well as stock solutions of PIPES, EGTA, and MgCl₂ at 4°C. The pH of the Pipes stock solution should be adjusted to 6.9 with 10 N HCl.
5. Phosphate-buffered saline (PBS) (10x stock solution): 50 mL 1.6 M Na₂HPO₄, 50 mL 0.4 M NaH₂PO₄ and 0.5 g NaN₃(Aldrich); store at room temperature, as well as stock solutions of Na₂HPO₄(place 1 h at 37°C before use if crystals are formed) and NaH₂PO₄. Working solution: 1 vol in 9 vol distilled water.
6. PBS + Triton: 44.75 mL distilled water, 5 mL 10x PBS, 250 µL 10% Triton.
7. Paraformaldehyde: Dissolve 1.85 g in 23.5 mL of PEM plus 1.5 mL of distilled water (7.4%), on stirring hot plate (60°C) in a chemical hood (toxic) until the solution becomes clear. Filter if slightly opaque. Cool to room temperature and store at 4°C for 14–20 d.
8. Post-extraction: 2 mg/mL myristoyl lysolecithin (Sigma) in 90 mM Pipes, 10 mM EGTA, 10 mM MgSO₄. Store at 4°C. Working solution: 30 µL stock solution in 970 µL PEM.

9. DAPI. Stock solution: 5 mg/mL in distilled water. Store in 40 µL aliquots at -20°C in Eppendorf tubes in the dark. Working stain: 0.5 µg/mL in PBS + Triton (keep in dark).
10. Antifade: Vectashield H100 (Biovalley or Vector laboratories) or DABCO: 10% w/v 1,4-diazobicyclo(2,2,2)octane in 90% glycerol, 10% 100 mM PBS, pH 8.7. Keep in dark at 4°C.
11. Tested antibodies: anti-β-tubulin (1:1,200, Amersham), anti-α-tubulin (1:500–1:1,200, Amersham), anti-actin (1:3,000–1:5,000, Amersham), MPM2, a mitotic phosphoprotein, which recognizes spindle-pole bodies and synaptonemal complex (1:300–1:750); anti-Rad51 (1:400; Oncogene); anti-GFP (1:500–1:250, Roche). For HA-tagged genes: anti-HA 3F10 (Boehringer Mannheim) at 1:4,000. When diluted, antibodies are conserved in small volumes at 4°C (2–6 months) and at -20°C (1–3 years). Do not refreeze, but keep at 4°C.

2.6. Drugs

1. Nocodazole (Aldrich). Stock solution: 5 mg/mL in DMSO. Working solution: dilute to 10 µg/mL in distilled water.
2. Cytochalasin D (CD, Sigma). Stock solution: 4 mM in 95% ethanol; working solution is 10 µM.
3. Latrunculin B (Calbiochem): 0.1–0.5 µg/mL in 0.5% DMSO.
4. 2,3-Butanedione monoxime (BDM, Sigma): 10–20 mM in distilled water.

2.7. Observation of Membranes, Mitochondria, and Peroxisomes

1. Plasma membrane: 25–30 µM FM 4-64 (Molecular Probes).
2. Mitochondria: 2-(4-dimethylaminostyryl)-1methylpyridinium iodide (DASPMI, Molecular Probes or Sigma) at 10–25 µM in water.
3. Peroxisomes: antibody against the trifunctional peroxisomal FOX2 enzyme of *Neurospora* (22) or the sequence encoding the SKL tripeptide (peroxisomal targeting signal 1).

2.8. Electron Microscopy

1. Phosphate buffer stock solutions. Solution 1: 15.6 g NaH₂PO₄·2H₂O in 250 mL distilled water. Solution 2: 35.8 g Na₂HPO₄·12H₂O in 250 mL distilled water (keep both at 4°C). Just before use, mix 16 mL of solution 1 with 84 mL of solution 2 (0.4 M) and add 100 mL distilled water (final 0.2 M).
2. Glutaraldehyde: 2–6% in 0.2 M phosphate-buffer or in 0.1 M cacodylate buffer, both at pH 7.2.
3. OsO₄: 2–4% in 0.2 M phosphate-buffer (pH 7.2).
4. Uranyl acetate: 5% in distilled water. Store at room temperature, in the dark and in thick glass container.

5. Lead citrate: 0.2 g in 50 mL distilled water plus 0.5 mL 10 N NaOH. Store at room temperature in the dark.
6. Resin: 81 mL Epikote 812 ($C_{12}H_{20}O_6$; Merck), 50 mL DDSA (Dodecyl succinic anhydride), 44.5 mL MNA (methyl nadic anhydride). Final use: add 0.4 mL DMP30 (dimethylaminomethyl phenol). Araldite (Merck).
7. Siliconized coverslips: Dimethyldichlorosilane 2% (Merk). Keep out of dust.
8. Formvar-coated slides. 1% formvar w/v in chloroform. Dip slide into solution and lift it as firmly as possible to avoid differences in thickness, and air dry. Discard if wrinkled or irregular.
9. Plastic-coated rings: In a tube with a diameter slightly larger than the diameter of an EM grid, cut several rings (1–2 mm high).

2.9. Silver Staining

1. Slides. Clean with 95% ethanol, dry and polish with lens paper. Coat slides with 0.9% (w/v) polystyrene from Falcon Petri dish dissolved in chloroform.
2. Silicon-treated coverslips: prosyl-28 (SCM).
3. Paraformaldehyde: 4% in distilled water (pH 8.2) plus 0.03% of sodium dodecyl sulfate (SDS).
4. Photoflo (Kodak): 0.4% in water.
5. Silver nitrate: 50% aqueous silver nitrate (Calbiochem) with a drop of gel developer (2% aqueous gelatin dissolved at 40°C and cooled).

3. Methods

3.1. Preparation of Strains for Meiosis and Microscopy

1. Store *Sordaria* wild-type and mutant strains either in tubes (4–5 years) or in small Petri dishes (1 year) at 4°C or at –80°C (see Sections 2.1 and 2.2 for media). For fresh cultures, take a 2–5 mm strip of agar plus mycelium with either a sterile needle or a drawing pen (through flame of Bunsen burner) and place it on fresh medium (small Petri dish) at 23–25°C in light. Avoid ethanol sterilization because it prevents *Sordaria* growth. Fruiting bodies form after four days and develop synchronously over the entire Petri dish. The first ascospores in each fruiting body are synchronous and are all in meiotic prophase the fifth day. Prophase takes roughly 10 h and ascospores are formed two days later. Cultures fruit at room temperature, but timing will be dependant on temperature.

2. Make *Neurospora* crosses for cytology at 25°C in Petri dishes on synthetic crossing medium (*see Section 2.1*, Step 3 and **Note 2**). For each cross, grow the “female” parent (*matA* or *matα*) for five days at 25°C and then fertilize by adding conidia (in distilled water) from the parent of the different mating type (23, 24). When used in a laboratory that cultivates other organisms, it is recommended to use the *fluffy* strain (from FGSC), which is perfectly fertile (by confrontation of two strains with opposite mating types), but which no longer makes asexual spores that easily contaminate other culture plates. The fruiting bodies develop synchronously and the first ascii in meiosis are found 3 days after fertilization. Ascus development is not synchronous and by 6–8 days all meiotic stages are present in each fruiting body and the early-developed ascii contain ascospores (24, 25).

3.2. The Iron-Hematoxylin Procedure

The propionic-iron-hematoxylin method (8, 10, 13, 18, 24–27) gives very reliable and accurate staining of all fungal nuclei (*see Note 3*). As illustrated in **Fig. 7.2**, pachytene chromosomes (**Fig. 7.2A**), mitotic metaphase chromosomes of ascospores (**Fig. 7.2B**) and chromosomes of anaphase II as well as the corresponding spindle-pole bodies (arrows in **Fig. 7.2C**) are brightly stained when observed in a light microscope.

1. For general observation of divisions in ascii, fix agar strips of 5–10 mm containing 10–20 fruiting bodies.
2. For detailed observations of meiotic stages fix only rosettes of ascii, this allows faster and better spreading. To do so, open each fruiting body with forceps (press the bottom to keep ascii together) and lift the rosette of ascii out on the agar plate. Accumulate four to five rosettes and transfer them into fixative (*see Section 2.3*, step 3). Cavity slides are well suited to accumulate the rosettes before storage in small tubes half filled with fixative. Once fixed, the material can be kept at room temperature for 10–20 d or for 1–2 months when stored in a freezer.
3. Hydrolysis of rosettes of ascii (*see Section 2.3*, Step 4 and **Note 4**): Transfer rosettes with fixative into a small centrifuge tube (glass). Remove fixative and add 5 mL of 1 N HCl. Heat 10 min at 60–70°C. Time is critical: to reach quickly the right temperature, heat the tube on a Bunsen burner (add thermometer into tube to control temperature) and stop hydrolysis rapidly by plunging the tube into ice. Rosettes can stay 1 d in 1 N HCl.
4. Hydrolysis of agar strips: Incubate at 70°C in a heater for 1 min, take out, let cool 2 min and wash the agar strips for 10 min in 3 vol of ethanol, 1 vol of acetic acid, and 1 vol of chloroform. They can stay 6–8 h in this solution before staining.

5. Staining. Take 5–10 rosettes of ascospores from HCl (use a Pasteur pipette; take care because they stick easily to glass), place on a clean slide and remove excess of acid with a filter paper (traces of HCl prevent staining). Add quickly a small drop of diluted ferric acetate solution (*see Section 2.3, Step 1*) and stir with a glass rod. Add two drops of hematoxylin (*see Section 2.3, Step 2*) and mix both solutions thoroughly with the glass rod (do not use metal, which can add iron). When the mixture turns brown, cover quickly with a coverslip (*see Note 5*). Gently tapping above rosettes spreads and breaks ascospores (best prophase figures are obtained when the ascus wall is broken). Press out excess stain with filter paper. The quality of the stain can be quickly assessed in a microscope and if necessary (i.e., if the nuclei are too pale), slight heating of the slide over flame improves staining (avoid cooking which precipitates hematoxylin). Seal with dental wax or glycerin.
6. Staining of agar strips: In a drop of diluted ferric acid solution, open perithecia one by one, squeeze out rosettes with needles and eliminate all wall debris. Add two drops of hematoxylin and proceed as above.
7. Observation. Chromosomes and spindle-pole bodies are normally highly contrasted in the light microscope (e.g., **Fig. 7.2**) but contrast can be improved when observed in phase contrast. Squashes remain good for several days. However, while only chromosomes (**Fig. 7.2A and B**) and spindle-pole bodies (**Fig. 7.2C**) are stained in fresh preparations, cytoplasm staining increases after two days.

3.3. The Acriflavine Procedure

Developed for *Neurospora* by Raju (28) this technique works for all fungi.

1. Hydrolyze fruiting bodies or rosettes of ascospores in 4 N HCl for 25 min at 30°C.
2. Wash samples in water.
3. Stain with acriflavine (*see Section 2.4, step 2*) 20–30 min at 30°C.
4. Wash three times in HCl-ethanol solution at 30°C, followed by three times in distilled water.
5. Squash rosettes in a drop of 10% glycerol.
6. When fruiting bodies are fixed, they must first be dissected in 10% glycerol and the wall removed before squashing ascospores in 10% glycerol.
7. Observe with an epifluorescence microscope at excitation 450 nm and emission 540 nm.

3.4. Immunocytology

The following protocols are identical for *Sordaria* (12, 18, 29, 30), *Neurospora* (10, 11, 31), and *Podospora* (32, 33) (see Notes 6 and 7 plus Section 2.5 for details). Examples of different antibodies and meiotic stages are shown in Fig. 7.3.

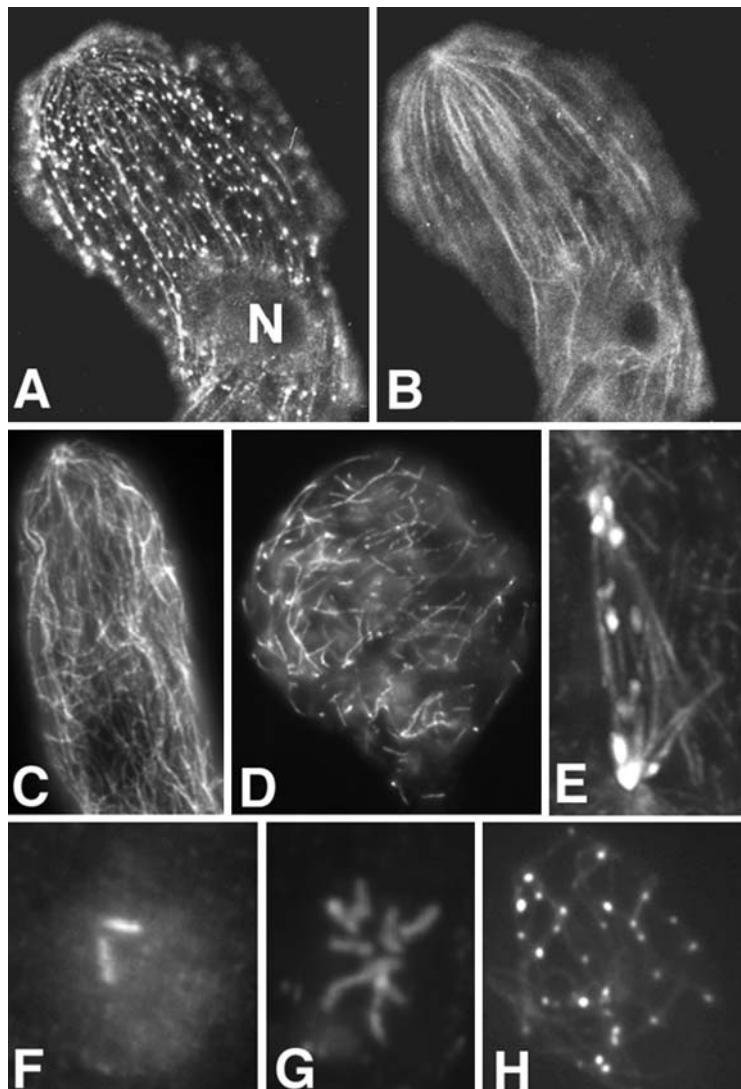


Fig. 7.3. Examples of antibody staining. (A and B) *Sordaria* prophase ascus, double stained with anti-actin (A) and anti-tubulin (B) and detected by confocal microscopy. (C and D) Untreated prophase ascus (C) shows dense layers of microtubules while an ascus after anti-actin drug treatment (D) shows aberrant shape (round instead of cylindrical) and aberrant microtubule organization. (E) Anaphase I spindle of *Neurospora* stained with anti-tubulin antibody merged with DAPI staining of chromosomes. (F and G) Prometaphase of post-meiotic mitosis: duplicated spindle-pole bodies are stained by anti-MPM2 (F) and chromosomes are stained by DAPI (G). (H) Leptotene nucleus double stained by Spo76-GFP (axes) and anti-Rad51 antibody shows numerous Rad51 foci located on unpaired chromosomes.

1. Fix rosettes of ascospores (*see Section 3.2, Step 2*) in fresh paraformaldehyde (3.7% for *Sordaria* and 7.4% for *Neurospora* and *Podospora*) 20–30 min at room temperature. Cavity slides are convenient because they allow easy removal of liquid with a glass Pasteur pipette that has been elongated in a flame to reduce diameter.
2. Wash with PBS: remove fixative with a pipette and add PBS. Keep rosettes in PBS while slides are made.
3. Place five to ten rosettes (with 7 µL PBS; use a P20 pipette) on a siliconized slide, add the polylysine-coated coverslip (polylysine side down) and, under a dissecting microscope, crush ascospores with a blunted hypodermic needle by tapping at the center of the rosette to disperse ascospores and finally break their bases.
4. With tweezers, remove the coverslip (to which ascospores remain attached because of the polylysine) from slide (watch polylysine side) and place it, ascospores up, in a large Petri dish (14 cm diameter, which allows easy handling) covered with parafilm.
5. Add 250 µL of PBS + Triton on the coverslip.
6. Prepare the next coverslip and again add 250 µL of PBS + Triton. Repeat until all needed coverslips are ready.
7. If different strains are used, mark location of each coverslip.
8. Add on each coverslip a large drop of lysolecithin and incubate at 37°C for 1 h.
9. Discard and rinse with PBS + Triton (250 µL per coverslip).
10. Discard and add primary antibody (*see Note 7*): first add 50 µL of PBS, make sure that the entire coverslip is covered, and then add 50 µL of antibody at correct concentration.
11. Incubate 12 h at room temperature and in dark (under aluminum foil or in box). To avoid drying, place wet filter paper around the coverslips.
12. Remove antibody and wash twice (15 min each) with PBS + Triton.
13. Incubate in secondary antibody + PBS (100 µL per coverslip) at 37°C for 45 min in the dark.
14. Remove and discard antibody, and wash with PBS + Triton.
15. Add 250 µL DAPI on each coverslip and stain for 10–15 min. Both DAPI solution and coverslips with DAPI must be kept in dark. Dim the room lights during handling.
16. Rinse each coverslip in distilled water and air-dry. Dab the last drop of liquid with filter paper.
17. Place each coverslip (polylysine side down) on a clean slide in a drop of antifade (*see Section 2.5, Step 10*), which reduces both fading and drying out of the preparations.

18. Seal the coverslip to the slide with nail polish and let dry in the dark for 1–2 h.
19. Store slides in a box either at 4°C (15–30 d) or at –20°C (up to two years).
20. View slides under fluorescent or confocal microscopy with excitation corresponding to the fluorophore on the secondary antibody. Use excitation at 364 nm for DAPI fluorescence. Software can be used to overlay the images (see Note 8). Examples are shown Fig. 7.3.

3.5. Observation of GFP- or RFP-Tagged Nuclei

The GFP coding sequence (p-EGFP-1, Clontech) is usually fused just after the last C-terminal amino acid predicted from the ORF (try amino-terminal fusions if no GFP is seen or if there is doubt concerning localization) (see Note 9).

1. Live-cell imaging of GFP-tagged vegetative cells: grow mycelia or protoplasts on sterile slides coated with a thin layer of medium, or use the inverted block method of Read's laboratory (34, 35).
2. Live-cell imaging of GFP-tagged ascospores: lightly squash in a drop of 10% glycerol (see Note 10).
3. Fixed samples: Fix rosettes of ascospores in paraformaldehyde (3.7% for *Sordaria* and 7.4% for *Neurospora* and *Podospora*) at room temperature for 20–30 min. Use cavity slides to allow easy removal of liquid from rosettes.
4. Wash with PBS: slowly remove fixative with a pipette and add PBS. Keep rosettes in PBS while next slides are made.
5. Place five to ten rosettes with 7 µL PBS (use a P20 pipette) on a siliconized slide, cover with a polylysine-coated coverslip (polylysine side down) and, under dissecting microscope, crush ascospores with a blunted hypodermic needle (as in Section 3.4, Step 3).
6. Remove the coverslip from the slide with tweezers and place it, ascospores up, in a large Petri dish covered with parafilm (as in Section 3.4, Step 4). The ascospores remain attached to the coverslip through the polylysine.
7. Place 250 µL of PBS + Triton on the coverslip and prepare each of the next coverslips in turn, until all coverslips are ready.
8. Discard and add 250 µL of DAPI for 10–15 min. Keep in dark (see Section 2.5, Step 9).
9. Rinse each coverslip in distilled water in dim light and dab the last drop of liquid with filter paper.
10. Place each coverslip (polylysine side down) on a clean slide in a drop of antifade (see Section 2.5, Step 10).
11. Seal the coverslip to the slide with nail polish and let it dry in the dark for 1–2 h.

12. Store slides in a box either at 4°C (two weeks) or at -20°C. Staining remains visible for one year, but the brightness of GFP, RFP, and DAPI decreases after 6 months.
13. View slides in a fluorescent microscope with a GFP filter for GFP samples and red emission for RFP. After control for GFP specificity, pictures are taken with an FITC filter, in which ascii are visible. This allows easier staging of nuclei. Deconvolution gives details not always seen with fluorescent microscopy (compare **Fig. 7.4B** with **Fig. 7.4A**). A confocal laser scanning microscope is necessary if two different structures are labeled (**Fig. 7.3A and B**). Prophase axes are less clear than with epifluorescence microscopy (compare **Fig. 7.4C** with **Fig. 7.4A and B**). Chromosomes seen by DAPI are less bright (**Fig. 7.4D**) than with GFP staining of axis proteins (e.g. cohesin Spo76/Pds5 in **Fig. 7.4A-C**). Also, GFP or RFP staining remains visible after antibody staining (e.g., anti-Rad51 in **Fig. 7.3H**).

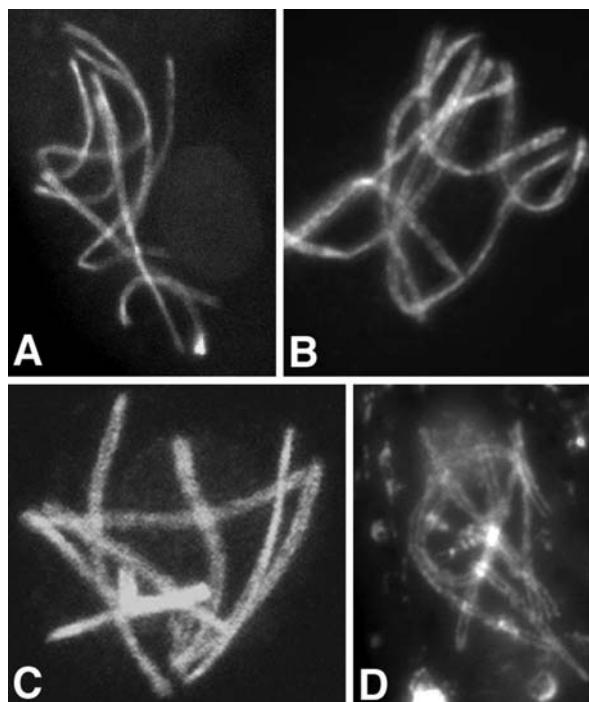


Fig. 7.4. Comparison of four methods to analyze pachytene synapsed chromosomes stained by Spo76-GFP. **(A)** Epifluorescent microscopy shows bright staining of the seven synapsed bivalents. **(B)** Deconvolution shows that Spo76 is not continuous but localizes as close, dense foci. **(C)** Confocal microscopy shows that staining is likely broader than axis. **(D)** DAPI staining is always less bright.

3.6. Drug Treatments

- As fruiting-body development is synchronous in each Petri dish, a third of the agar containing fruiting bodies is dipped (fruiting bodies down) in drug solution, while the second third is treated with distilled water containing the appropriate concentration of solvent. The third part remains in the dish as a control (29–31).
- Apply nocodazole to fruiting cultures for 2–12 h. Cortical microtubules (examples in **Fig. 7.3B and C**) begin depolymerization after 15 min and spindle microtubules (**Fig. 7.3E**) after 12 h.
- Treat with cytochalasin D overnight. Cortical actin filaments (**Fig. 7.3A**) are disrupted after 10 h. An example is given in **Fig. 7.3C and D**: in untreated asci, dense arrays of microtubules lie parallel to one another and converge at the ascus tip (**Fig. 7.3C**). After 10 h drug treatment, instead of being cylindrical, asci are round-shaped and microtubules are randomly arranged (**Fig. 7.3D**).
- Latrunculin B arrests homolog movement after 2 h treatment.
- Overnight treatment with BDM arrests spindle-pole body movement after spindle-pole body duplication (e.g., **Fig. 7.3F and G**).
- After washing off the drug with water, recovery of the cytoskeleton network at different times, followed by fixation, can be monitored because asci develop normally in the treated fruiting bodies (see Note 11).

3.7. Organelles and Membrane Staining

- Isolate asci as described in **Section 3.2**, Steps 1 and 2.
- Plasma membrane: Place rosettes of asci in one drop of FM 4-64 for 1–2 h and squash.
- Mitochondria: Add a drop of DASPMI on unfixed rosettes of asci, and squash.
- Double staining with DAPI allows overlap of the blue DNA stain (blue emission) with the orange DASPMI stain (FITC filter).
- Peroxisomes: prepare asci as in **Section 3.4**. Use antibody against the trifunctional peroxisomal FOX2 enzyme (22) at 1:200 dilution.
- Add the sequence encoding the SKL tripeptide (peroxisomal targeting signal 1) at the end of the GFP open reading frame (see Note 9). Transform wild-type or mutant strain. Prepare slides as in **Section 3.5**. SKL1-GFP brightly stains all peroxisomes of both vegetative and sexual cycles. Observe with GFP and FITC filter (32, 33).

3.8. Electron Microscopy

- Fix rosettes of asci as described in **Section 3.2**, Step 2 except using 2–5% glutaraldehyde in phosphate-buffer (pH 7.2) or in cacodylate buffer (pH 7.2) for 3 h at room temperature. Use a cavity slide because it allows easy removal of liquid from rosettes.

2. Wash three times with 0.2 M phosphate buffer or cacodylate buffer.
3. Transfer to a tube, and replace buffer with 2–4% OsO₄ in the same buffer. Postfix at room temperature for 1 h under a fume hood and in closed tubes.
4. Wash rosettes once in buffer and three times in water.
5. Stain in 5% aqueous uranyl acetate overnight. This step reinforces the contrast of synaptosomal complex components. Omit this step for cytoplasmic or cytoskeleton studies.
6. Dehydrate rosettes through an ascending ethanol series from 5 min at low concentration to 20 min at higher concentrations.
7. Dehydrate further by incubating in a fume hood twice for 10 min in 1, 2-propylene oxide (C₃H₆O).
8. Infiltrate sample through a graded series of 1, 2-propylene oxide and low-viscosity epoxy resin (or Epon or araldite): 1 h in a 1:1 mixture of 1,2-propylene oxide and resin, then overnight in resin alone.
9. Discard resin, replace with fresh resin (plus dimethylaminomethyl phenol if using Epon), and incubate for 1 h.
10. Embed rosettes or parts of rosettes in capsules filled with resin and polymerize 12–15 h at 60°C.

3.9. Single Ascus Embedding

1. Prepare siliconized coverslips the day before and keep out of dust.
2. Rosettes of ascii are fixed until Step 8 of **Section 3.8** (see **Note 12**).
3. Prepare slides: on each slide, glue two siliconized coverslips with small ribbons of magic tape (which is easy to remove) along two sides and put four small drops of resin on each coverslip (towards corner for easy handling of capsules, see below).
4. Prepare rosettes of ascii: Put a large drop of resin on a clean slide and, with a needle or pen, add one to three rosettes of ascii taken from Step 8 of **Section 3.8**.
5. Separate single ascii from the rosettes under a dissecting microscope. The presence of resin allows easy dissection of rosettes with insect needles. Keep ascii in resin while next slides are made.
6. Transfer each single ascus into one of the drops already prepared on coverslips and push it toward the surface of the coverslip, which saves time during sectioning because the ascus will be present in the first sections.
7. When all ascii are transferred, polymerize slides at 60°C for 12–15 h.
8. After polymerization, turn the coverslips over and reattach with magic tape. The drop of resin will now be sandwiched between the slide and coverslip, which allows an easy screen of meiotic stages.

9. Select meiotic stage in a phase-contrast microscope. After osmium fixation, chromosomes and spindle-pole bodies are visible. Mark the coverslip to note the meiotic stage.
10. Turn coverslips over again and reattach with magic tape.
11. Fill capsules with resin. Add a strip of paper indicating the meiotic stage plus strain in each. (Use a lead pencil) Turn capsule onto the selected drop.
12. Polymerize slides with capsules at 60°C for 12–15 h.
13. Remove capsules from coverslips (they slide on silicon) and store at room temperature. Capsules we have prepared are still good after 30 years.

3.10. Serial Sectioning

1. Dip a clean slide into formvar solution, lift it as firmly as possible and air dry.
2. Cut the upper edge of the formvar film with a razor and float the formvar film off onto a clean water surface in a large glass bowl. The film will be clearly visible in oblique light.
3. Place single-slot EM grids onto the formvar film.
4. Pick up the film plus 10–12 grids on a parafilm sheet: use the parafilm to push the film plus grids underneath the water surface, and then pick up. Air-dry the grids sandwiched between the formvar and the parafilm.
5. Cut each grid out from the formvar with a thin needle: make several holes in the formvar around the grid and lift with tweezers without tearing the formvar film.
6. Trim the capsule face to the ascus wall. With the single ascus embedding method, asci will be located at the flat surface of the capsule.
7. Cut sections (30–80 nm thick) with a diamond knife. Cut individual ribbons of sections that correspond to the length of the single-hole grid (when too small, ribbons have a tendency to turn in water). Detach the ribbon by running an eyelash (mounted with tape or nail polish on a wooden stick) along the outside of the diamond edge. Turn off microtome momentarily.
8. During sectioning, note the number of sections in each ribbon and note also the place of single, missing, or folded sections. This helps later for nuclear reconstruction.
9. Pick up ribbons of serial sections with an uncoated single-hole grid: Hold the grid edge with tweezers, lower the grid (dull surface down) onto the water over the ribbons and pick up quickly with drop of water.
10. Lower the grid with ribbons either onto a formvar-coated single-hole grid (prepared in advance and hold with tweezers under a dissecting microscope) or onto a formvar-coated ring (*see Note 13*) and let dry.

11. As quickly as possible, turn the microtome back on: thermal drift during the time the microtome is turned off will cause variable thickness for the first section.

12. Stain grids with uranyl acetate for 30 min at 60°C followed by lead citrate for 20 min at room temperature (use plastic kit with slots).

3.11. Three-Dimensional Reconstitution of Meiotic Nuclei

1. In transmission EM (TEM), observe grids at low magnification to survey possible holes in the formvar. Minimize exposure near holes and in thick sections, because prolonged exposure in these regions will lead to rupture of the formvar and loss of the grid.
2. Take pictures from all sections in which the nucleus is visible (from 50 to 80 for prophase nuclei, depending on the nuclear shape and volume). Bookkeeping of the order of sections and the place where sections were single or missing helps greatly. Print all pictures of the nucleus.
3. Reconstruct chromosomes: starting with section 1, trace each synaptonemal complex (SC) component visible on the picture on a thin plastic sheet with an ink pen. Addition of the section number allows easy checking of SCs or lateral/axial elements (LE) that cross the nucleus several times and that therefore are found in several places on each section. Trace the nuclear membrane as an outside marker.
4. Always trace the next section in overlap with the previous one. This corrects the possible distortion due to formvar and/or differences in section thickness. Ideally no section should be missing, but when SCs or LEs are cut perpendicularly, a lost section will not interfere with reconstruction or even with length measurement if it was noticed previously where the section was missing.
5. Once all SC components are traced (e.g., **Fig. 7.5A**), the nucleus can be analyzed for initiation of homolog recognition (e.g., **Fig. 7.5B** which shows homologous pair 3 aligned, and homologous pair 1 far apart), synapsis, recombination nodules (**Fig. 7.5C and D**), telomere position on the nuclear envelope, etc.
6. 3D rendering can be achieved by, for example, Vector Works and Zoom software (**Fig. 7.5A and B** and Refs. 8, 12, 36). With Zoom software, the nucleus can be turned, the path of each single SC or chromosome axis can be followed and the synapsis behavior of the different homolog pairs can be compared (8, 36). *Movies showing rotations of 3D reconstructions of individual chromosome pairs and of an entire nucleus are provided on the companion CD for this volume.*

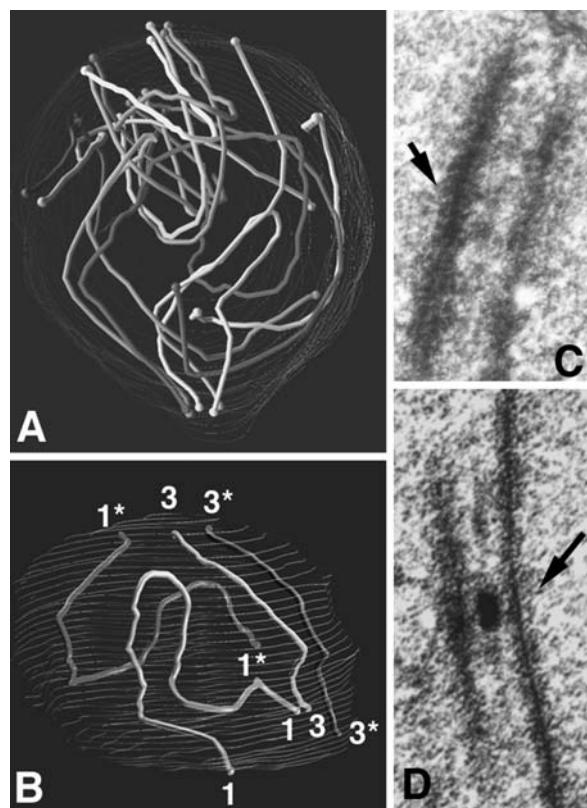


Fig. 7.5. Electron-microscope analyses of synaptonemal complex. (A) Early bouquet nucleus reconstructed from serial sections and treated by Zoom software to render a 3D image. *Movies showing rotations of 3D reconstructions of individual chromosome pairs and of an entire nucleus are provided on the companion CD for this volume.* (B) Early leptotene nucleus reconstructed from serial sections. The two pairs of homologous chromosomes show clearly that shorter pair 3 is already aligned when long pair 1 is still far apart. (C) *Neurospora* SC with small recombination nodule (arrow). (D) *Sordaria* SC with a large late recombination nodule (arrow).

3.12. Spreading and Silver Staining

1. Place five to six rosettes of ascospores on a silicon-treated coverslip in a drop of ice-cold paraformaldehyde for 4–5 min (*see Section 2.9*). Fixation time is critical.
2. Pick up the coverslip with a plastic-coated slide and knock the nuclei out of ascospores by tapping over the rosette with pencil or rubber hammer (*see Note 14*).
3. Freeze the slide on a cold plate or dry ice and quickly flip off the coverslip with a razor blade: some cells and nuclei will stick to the slide (freezing timing is critical).
4. Add a few drops of paraformaldehyde on the area previously covered by the coverslip and fix for 20–40 min. Mark the edges of the slide with an ink pen to indicate the area.

5. Remove the fixative, dip the slide into 0.4% Photoflo and let air-dry.
6. Mix one drop of silver nitrate with one drop of gel developer on a large coverslip.
7. Pick up the coverslip plus stain using the slide to be stained. Use gloves and use quick movement to avoid letting the stain run off. Incubate on a hot plate at 55°C for 2–3 min. (Higher temperature induces cracks in the plastic membrane.)
8. Wash off the coverslip with distilled water, taking care not to damage the plastic membrane, and wash the slide two to three times more in fresh water.
9. Dip the slide into 0.4% Photoflo and let air-dry.
10. Examine slides in light microscopy (phase contrast) to locate good SCs and mark their places with permanent-ink pen to guide where grids must be placed.
11. Cut the edge of the plastic membrane with a razor blade to lift one end, and float the plastic membrane off the slide carefully on a clean water surface in a large glass bowl. Lay single-slot grids on the marked areas. A tiny drop of superglue on the grid edge helps to keep the grid on the plastic membrane.
12. Pick up the membrane with a sheet of parafilm (*see Section 3.10, Step 4*) and let dry, covering to avoid exposure to dust.
13. Cut each grid out of membrane with a thin needle (*see Section 3.10, Step 5*) and store in a grid box before examination.

4. Notes



1. *S. macrospora* and *N. crassa* can also be collected in nature. *Sordaria* grows on dung and is easily isolated by placing small pieces of dry horse or cow dung on agar plates (*see Section 2.1, Steps 2 and 3 for medium*). After a few days, several fungi grow and fruit, among which *S. macrospora* and *S. fimicola* are the most common. As can be deduced from its name, *S. macrospora* makes larger ascospores than *S. fimicola*. *N. crassa* grows mostly on burned wood and sugar canes, but can also be found on bread when humidity is high. It produces large amounts of two types of powdery asexual spores (bright orange when made in light), which can be easily collected. *S. macrospora* does not produce asexual spores and can thus be cultivated with other organisms.
2. *Neurospora* fruiting is more strictly under nutritional control (nitrogen starvation) than for *Sordaria*, which can fruit in water (*for more details see Ref. 21*). Addition of filter paper

strips as a carbon source helps fruiting. Also, the concentration of amino acid supplements must be minimized because amino acid nitrogen inhibits crossing. Ascospores must be activated by heat before germination (21).

3. The propionic-iron-hematoxylin stain was first developed by Lu to describe the course of meiosis in the ascomycete *Gelasinospora* (26) and further adapted by Lu and Raju (27) to stain meiotic nuclei of *Coprinus*. It is also perfect for *Podospora*, *Neurospora*, *Aspergillus*, *Ascobolus*, and *Sordaria* nuclei (e.g., 12, 13, 18, 20, 24, 25, 32).
4. Because hematoxylin stains any acidic component of the cell, hydrolysis is necessary to lessen the cytoplasmic staining of the numerous ribosomes present in ascii.
5. Hematoxylin alone does not stain, but becomes a stain by lake formation with ferric ion (the mordant). The quality of staining therefore depends on the proportion of the two components. Insufficient ferric ion makes faint stains and too much causes hematoxylin to precipitate. The usual proportion is a small drop of iron and two drops of hematoxylin, but this depends on the concentration of the ferric acetate solution. Try out the proportion on a slide before use: the mix must be dark brown.
6. The cell wall of intact ascii is impermeable and thus interferes with antibody penetration and visualization. Ascii can be exposed to wall-digesting enzymes (e.g., Novozyme) to facilitate antibody penetration, but this often results in the loss of some components (e.g., actin microfilaments or even microtubules) and is difficult to reproduce routinely. Therefore, ascii are placed between the surface of a siliconized glass slide and a polylysine-coated coverslip and crushed with a blunted hypodermic needle. Ascii remain mostly complete but their bases are open, which is enough to allow antibodies to enter the cell without, apparently, damaging the complex cytoskeleton network (**Fig. 7.3A and B** and Refs. 29, 30 for *Sordaria*; Refs. 10, 11, 31 for *Neurospora*).
7. For antibody staining, controls include the use of primary or secondary antibodies alone, or use of an inappropriate secondary for a given primary antibody. Double staining: always use the red secondary antibody to label the less abundant nuclear component (e.g., actin in **Fig. 7.3A**) and fluorescein for the other (e.g., tubulin in **Fig. 7.3B**). For capturing images, start with the red filter labeling, then fluorescein and end with DAPI (exposure to UV wavelengths fades sample quickly). When possible, use antibodies from mouse or rat. Rabbits routinely make antibodies against fungal cell walls and therefore antibodies made in rabbits also stain ascus walls.

8. Fluorescence from FITC or rhodamine-tagged secondary antibodies as well as from GFP- or RFP-tagged proteins is scanned with an epifluorescence photomicroscope equipped with appropriate filters. Images are captured by CCD camera. Pictures are accurate enough for most analyses (e.g., **Fig. 7.1B –D**, **Fig. 7.4A**). Z-sections help follow each chromosome/bivalent and allow 3D reconstructions (e.g., by ImageJ software). Series of optical pictures can be done “by hand” in the absence of a Z-sectioning mechanism: they are less regular but good enough to locate bivalents and see which is lying in back of the others.
9. For *Sordaria*, all GFP-tagged genes are inserted ectopically because homologous recombination is a rare event (8, 12, 18). To be fully reliable, each tagged gene must complement the meiotic and sporulation defects with exactly the same efficiency as the complementing sub-clone when introduced in the corresponding mutant or deleted strain (8, 12, 18). Also, ascus sizes, synapsis and ascospore formation of each strain containing a GFP-tagged protein in a wild type or a mutant background are compared to an isogenic strain lacking the GFP tag. Tagged constructs are first introduced by transformation in a wild-type strain and further crossed to the strain of interest (using spore-color markers to recognize hybrid fruiting bodies). Analysis of the 8-spored asci allows easy recovery of double mutants (in four wild-type : four mutant asci) with GFP or RFP-tags. Protocols for GFP or RFP tagging are identical for *Neurospora*, except that transformation must be done in strains lacking both the *rid* and *sad-1*(or *sad-2*)genes in order to avoid respectively RIP and MSUD silencing (see **Section 1**) of the duplicated sequences (see 10, 11, 34 for details and plasmids).
10. Contrary to mycelia or protoplasts, it is difficult to keep isolated rosettes of asci alive for more than 2 h. After 1 h, asci start developing vacuoles and slowly die away. This is enough to screen GFP localization or to follow post-meiotic mitoses but for detailed analyses of meiotic prophase, asci must be fixed (examples in **Fig. 7.1B –D**). GFP and RFP staining is brighter when strains are maintained in the cold for a couple of hours before cytology. Also, because fruiting bodies are thick-walled and black, strains can be grown in the light. In contrast, for mycelial or protoplast observations, grow strains in dark or reduced light.
11. GFP and RFP staining (e.g., cohesins Rec8 and Spo76/Pds5, or Rad51, Msh4) remain visible after overnight drug treatment.
12. First developed for *Neotiella* (37), single-ascus embedding has since been used for both *Neurospora* and *Sordaria* (6, 7,

8, 12–16, 18, 19, 36). Ascus growth during prophase (see Section 1) is advantageously used for selection and storage of specific meiotic or mutant stages in large quantities and for several years. Another advantage comes from the fact that asci are close to the surface before sectioning, which saves a lot of time at the microtome.

13. Lowering a single-hole grid with ribbons over a formvar-coated ring with a hole larger than the grid is easier than overlap of coated and uncoated single-hole grids because the single-hole grid with ribbons often turns, provoking the loss of part of the sections. When dry, the grid is pushed out of the ring hole with a nail head that is the same size as the grid. First make several holes around the grid, then push with the nail head and take off with tweezers without tearing the formvar membrane.
14. A protocol for spreading SC of *Neurospora* was developed by Lu (17). It also works for *Sordaria*, but in both organisms the breakage of meiotic cell walls remains a difficult step, which limits the routine use of the technique.

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Chapter 8

Meiotic Cytogenetics in *Coprinus cinereus*

Miriam E. Zolan and Patricia J. Pukkila

Abstract

The basidiomycete fungus *Coprinus cinereus* has naturally synchronous meiosis and is amenable to analysis using an array of well-developed genetic and molecular tools. In this chapter, we explain in detail the two methods most commonly employed for *C. cinereus*, staining of intact gill segments and chromosome spreads, with an example of the application of each. We describe iron-hematoxylin staining of intact gill segments for the brightfield examination of meiotic progression, and the use of surface spreads and fluorescence in situ hybridization (FISH) to investigate meiotic chromosome pairing. Gill segments can alternatively be stained with DAPI for the determination of meiotic stage, or propidium iodide for the quantitation of nuclear DNA content, and the chromosome fixation and spreading techniques used for FISH are also suitable for immunolocalization studies of chromosomal proteins.

Key words: Coprinus, fluorescence in situ hybridization, meiosis, chromosome pairing.

1. Introduction

In contrast to most mushrooms, the basidiomycete *Coprinus cinereus* can be cultured easily in the laboratory on defined medium. The entire life cycle can be completed in just two weeks in vigorous strains. Accordingly, *C. cinereus* has become an important experimental system for studies of multicellular development in fungi. Following mating of two compatible strains, the resulting dikaryotic hyphae, which maintain a single haploid nucleus from each parent in every cell, respond to light and nutritional signals by forming small aggregates (hyphal knots) on the surface of the culture dish. After 2–3 days at 25°C, some of the knots develop further into cylindrical primordia (2 mm in diameter). If one slices a primordium in the vertical plane, distinct tissues (vertically oriented stipe below and horizontally-oriented gills above, with

an exterior layer of large veil cells) are obvious. After another 2–3 days, the primordia (now 0.5–1.5 cm in diameter) contain about 100 double-sided gills, and each gill surface is composed of thousands of club-shaped cells (basidia).

A striking feature of this species is that nuclear fusion (karyogamy) and each subsequent stage of meiosis occur synchronously in all basidia of a fruit body (and all fruit bodies of similar size in the culture). The time of karyogamy can be controlled by the light cycle. Small pieces of gill tissue can be removed from a fruit body without affecting development of the remaining tissue. Following meiotic prophase (8 h) and the two meiotic divisions (4 h), four spores develop on the surface of each basidium (the developing spores are attached to the basidium by small surface projections called sterigmata). The nuclei migrate through the narrow sterigmata into the developing spores. The stipe elongates and the cap expands, opening up like an umbrella with the spores on the lower surface. The spores are then actively discharged from the cap, which deliquesces, leaving an inky puddle filled with black spores on the surface of the medium. Prior to deliquescence, gill pieces with attached spores can be easily removed, dried down, and subjected to tetrad dissection using a micromanipulator.

Cytogenetic approaches are of three general types, which utilize either intact gill segments, sections of embedded material, or surface spreads of meiotic chromosomes. Each approach has its strengths. For intact gill segments, mushroom caps can be sampled quickly, samples can be stored in fixative essentially indefinitely before slides are prepared, and hundreds or thousands of cells can be viewed at relatively low resolution (1). The examination of sections of embedded material using electron microscopy (EM) allows higher-resolution analysis of meiotic chromosomes. However, while sections can be employed to examine many nuclei, each section samples only a segment of each nucleus. Serial sectioning with three-dimensional reconstruction allows the complete analysis of the meiotic karyotype, but is impractical for the examination of large numbers of nuclei (2, 3). In contrast, hundreds of nuclei can be examined in their entirety by the use of “chromosome spreads,” in which disrupted nuclear contents are spread onto the surface of slides. The spreading procedures for *C. cinereus* were developed to examine the synaptonemal complex using silver staining and EM (3, 4) but are easily adapted for brightfield and fluorescence microscopy applications.

In this chapter, we explain in detail the two methods most commonly employed for *C. cinereus*, staining of intact gill segments and chromosome spreads, with an example of the application of each. We describe iron-hematoxylin staining of intact gill segments for the brightfield examination of meiotic progression (1) and the use of surface spreads and fluorescence *in situ* hybridization (FISH) to investigate meiotic chromosome pairing (5).

Gill segments can alternatively be stained with DAPI for the determination of meiotic stage, or propidium iodide for the quantitation of nuclear DNA content (6) and the chromosome fixation and spreading techniques used for FISH are also suitable for immunolocalization of chromosomal proteins (7).

2. Materials

2.1. Fruit Body Formation

1. YMG Medium: 4 g/l yeast extract (Difco), 10 g/l malt extract (Difco or Oxoid), 4 g/l dextrose, and 15 g/l agar (*see Note 1*).
2. Pyrex 90 × 50 cm dishes with Pyrex covers, or test tubes with loosely fitting caps (*see Note 2*).

2.2. Tissue Harvesting and Fixation

1. 10% chromic acid: 10 g chromium trioxide in 100 mL H₂O. Store at room temperature.
2. Fixative (use immediately after preparing): 9 vol absolute ethanol, 6 vol propionic acid, 2 vol 10% chromic acid (8).

2.3. Gill Dissection, Acid Hydrolysis, and Iron-Hematoxylin Staining

1. 70% ethanol.
2. Hydrolysis solution (use immediately after preparing): 1 vol HCl, 1 vol absolute ethanol.
3. Carnoy's rinse (use immediately after preparing): 3 vol ethanol to 1 vol glacial acetic acid.
4. 3% (w/v) ferric ammonium sulfate in 50% propionic acid (*see Note 3*).
5. 2% Hematoxylin in 50% propionic acid (store at room temperature in the dark).
6. Glycerol.

2.4. Chromosome Spreads

1. Slides: for immunofluorescence or FISH, use either plain glass slides or aldehyde-coated slides (CEL Associates, Inc., Pearland, TX). Clean plain slides with Sparkle (A.J. Funk & Co., Elgin, IL) and wipe dry with Kimwipes. Clean aldehyde slides with compressed air.
2. Coplin staining jars with screw caps (used for both spreads and for FISH).
3. Borate buffer: 0.02 M sodium borate adjusted to pH 9.22 with 0.5 N NaOH. Filter through 0.2 μm filter and store at 4°C.
4. Paraformaldehyde (4%): add 4 g powder to 100 mL H₂O. Heat to 40°C. Add 10 N NaOH (about 18 drops) drop by drop, until solution is clear. Cool to room temperature. Adjust pH to 6.5–7 using concentrated HCl (drop by drop,

10–14 drops). Adjust pH back to 8.2 using 0.02 M borate buffer (pH 9.2). Filter through 0.2 µm filter. Store at 4°C for up to two weeks. Check pH at least every other day and discard if pH falls below 7.9.

5. 4% Paraformaldehyde + 0.03% SDS: 50 mL 4% paraformaldehyde + 75 µL 20% SDS.
6. 0.4% Photoflo rinse solution: 4 mL Photoflo 200 (Kodak) + 1,000 mL H₂O. Adjust to pH 8.2 with 0.02 M borate buffer; filter through 0.2 µm filter.
7. 0.9% Optilux in chloroform: dissolve 0.45 g Optilux plastic (Falcon Petri Dish #1001; *see Note 4*) in chloroform, using a glass container with lid. Do this in a fume hood.
8. Spreading dishes: use 2 cm (inner diameter) thick-walled glass dishes. The ones we use have a volume of 1.5 mL and hold about 1.8 mL when a convex surface is made (**Section 3.4**, Step 5). Soak for 2 h in 95% ethanol. Dry in the hood on paper towels. Coat in Optilux by immersing in 0.9% Optilux solution (use large tweezers and let go completely for 1 s). Tap excess off dishes, arrange them on their sides inside a glass beaker, and let them dry overnight in the hood. Soak used dishes overnight in chloroform, using a beaker with a glass lid.
9. 0.5% NaCl: filter through 0.2 µm filter and store at room temperature.
10. Drill: 6–18 V, portable, max. speed 600 rpm, plus plastic drill pestles (USA Scientific #1415-5390, Ocala, FL)

2.5. FISH

1. Probes: We routinely use two overlapping cosmids, with total complexity >30 kb, for each locus to be examined (*see Note 5*), but smaller probes can be used (the complexity of the probes used in the experiments shown in **Fig. 8.2** is about 30 kb). Label probes in large batches with either biotin or digoxigenin using commercially available nick translation kits (BioNick: Invitrogen, Carlsbad, CA; DigNick: Roche, Mannheim, Germany). Purify probes with Qiagen's PCR Purification Kit (Valencia, CA), using RNase-free sterile water for elution. After elution, concentrate probes using a Speed-Vac (no heat – do not let the DNA dry), dissolve in 10 µL of the hybridization solution per 1 µg DNA (add hybridization solution to the tube with concentrated probe to eliminate DNA loss), and store at –20°C until use.
2. RNase A: 1 mg/mL stock solution, stored at –20°C. Dilute to 0.1 mg/mL in 2x SSC. Make up a fresh dilution for each experiment.
3. 0.1 M Triethanolamine-HCl, pH 8: Filter through 0.22 µm filter. Add 5 µL of acetic anhydride per mL; add the acetic anhydride fresh to the triethanolamine each time.

4. Formamide (ultra-pure grade): Denaturation solution should be made fresh each time. Other solutions containing formamide should be relatively fresh (used within a few months) to prevent background. Formamide should be kept at 4°C when not in use.
5. Denaturation solution: 70% formamide in 2X SSC. Prepare fresh using sterilized SSC.
6. Hybridization solution: 2X SSC, 50% formamide, 10% dextran sulfate (from a 50% stock solution in water, filtered using 0.2 µm filter, stored at -20°C), 1 mg/mL sonicated salmon sperm DNA (from 10 mg/mL stock).
7. Blocking solution: 5% BSA plus 0.1% Tween-20 in 4X SSC. Prepare fresh each time.
8. UltraAvidin-rhodamine (5 µg/mL; Leinco Technologies, Inc., St. Louis, MO): 5 µL of 1 µg/µL stock in 1 mL blocking solution.
9. Wash solution: 4X SSC + 0.1% Triton X-100.
10. Biotinylated anti-avidin D (5 µg/µL, from goat; Vector Laboratories, Burlingame, CA): 10 µL of 500 µg/µL stock in 1 mL blocking solution.
11. Anti-digoxigenin-fluorescein, Fab fragments (10 ng/µL, from sheep; Roche, Mannheim, Germany): 10 µL of 100 µg/mL stock in 100 µL blocking solution.
12. Anti-fluorescein antibodies (10 ng/µL, from mouse; Roche, Mannheim, Germany): 40 µL of 50 µg/mL stock into 200 µL blocking solution.
13. FITC-conjugated rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark): 4 µL in 200 µL blocking solution.
14. FITC-conjugated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark): 4 µL in 200 µL blocking solution.
15. 5% Normal goat serum (Vector Laboratories Inc., Burlingame, CA) in 2X SSC.

3. Methods

Meiosis occurs in a highly synchronous fashion in *C. cinereus*, and it is often useful to store samples of material collected at particular times for subsequent verification of meiotic stage. The procedure for iron-hematoxylin staining of intact gill segments described here (1) provides adequate resolution of chromosome morphology to determine the proportion of basidia that have completed karyogamy (nuclear fusion), the proportion with synapsed chromosomes,

and the proportion that have completed either the first or second meiotic division. Chromosomes, nucleoli, and spindle-pole bodies can be observed by these methods using simple microscopes equipped with only bright-field illumination (**Fig. 8.1**), and because the gills remain intact, there is no possibility of sampling bias that can occur in squashed material if nuclei in different stages adhere differentially to the underlying substrate. The fixation step is rapid and simple, and samples from many experiments can be stored at -20°C indefinitely until it is convenient to perform the more lengthy hydrolysis, staining, and analysis steps.

Surface spreads of meiotic chromosomes must be made using freshly fixed tissue. However, slides containing spread preparations can be stored for months at -20°C before the hybridization steps described for FISH. Two cautions must be kept in mind when surface spreads are used for any meiotic analysis. First, sample bias will occur if nuclei at different stages adhere differentially to slides. In practice, nuclei of all stages are observed, and the proportion of each stage at any timepoint is generally consistent (5). Second, weak, dynamic interactions may be disrupted. Therefore, spreads serve well only for the examination of stable

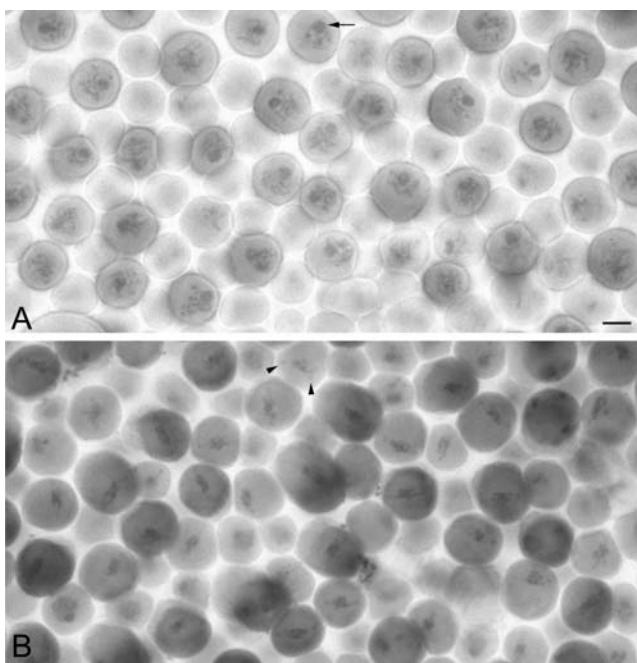


Fig. 8.1. Nuclei stained with iron-hematoxylin in intact gill segments. **Panel A** shows a gill segment that was fixed 6 h after nuclear fusion (karyogamy), and chromosomes in mid-pachytene are seen in all nuclei. **Panel B** shows a gill segment that was fixed 10 h after karyogamy, and chromosomes in metaphase of the first meiotic division are seen in the majority of the nuclei. The *arrow* in **A** indicates a nucleolus, and the *arrowheads* in **B** indicate spindle-pole bodies. Since adjacent basidia (*circles*) are often of unequal height, not all nuclei can be viewed at a single focal plane. Bar = 5 μm .

chromosomal interactions. These disadvantages are balanced by the utility of surface spreads for the facile examination of hundreds of nuclei and the relatively high-resolution analysis they afford. Depending upon the probe sequences chosen, FISH can be used to examine homolog pairing, chromosome compaction, or sister-chromatid cohesion for multiple chromosomal loci (Fig. 8.2).

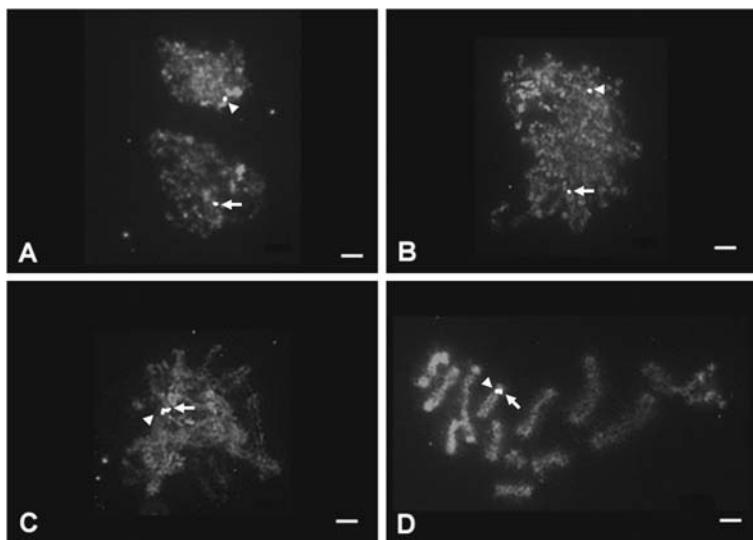


Fig. 8.2. FISH analysis of homolog pairing. Chromosome spreads were hybridized as described in this chapter, using probes for the *B42* and *B43* mating type loci. The *B* locus sequences are allelic in chromosomal position but dissimilar in DNA sequence (9) and therefore serve as homolog-specific probes. **Panel A** shows the two nuclei of a dikaryotic basidium, pre-fusion. **Panel B** shows a pre-leptotene nucleus after karyogamy (note that the *B* loci are not paired). **Panel C** shows a zygotene nucleus (the *B* loci have paired), and **panel D** shows a pachytene nucleus. *Arrows* and *arrowheads* indicate *B42* and *B43* probes, respectively. Bar = 2 μ m.

3.1. Fruit Body Formation

1. Inoculate compatible monokaryotic strains side-by-side on YMG medium and culture at 37°C where mating and dikaryon formation will occur. Use this plate, or subcultures from it, to inoculate fruiting dishes or tubes (see Note 6). Dikaryons can be stored for several weeks at 4°C.
2. Place a small piece of the dikaryotic culture on 125 mL of YMG medium in the 90 X 50 Pyrex dish with a loosely fitting lid. Incubate the dishes at 37°C in the dark for 5 days, which is sufficient time for the dikaryon to cover the medium.
3. Transfer the dishes to a humid room at 25°C and induce synchronous fruiting by illuminating the cultures for 16 h in each 24-h period. Hyphal knots develop into primordia with

recognizable gill and stipe tissue. The primordia increase in size, and meiosis is initiated immediately following nuclear fusion (karyogamy) in the basidial cells of the gill. Under our culture conditions, karyogamy is complete by 1 h prior to the start of the last light cycle before spores are shed. Thus, the stages of meiosis can be moved around the clock by the timing of the light and dark periods. A typical fruit body contains 100 double-sided gills, and each has about 5×10^5 basidia lying side-by-side on the surface.

3.2. Tissue Harvest and Fixation

1. Remove a few gills from a developing fruit body by slicing a wedge-shaped piece of the cap with an X-acto knife or scalpel. Multiple samples can be removed from the same mushroom without disturbing its subsequent development if the mushroom remains attached to its underlying hyphal substrate (*see Note 7*). Use forceps to strip away the overlying veil cells, and remove any stipe tissue.
2. Place the gills in freshly prepared fixative and hold at room temperature overnight. Then they can either be stored at -20°C or stained.

3.3. Gill Dissection, Acid Hydrolysis, and Iron-Hematoxylin Staining

1. Rinse the fixed gill tissue in 70% ethanol and transfer to 70% ethanol in a shallow depression slide and examine under a dissecting microscope. Use forceps to peel away single layers of tissue (one half of the double-sided gill).
2. Heat 1 mL of the hydrolysis solution to 70°C in a glass tube using a Temp-Blok module heater. Add the gill tissue to the solution and incubate for exactly 1 min at 70°C .
3. Remove the tube from the Temp-Blok and hold in the air for exactly 2 min.
4. Plunge the tube into an ice bucket and cool to 4°C .
5. Remove the tissue from the cold hydrolysis solution and place into a clean tube containing Carnoy's rinse. Flick the tube several times to facilitate the rinse.
6. Transfer the tissue to a fresh tube of Carnoy's rinse (*see Note 8*).
7. Place the tissue in a small drop of ferric ammonium sulfate or saturated ferric acetate (*see Note 3* and Acknowledgments) on a microscope slide.
8. Add an equal-sized drop of 2% hematoxylin, and mix the drops thoroughly.
9. Check the staining by placing a small drop of glycerol on a slide, adding a piece of gill arranged with the basidia facing up, and then adding a coverslip. If staining is too intense, a precipitate may be observed, which can be prevented by

diluting the hematoxylin and/or rinsing the gill in 70% ethanol before mounting. If staining is too faint, add a coverslip and seal it with dental wax. Leave the slide overnight at room temperature. The next day, remove the coverslip, rinse the gills in 70% ethanol, mount in glycerol, and observe using bright-field microscopy.

3.4. Chromosome Spreads

1. Place drops (~ 0.1 mL) of 4% paraformaldehyde into 1.5 mL microcentrifuge tubes and hold on ice.
2. Remove a sliver of gills (about 1 mm) from a mushroom cap using either a scalpel or an inoculating needle. Then, either scoop gills away from veil cells using a scalpel, or peel the veil off with forceps. Also remove any stipe tissue.
3. Place gills in paraformaldehyde (in a microcentrifuge tube from step 1) and set at room temperature. Start a timer for 2 min.
4. At 1.5 min, begin drilling, in the microcentrifuge tube. Drill samples 7–12 times with four layers of paper towels under the microcentrifuge tube as a cushion during drilling; the solution should be milky, and the drilling should be completed within 30 s. After drilling, place tubes on ice. A tube can sit on ice as long as 20 min, but it is also fine to proceed to the next step right away.
5. Fill spreading dishes with 1.8 mL 0.05% NaCl (until moderately convex). *See Note 9.*
6. Using a Pipetman P20, drop/touch 13 μ L of the cell suspension to the surface of the 0.05% NaCl. Turn a cleaned slide and touch to the surface of the solution. A circle of solution should be apparent on the underside of the slide.
7. Quickly lift the slide straight up and turn it back over. Some solution (a circle) should remain on the surface of the slide.
8. Repeat with two more slides, using a fresh spreading dish and fresh 13 μ L aliquot of cell suspension for each slide.
9. Set down the slides face up, and add 8–10 drops of 4% paraformaldehyde + 0.03% SDS to each (enough to create large ovals on the slides).
10. Leave slides for 20 min, then drain, dip into H₂O seven times each to rinse off the NaCl, and tap the short edges of the slides on paper towels to remove excess H₂O. Dip slides into 0.4% Photoflo, seven times in two different jars, and prop each on a short edge to dry for 1 h or overnight.

3.5. FISH

1. Spread chromosomes onto plain or aldehyde-coated microscope slides, which can be stored at -20°C until use (slides can be stored for at least 6 months).
2. Bake slides at 65°C for 2–3 h (we use an upside-down temp-block).

3. Add 150 μ L of 0.1 mg/mL RNase A in 2X SSC to the slide, cover the slide with a 22 \times 40 mm coverslip (for short incubations, we have used pieces of parafilm or other plastic cut to size) and incubate at 37°C for 30 min. Slide incubations such as this are done in a tightly closing box with a moist sponge inside.
4. Wash slides three times in 2X SSC at room temperature, 2 min. each wash. Perform all wash steps by soaking slides in a container filled with the appropriate solution. We use lidded glass jars designed so that the slides stand vertically. (For more effective washing, jars with slides can be put on a low-speed shaker, but this creates a higher risk that DNA spreads will be washed away.) All wash solutions (SSC, etc.) should be made with MilliQ or nanopure H₂O and sterilized through a 0.2 μ m filter. After each wash, blot slides lightly by touching a short edge to a paper towel.
5. Dehydrate slides in a room temperature ethanol series (2 min each in 70, 85, then 100% ethanol), and air dry completely.
6. Denature chromosomes by immersing slides in 70% formamide in 2X SSC (made fresh using sterilized SSC) at 70°C for 2 min. For this and all warm wash steps, we place the wash container in a water bath or constant-temperature room (37°C). Warm the formamide solution in a 75°C water bath (the temperature of the solution inside container will be cooler). Do not let the formamide solution sit at the high temperature for an extended period of time (15–20 min before use is adequate).
7. Dehydrate slides in a 4°C ethanol series (2 min each in 70, 95, then 100% ethanol). It is not necessary to dry them completely.
8. Acetylate slides (to reduce background) for 1 min in 0.1 M triethanolamine-HCl, pH 8 plus acetic anhydride (5 μ L of acetic anhydride per mL, added fresh to the triethanolamine each time). Shake the jar lightly during this wash.
9. Wash slides twice in 2X SSC at room temperature for 5 min. Then drain excess solution, but do not allow slides to dry completely.
10. Denature biotinylated and digoxigenin-labeled probes (1 μ g of each probe per slide) at 75°C for 5 min, using either a water bath or a heat block.
11. Add probes to slides, and then cover slides with glass coverslips. Seal coverslips with rubber cement, and incubate slides for approximately 36 h (over two nights) at 37°C, in a box with a moist sponge to keep slides from drying out.

12. Wash slides as follows:
 - a. 50% formamide in 2X SSC at 37°C for 30 min.
 - b. Twice in 2X SSC at 37°C, 10 min each wash.
 - c. Twice in 0.5X SSC at 50°C, 10 min each wash.
13. Block slides with 150 µL blocking solution at room temperature for 20 min (added to slide and covered with a coverslip).
14. Drain slides but don't rinse, dispense 75 µL UltraAvidin-rhodamine, add a coverslip (but don't seal), and incubate slides at 37°C for 30 min in a black box (black electrical tape works well) containing a moist sponge. Do all steps involving fluorescent reagents out of direct light, and store reagents containing fluorescent dyes in dark-tinted microfuge tubes.
15. Wash slides three times in wash solution at 37°C, 10 min each wash.
16. Cover spreads with 75 µL 5% normal goat serum in 2X SSC, add a coverslip, and incubate slides at room temperature for 10 min.
17. Drain slides but don't rinse, dispense 50 µL of biotinylated anti-avidin and 50 µL of anti-digoxigenin-fluorescein, and cover with a coverslip. Incubate slides at 37°C for 30 min.
18. Wash slides three times in wash solution at 37°C, 10 min each wash.
19. Dispense 75 µL blocking solution, and incubate slides at room temperature for 10 min.
20. Drain slides but don't rinse, dispense 75 µL UltraAvidin-rhodamine and 50 µL anti-fluorescein antibodies, add a coverslip, and incubate slides at 37°C for 30 min in a black box containing a moist sponge (as in Step 14).
21. Wash slides three times in wash solution at 37°C for 10 min each wash. If the signal is bright enough, the procedure can be stopped here. We have done up to two additional fluorescein applications (below) although we have had good luck stopping after Step 23. This results in two layerings of both rhodamine and fluorescein. If stopping here or after Step 23, proceed to Step 26.
22. Dispense 75 µL FITC-conjugated rabbit anti-mouse antibody, cover spreads with a coverslip, and incubate slides at 37°C for 30 min.
23. Wash slides three times in wash solution at 37°C, 10 min each wash.
24. Dispense 75 µL FITC-conjugated swine anti-rabbit antibody, cover spreads with a coverslip, and incubate slides at 37°C for 30 min.
25. Wash slides three times in wash solution at 37°C, 10 min each wash.

26. Mount slides in one drop (~10 μ L) of Vectashield plus DAPI (Vector Laboratories; coverslips should also be cleaned with Sparkle or compressed air).
27. Seal slides with nail polish and store at 4°C. Slides are good for at least several weeks.
28. View slides using a fluorescence microscope equipped with appropriate filters for fluorescein, rhodamine, and DAPI. An ideal system employs a double filter wheel or other adaptation so that filter cubes are not switched; otherwise, the different wavelength images captured per image might not be in perfect registration.

4. Notes



1. Some strains fail to make fruit bodies unless Difco agar is used. Other strains prefer Sigma agar (St. Louis, MO).
2. Air circulation within the dish is critical for fruit body development and can be accomplished by placing two small folds of tape on each side of the dish so that the lid rests on the tape and is held slightly above the dish. Air circulation within tubes is adequate with standard caps. Dishes are used when large numbers of fruit bodies from the same strain are needed, and tubes are used when many different strains are cultured simultaneously.
3. Although staining is reasonable with ferric ammonium sulfate, superior staining is obtained with saturated ferric acetate in 50% propionic acid that has been stored in the dark for several months before use.
4. Optilux Petri dishes are now sold by BD Biosciences, San Jose, CA, but currently available plates have not been tested.
5. The choice of probes for FISH should be carefully considered. A comparison of mutant and wild-type strains is best served by the analysis of multiple chromosomal regions, including telomeric and interstitial sites.
6. Serial subcultures should be avoided, since spontaneous variants may be propagated instead of the original strain or cross.
7. Each mushroom is monitored for the completion of development (stipe elongation, cap deliquescence, and spore formation in the case of wild-type strains). Discard samples taken from any mushroom that fails to complete development.
8. Chloride ions interfere with the staining reaction, so the washes after hydrolysis are important.

9. Alternatively, Steps 5 and 6 can be eliminated and the homogenate can be deposited in a drop directly on the cleaned slide and flooded with the 4% paraformaldehyde + 0.03% SDS as in Step 7. This method allows recovery of well-spread nuclei primarily at the margins of the oval.

Acknowledgments

The authors would like to dedicate this chapter to Dr. Ben Lu, who introduced each of them to *C. cinereus* cytology. He first provided P.J.P. with some aged ferric acetate so she could use the optimal iron-hematoxylin method right away. (In turn, she would be happy to supply aged ferric acetate to anyone who would like to try this method.) He also taught the techniques of chromosome spreading and silver staining to two generations of Zolan lab students and post-docs, who bestowed on him the title of Zolan-lab “fairy godfather.” M.E.Z. also thanks Sasha Savitskyy for help on this manuscript and for the data presented in Fig. 8.2, and Heather Palmerini, Felicia Kennedy, Claire Burns, and Elizabeth Sierra Potchanant for helpful comments on the manuscript. The work in our laboratories is supported by NSF grant EF-0412016 (to P.J.P.) and NIH grant GM43930 (to M.E.Z.).

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Chapter 9

Cytological Analysis of *Arabidopsis thaliana* Meiotic Chromosomes

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Abstract

Advances in molecular biology and in the genetics of *Arabidopsis thaliana* have led to this organism becoming an important model for the analysis of meiosis in plants. Cytogenetic investigations are pivotal to meiotic studies and a number of technological improvements for *Arabidopsis* cytology have provided a range of tools to investigate chromosome behaviour during meiosis. This chapter includes protocols on basic cytology, FISH analysis, immunocytology, a procedure for a meiotic time course and electron microscopy.

Key words: *Arabidopsis thaliana*, meiosis, cytogenetics, FISH, immunocytology, meiotic time course, electron microscopy.

1. Introduction

Flowering plants possessing large chromosomes have been frequently used as material for cytogenetic investigations of the meiotic pathway. However, many of these species often have limited molecular genetic tools to enable our understanding of meiosis. Linking cytogenetical observation with molecular genetics is essential if we are to understand the mechanisms of meiosis. The developments in cytological techniques described in this chapter, combined with the extensive range of genomic resources that are available, have established *Arabidopsis thaliana* as an excellent system for the analysis of meiosis (1).

One of the reasons that *Arabidopsis* was originally selected for genome sequencing was that it has a relatively small genome (120 Mbp) and possesses only a small proportion of highly repetitive

and moderately repetitive sequences (2). Because of this, *Arabidopsis* chromosomes ($2n = 10$) are particularly small compared to those used in earlier cytogenetic studies, e.g., the common onion (*Allium cepa*, $2n = 16$) whose genome is around 30-times greater than that of *Arabidopsis*. This difference necessitated the adaptation and development of cytogenetic techniques to overcome this apparent drawback. During the last decade, our group has developed a range of techniques and resources in the application of molecular cytogenetics and molecular biology to the study of meiosis in *Arabidopsis*. As a result, we have shown that this model plant is an excellent system for the study of meiosis by combining the accessibility of plants for cytogenetical studies with the molecular approaches that have been developed in yeast (3, 4).

This chapter describes our current protocols, including troubleshooting, for the cytological analysis of *Arabidopsis* chromosomes. The techniques described here include the basic preparation of meiotic chromosomes, fluorescent in situ hybridisation (FISH) analysis, immunolocalisation of chromosome associated and meiotic proteins, time course of meiosis and ultra-structural chromosome analysis by electron microscopy (EM).

2. Materials

Arabidopsis Growth Conditions: Sow seeds of *Arabidopsis* in 6 cm diameter pots in soil-based compost. Grow the plants in dedicated growth chambers maintained at 18°C with a 16 h light cycle.

2.1. Fixation and Preparation of Slides for Basic Cytology, FISH and Time Course Studies

1. Fixative: Three parts absolute ethanol: one part glacial acetic acid, keep on ice. Prepare fresh fixative as required and discard at the end of the day. (See Note 1).
2. 0.01 M Citrate Buffer: Prepare a working solution of the buffer (pH 4.5) by using 4.45 mL 0.1 M sodium citrate, 5.55 mL 0.1 M citric acid, made up to 100 mL with sterile deionised water.
3. Stock digestion medium: Dissolve 1% cellulase, 1% pectolyse in a working solution 0.01 M citrate buffer, pH 4.5. Store in aliquots at -20°C.
4. Digestion medium: Mix 333 µL of the stock digestion medium with 667 µL 0.01 M citrate buffer, pH 4.5.
5. 60% Acetic acid: Dilute glacial acetic acid with sterile deionised water.

6. Counterstaining solution: 10 µL/mL 4, 6-diaminido-2-phenylindole (DAPI) at 1 mg/mL in an antifade mounting medium, Vectashield (Vector). Store DAPI as a stock solution at 1 mg/mL in sterile deionised water. Dispense in aliquots and store at –20°C.

2.2. FISH Analysis

1. Pre-treatment washing solution: 2 × SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). Prepare a stock solution 20 × SSC and store at room temperature.
2. Digestion medium: 0.01 g pepsin in 100 mL 0.01 N HCl. Prepare freshly and pre-heat for a few minutes at 37°C before use.
3. Paraformaldehyde fixative: Weigh out 4 g paraformaldehyde (EM grade) in the fume hood. Dissolve in 100 mL of sterile deionised water and four drops of 1 N NaOH, pre-warmed to 60°C in the microwave. Stir the mixture on a magnetic stirrer for 1 h, or until it dissolves before filtering through Whatman paper. Adjust the pH to 8.0. The fixative can be stored for up to one week at 4°C.
4. Alcohol series with absolute ethanol and sterile deionised water: 70, 90 and 100% ethanol for slide dehydration.
5. Probe labelling: Use the nick translation labelling kit (Roche) following the manufacturer's instructions. Use either Biotin-16-dUTP or digoxigenin-11-dUTP (Roche) as nucleotide conjugates for DNA labelling.
6. Hybridisation mix: Weigh out 1 g dextran sulphate (use high MW 500,000), 5 mL deionised formamide, (*see Note 2*) and 1 mL 20 × SSC made up to 7 mL with sterile deionised water. Dissolve at 65°C, cool and adjust pH to 7.0. Aliquot into 1 mL microfuge tubos and store at –20°C.
7. Prepare 20 µL of probe mixture per slide: 14 µL of hybridisation mix, 0.5–2 µL of labelled probe, and if necessary add sterile deionised water to 20 µL. Thus, the final hybridisation mix consists of 50% deionised formamide, 2 × SSC and 10% dextran sulphate pH 7.0.
8. Vulcanising rubber solution (e.g., as found in bicycle wheel repair kits).
9. Post-hybridisation washes : Prepare three Coplin jars of 50% formamide-2 × SSC pH 7.0 (150 mL deionised formamide, 30 mL 20 × SSC and 120 mL sterile deionised water); 1 Coplin jar of 2 × SSC; and 1,000 mL of wash (200 mL 20 × SSC, 800 mL sterile deionised water, and 0.5 mL Tween 20) for all subsequent washes.
10. For detection of digoxigenin probes, make up antibodies as either anti-digoxigenin-FITC or anti-digoxigenin rhodamine at (5 ng/µL) made up in digoxigenin blocking solution shortly

before use. The blocking solution is made from 100 mL 4 × SSC, 0.05% Tween 20 and 0.5% Roche Digoxigenin blocking reagent. Centrifuge at 19,000*g* for 5 min and store the supernatant in 1 mL aliquots at –20°C.

11. For biotin-labelled probes use Streptavidin-Texas Red/Cy3/FITC (Roche) made up in biotin blocking solution. Prepare from 100 mL 4 × SSC, 0.05% Tween 20 and 5 g dried skim milk. Centrifuge at 19,000*g* for 5 min and store the supernatant in 1 mL aliquots at –20°C.
 12. Counterstaining solution: *See Section 2.1*, Step 6.
- 2.3. Time Course**
1. BrdU labelling kit (Roche). (*See Note 3*).
 2. Counterstaining solution: *See Section 2.1*, Step 6.

2.4. Immunocytology

2.4.1. Spreading Technique

1. Digestion medium: Dissolve 0.1 g (0.4%) cytohelicase (Sigma) with 0.375 g (1.5%) sucrose and 0.25 g (1%) poly-lvinylpyrrolidone in 25 mL sterile deionised water. Dispense aliquots of 1 mL and store at –20°C.
2. Spreading medium: 0.05% Triton X-100 in freshly distilled water.
3. Paraformaldehyde fixative: *See Section 2.2*, Step 3.
4. Blocking buffer: Dissolve 1% Bovine Serum Albumen (BSA) in phosphate buffered saline, PBS. (We use prepared PBS tablets, but PBS can also be made up from 10 × stock consisting of 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄, adjusted to pH 7.4 if necessary. Autoclave before storage at room temperature. The working solution of the buffer is 1:10 with sterile deionised water.)
5. Primary antibodies: Make up to the relevant dilution, e.g. 1:50, 1:100, 1:500, 1:1,000 in 1% BSA and PBS with 0.1% Triton X-100.
6. Washing solution: PBS + 0.1% Triton X-100.
7. Secondary antibodies: Make up to the relevant dilution (generally 1:50) in 1% BSA and PBS with 0.1% Triton X-100 (e.g., anti-rat, anti-rabbit, anti-guinea pig, or anti-mouse, conjugated to FITC, Texas red, Cy3 or Cy5).
8. Counterstaining solution: *See Section 2.1*, Step 6.

2.4.2. Squash Technique

Prepare enzyme digestion medium, spreading medium, paraformaldehyde fixative, blocking solution, primary antibodies, washing solution, secondary antibodies, and counterstain solution as described in **Section 2.4.1**, except that pH of the paraformaldehyde fixative should be adjusted to 7.3.

2.5. Electron Microscopy

We use sections and spreads both for synaptonemal complex and immunolocalisation for EM analysis.

2.5.1. Sections

1. Fixative: Prepare in fume hood: 2% glutaraldehyde, 2% paraformaldehyde (both EM grade) and 0.1 M sodium cacodylate in sterile deionised water. Prepare fresh.
2. Alcohol series: 70, 85, 90% absolute ethanol in sterile deionised water.
3. 50% LR White: Mix 1 vol LR White (Agar Scientific) with 1 vol 90% ethanol.
4. 100% LR White.
5. Gelatine capsules (Agar Scientific).
6. Formvar-coated grids: For standard EM, copper grids may be used. For immunolocalisation, we use nickel grids. Prepare Formvar by dissolving 0.75 g in 100 mL chloroform. Using a pair of fine forceps, dip the grids into the solution, air dry and store in a Petri dish.
7. Staining solution for sections: Dissolve either 30% uranyl acetate in methanol or 2% uranyl acetate in water.

2.5.2. Spreads

1. Enzyme digestion medium: *See Section 2.4.1.*, Step 1.
2. Spreading medium: *See Section 2.4.1.*, Step 2.
3. Paraformaldehyde fixative: *See Section 2.2.*, Step 3. Adjust the pH to 7.3.
4. Plastic-coated slides: Dip slides in a solution comprising 0.75 g plastic Petri dishes cut up and dissolved in 100 mL chloroform. The solution is satisfactory for one month, after which it needs to be replaced. The slides are glow discharged in order to remove static charge.
5. Make up a 50% silver nitrate solution for staining of axial/lateral elements immediately before using.
6. Cut up rectangles of nylon mesh to cover the slides.
7. Finder grids (Agar Scientific). For analysis of spreads we use finder grids to facilitate their location with EM.

2.5.3. EM Immunocytology

The following protocols are suitable both for sections and spreads.

1. Blocking buffer: *See Section 2.4.1.*, Step 4.
2. Primary antibodies: *See Section 2.4.1.*, Step 5.
3. Washing solution: *See Section 2.4.1.*, Step 6.
4. Secondary antibodies: Make up at 1:50 using conjugates of 5 nm gold or 10 nm gold (BB International).

3. Methods

To study chromosome dynamics during meiosis microscopically, meiocytes have to be preserved without causing any appreciable distortion through a process known as “fixation”. In *Arabidopsis* cytogenetics we use different fixation techniques according to the type of study, namely, chromosome structure or protein dynamics.

3.1. Fixation and Preparation of Slides for Basic Cytology, FISH and Time Course

Visualisation of *Arabidopsis* chromosomes has been achieved by specific DNA staining with DAPI and the utilisation of fluorescence microscopy (5).

1. Cut terminal inflorescences with one or two open flowers only and immediately place in freshly made ice-cold fixative. Leave them on the bench at room temperature and replace the fixative after 2–3 h. The fixed material is suitable for digestion 24 h after fixation, and can be stored at –20°C for up to 6 months.
2. Place single fixed inflorescences in watch glasses (preferably coloured black) with fresh fixative. Using a mounted needle and fine forceps (e.g., watchmaker’s forceps), divide the inflorescence into individual buds. Discard buds with yellow anthers (these contain pollen and are therefore post-meiotic), as well as any larger buds. The remaining buds are retained.
3. Replace the fixative with the working citrate buffer (twice for 5 min each) and then replace with the working solution of the enzymes for 75–90 min (shorter times preserve the organisation of the meiocytes in early meiosis) and incubate in a humidified atmosphere (e.g., a sandwich box containing damp tissues) at 37°C for 30 min. Remove the enzyme solution and replace with the working citrate buffer at 4°C in order to stop the reaction.
4. Place a single bud onto a slide with a minimum of buffer. Quickly macerate the bud with a mounted needle, ensuring that the material does not dry out.
5. Add 10 µL of 60% acetic acid, mix with the material on the slide, and place the slide on a hot block at 45°C for up to 30 s (see Note 6). Circle the region of the slide containing the material with a diamond pen.
6. Place the slide on the bench and add 100 µL of ice-cold fixative as a ring around the material. Dry the preparation. We find that using a commercial hair drier is suitable for this purpose.

7. The slides are now ready for basic cytology after mounting in 7 µL DAPI in Vectorshield. View with a fluorescence microscope and capture images with an image analysis system. (See Fig. 9.1).

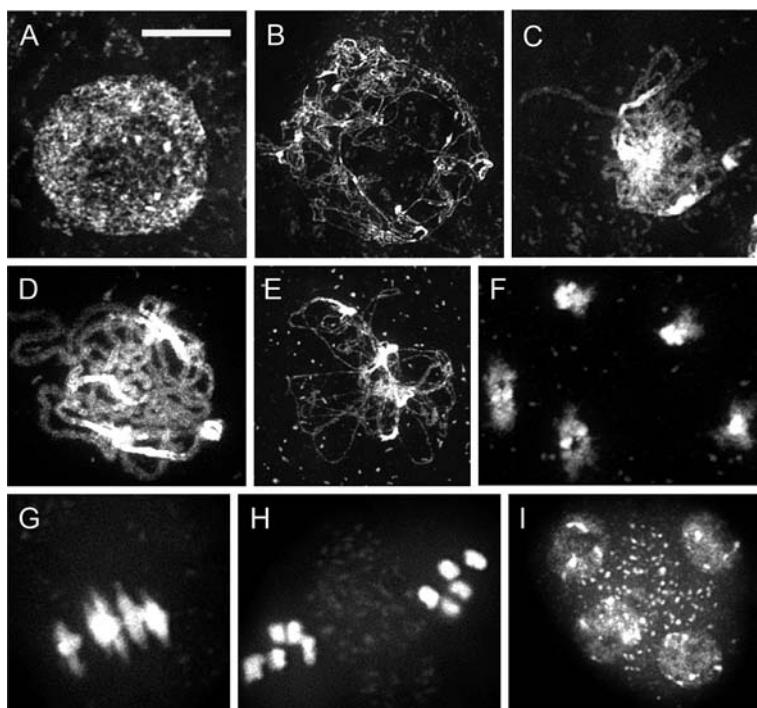


Fig. 9.1. Male meiosis in wild-type pollen mother cells (PMCs) of *Arabidopsis* prepared by the spreading technique and stained with DAPI. (A) Premeiotic interphase. (B) Leptotene showing unsynapsed chromosome axes. (C) Late zygotene, showing mostly synapsed axes and a few unsynapsed regions. Note the typical distribution of mitochondria in zygotene which are distributed around one of the poles only. (D) Pachytene, showing full synapsis. (E) Diplotene, showing homologue separation. Note distribution of the mitochondria, randomly distributed throughout the cytoplasm. (F) Diakinesis showing five moderately condensed, unaligned bivalents. (G) Metaphase I, showing five condensed and aligned bivalents. (H) Metaphase II showing two groups of five chromosomes. (I) Tetrad stage showing four haploid nuclei at the end of meiosis. Bar = 10 µm.

3.2. FISH

In order to distinguish between the different chromosomes in *Arabidopsis*, FISH with different DNA probes has been used (6, 7). We usually take between five and eight slides through the pre-treatments using an appropriate Coplin jar.

1. Wash the chromosome preparations in 2 × SSC at room temperature for 10 min.
2. Digest the preparations with pepsin at 37°C for 45–90 s. Do not overdigest or you will lose the material from the slide.

3. Wash in 2 × SSC at room temperature twice for 5 min.
4. Fix the material in paraformaldehyde in the fume hood for 10 min.
5. Wash twice in sterile deionised water.
6. Dehydrate the preparations by passing them through an alcohol series: 70, 85 and 100% ethanol, 2 min each.
7. Drain the slides. Check if the material is still on the slides using a phase contrast microscope.
8. Denaturation of the probe and chromosomes: Place 20 µL of the probe mixture on the slide and seal with a coverslip (22 × 22 mm) and vulcanising rubber solution. Heat the slides on a hotplate at 75°C for 4 min.
9. Hybridisation: Incubate the slides in a humidified atmosphere at 37°C overnight.
10. Post-hybridisation washes: remove the rubber solution and coverslips using fine forceps. Wash the slides three times, 5 min each, in 50% formamide-2x SSC at 45°C; then once in 2x SSC at 45°C, 5 min; then once in 4x SSC-0.05% Tween 20 at 45°C, 5 min; then once in 4x SSC-0.05% Tween 20 at room temperature for 5 min.
11. Labelling detection: Add fluorescent antibodies (either anti-dixoygenin- or streptavidin-conjugated) to the slides (100 µL per slide), cover with a rectangle of parafilm to fit the slide and incubate in a humidified atmosphere at 37°C in the dark for 30 min. Afterwards, remove the parafilm and wash the preparations in the washing solution three times for 5 min each.
12. Counterstain the slides with 7 µL DAPI staining solution.
13. View the FISH preparations with a fluorescence microscope that has filters for DAPI, TRITC and FITC and that is equipped with an image capture and analysis system.

3.3. Time Course

The thymidine analogue bromodeoxyuridine (BrdU) can be used to label replicating DNA at meiotic S phase. BrdU can be detected immunocytoologically by an anti-BrdU antibody carrying a fluorescent tag. This BrdU labelling has been used to produce a temporal framework for meiosis in *Arabidopsis* (8).

1. Cut terminal inflorescences from well-grown plants under water, using sharp dissecting scissors, retaining a stem of around 4 cm.
2. Quickly transfer the cut ends to a BrdU solution (provided by the kit; use undiluted). Alternatively, a freshly made up BrdU solution can be substituted for this step (*see Note 3*).
3. Leave the cut stems in the BrdU solution for 2 h for uptake of BrdU via the transpiration stream and incorporation into cells in S phase.

4. Fix the inflorescences after this time (time 0) and subsequently at 2 h intervals. Prepare slides as described above for basic cytology (see **Section 3.1**, up to Step 6).
5. Detect the incorporated BrdU as described by manufacturer of the BrdU kit. (Alternatively, see **Note 3**.)
6. Mount the preparation in 7 µL DAPI in Vectorshield and view with a fluorescence microscope equipped with an image capture and analysis system.

3.4. Immunocytology

Immunolocalisation of meiotic and chromosome associated proteins has been performed by two different techniques: spreading and squash techniques. The latter is more efficient for the localisation of chromosome associated proteins that need a slight pre-fixation to avoid cross-reaction and therefore background signal.

3.4.1. Spreading Technique

1. Collect about five buds around 0.2–0.4 mm on damp filter paper. Using a dissecting microscope, quickly remove the anthers from the buds using a mounted needle and fine forceps, yielding a maximum of 30 anthers. Discard any yellow anthers as these will contain pollen.
2. Place approximately 30 anthers into 10 µL of digestion medium, directly onto a clean glass slide. Tap the anthers out in the digestion medium with a thin brass rod to release the pollen mother cells (PMCs) from the anthers. Incubate the suspension at 33°C in a humidified atmosphere for 4 min.
3. Add 10 µL spreading medium to the suspension. Monitor the slide carefully using a phase contrast microscope for bursting of the cell walls and fix when this occurs (maximum time 4 min).
4. Mark the area of the suspension with a diamond pen and fix with 20 µL paraformaldehyde fixative in the fume hood.
5. When dry, briefly rinse in wash solution, rinse in water and dry.
6. Immerse the slides in wash solution twice for 5 min.
7. Block non-specific binding by incubation with the blocking buffer (100 µL is sufficient). Cover with parafilm and incubate in humidified atmosphere at room temperature for 45 min.
8. Make up primary antibodies to the appropriate dilution. Add 100 µL diluted antibody and cover with parafilm. Incubate in a moist box either overnight at 4°C, or at 37°C for 30 min.
9. Wash twice in the washing solution for 5 min.
10. Apply secondary antibody and cover with parafilm. Incubate for 30 min at 37°C in a moist box in the dark. Keep the preparations in the dark from this stage onwards.

11. Wash twice in the washing solution for 5 min.
12. Mount the preparation in 7 µL DAPI in Vectorshield and view with a fluorescence microscope equipped with an image capture and analysis system.
13. Optional: Immunocytology can be combined with FISH (*see Note 4*) or with BrdU (*see Note 5*)

3.4.2. Squash Technique

1. Collect about five buds around 0.2–0.4 mm, depending on the meiotic stage required for the study. A total of five buds per slide is a good quantity to work with.
2. Fix the flower buds in the paraformaldehyde fixative on a slide for 20 min in the fume hood. Discard the fixative by pipetting it out carefully from the slide.
3. Digest the flower buds in the digestion medium on a slide at 37°C for 90–120 min in a moist box. Shorter times of digestion will preserve the bud structure better for dissection later and longer digestion periods will be better for faster dissection of the anthers. In large flower buds (later stages of meiosis) longer digestion helps the dissection of the anthers. In smaller flower buds (early stages of meiosis) long digestion time can over digest the bud tissues including the anthers and dissection is difficult later on.
4. Wash three times in the washing solution. Use a pipette to change the buffer.
5. Dissect the anthers out of the flower buds using a dissecting microscope, a mounted needle and fine forceps. Each flower bud contains six anthers, thus a maximum of 30 anthers per five buds will be placed on each slide. Any yellow coloured anthers contain pollen grains, so they should be discarded.
6. Digest the anthers in the enzyme digestion mix at 37°C for 10 min.
7. Add 10 µL of 0.05% Triton X-100 to the slide with the anthers and cover with a cover slip (22 × 22 mm).
8. Squash the cover slip onto the slide by gently pressing the cover slip.
9. Immerse the slide in liquid nitrogen for 1 min. Remove from liquid nitrogen and quickly remove the cover slip from the slide with a razor blade.
10. Leave the slides on the bench to dry.
11. Wash the slides three times in the washing solution.
12. Block the preparations in the blocking solution at room temperature for 10–20 min.

13. Incubate with the primary antibody at 4°C overnight in a humidified atmosphere. Some primary antibodies require longer periods of incubation for better results, and using a new antibody it would be better to leave for both 24 and 48 h in order to optimise this step.
14. Wash the slides three times in the washing solution.
15. Incubate with the secondary antibody in a humidified atmosphere at room temperature for 45 min in darkness. Keep the preparation in the dark from now onwards.
16. Wash three times in the washing solution.
17. Mount the preparation in 7 µL DAPI in Vectorshield and view with a fluorescence microscope equipped with an image capture and analysis system.

3.5. Electron Microscopy

Electron microscopy studies of meiosis in *Arabidopsis* have allowed us to analyse the pairing and synapsis of homologous chromosomes at an ultrastructural level (9, 10). We also have developed techniques for immunolocalisation (11) (see Fig. 9.2).

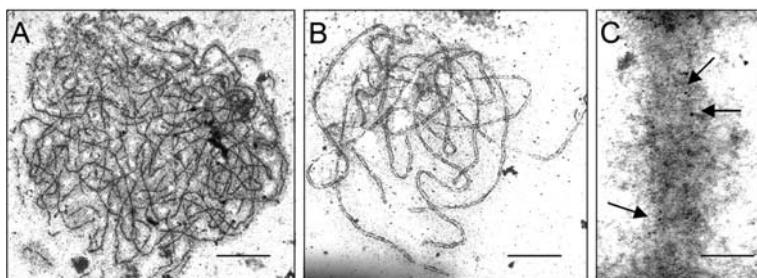


Fig. 9.2. Electron micrographs of spread and stained *Arabidopsis* PMCs. (A) Silver-stained leptotene stage showing unsynapsed chromosome axes. (B) Silver-stained pachytene stage. The chromosome pairs are fully synapsed, with the appearance of typical tripartite synaptonemal complexes. Bars in A and B = 1 µm. (C) Immuno-gold localisation of anti-ZYP1 antibody. The distribution and spacing of the gold particles is consistent with localisation to the inner surface of the lateral elements. Bar = 100 nm. Figure 9.2C reproduced from Ref. (11) with permission from Cold Spring Laboratory Press.

3.5.1. Sections for EM, Also Suitable for Immunocytochemistry

1. Trim terminal inflorescences to contain buds at stage 9 and smaller. At this stage the petal primordia are stalked at their base, and are around 0.2–0.4 mm (3). Around ten buds attached to the stalk will remain. It is useful to leave a stalk as long as possible, at least 1 cm, as this facilitates handling. Fix the inflorescences overnight.
2. Dehydrate through an alcohol series: 70, 85, 90% ethanol for 5 min each.
3. Place in 50% LR White for 2 h.

4. Replace with fresh 50% LR White for a further 2 h.
5. Replace the solution with 100% LR White and incubate overnight to 48 h.
6. Place the specimen in gelatine capsules and place in a vacuum to drive out any air bubbles.
7. Polymerise under UV light for 2–3 days.
8. Cut semi-thin sections (10–20 µm). Pick up sections onto formovar-coated copper grids.
9. Stain with 30% uranyl acetate in methanol for 7 min or 2% uranyl acetate in water for 30 min at RT.
10. Examine grids in an electron microscope.

3.5.2. Spreads for EM

Five buds between 0.2 and 0.3 mm, containing leptotene to pachytene are used.

1. Using a dissecting microscope, dissect individual anthers onto damp filter paper, producing a pool of around 30 anthers.
2. Place approximately 30 anthers together in a cavity slide containing 10 µL digestion medium. Gently tap the anthers with a brass rod to release meiocytes and leave for 4 min in a moisture box at 33°C.
3. Place 2 µL of this solution onto 10 µL drops of spreading medium on glow-discharged plastic-coated slides.
4. Incubate the suspension for a further 4 min, and monitor with a phase contrast microscope until the meiocytes can be seen to be bursting.
5. Spread the suspension over the slide using a glass spreader and fix by adding 20 µL paraformaldehyde fixative in the fume hood. Allow to dry.
6. Wash the dry slides in sterile deionised water and stain using silver nitrate. Place approximately 200 µL of silver nitrate solution on the slides and cover with nylon mesh. Incubate in a moist box for 20–30 mins at 60°C until the mesh appears to be golden brown. Wash the slides and nylon mesh in a large volume of water. The nylon mesh will float away. Rinse the slides in sterile deionised water and dry.
7. Examine the stained slides with a light microscope and mark well-spread cells with black ink before floating the plastic supporting film onto a clean water surface.
8. Place copper finder grids onto the marked area, and pick up the plastic film on clean paper and dry.
9. Examine grids in an electron microscope.

3.5.3. EM**Immunolocalisation (LR
White Sections)**

1. Prepare the material for sections as described in **Section 3.5**, Steps 1–7.
2. Cut semi-thin sections (10–20 µm). Pick up sections onto Formvar-coated nickel grids. The sections should be on dull sides of the grids.
3. Block non-specific binding by incubation with the blocking buffer: place the grid, dull side down, into a drop (~200 µL) of blocking buffer in a Petri dish or on dental wax.
4. Transfer grids to primary antibody. Incubate overnight at 4°C.
5. Wash three times for 5 min each in washing buffer.
6. Apply secondary antibody for 90 min at room temperature.
7. Wash three times for 5 min each in washing buffer.
8. Wash three times for 1 min each in sterile deionised water.
9. Stain with 30% uranyl acetate in methanol for 7 min or 2% uranyl acetate in water for 30 min at RT

**3.5.4. Immunolocalisation
on Spreads for EM**

1. Prepare spreads as described for EM, to the stage before silver staining: See **Section 3.5.2**, Steps 1–7.
2. Wash the slides in sterile deionised water once. Examine the slides with phase contrast. Mark suitable spreads with black ink and float the plastic supporting film onto a clean water surface. Pick up on Formvar-coated nickel grids. Spreads should be on dull sides of the grids.
3. Block non-specific binding by incubation with a blocking buffer: place grid, dull side down, into a drop (~200 µL) of blocking solution in a Petri dish or on dental wax.
4. Transfer grids to primary antibody. Incubate overnight at 4°C.
5. Wash three times for 5 min each in washing buffer.
6. Apply secondary antibody for 90 min at room temperature.
7. Wash three times for 5 min each in washing buffer.
8. Wash three times for 1 min each in sterile deionised water.
9. Stain with 30% uranyl acetate in methanol for 7 min or 2% uranyl acetate in water for 30 min at RT.

4. Notes

1. As an alternative, buds can be fixed in Carnoy's fluid II (6 vol absolute ethanol, 3 vol chloroform, 1 vol glacial acetic acid). Keep this fixative on ice. Dissect the flower buds from the plants and immerse in the solution for fixation. Replace the fixative the following day with fresh fixative. This fixative is

considered to be better for long-term storage of material, but needs to be removed and replaced with 3:1 fixative (*See Section 2.1*, Step 1) before enzyme digestion. Discard unused fixative at the end of the day.

2. We deionise our formamide by mixing 200 mL formamide with 10 g mixed resin beads (e.g., Amberlite, Pharmacia Biotech) on a stirrer for at least 1 h. Filter and store at 4°C.
3. As an alternative to using a commercial kit you can make solution of 10 mM BrdU, and detect its incorporation using monoclonal anti-BrdU (Sigma) at a concentration of 1:500 in TNB buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% BSA). Detect anti-BrdU with rabbit anti-mouse FITC (Sigma) at a concentration of 1:50 in TNB buffer. The washing solution for this is TNT (100 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5%). Place the slides in a Coplin jar containing 70% formamide in 2x SSC (pH 7.0) at 75°C, thereby denaturing the DNA and allowing access to the antibodies. After 2 min, snap chill the slides by briefly placing them in TNT buffer at 4°C. Detect BrdU by applying 100 µL of anti-BrdU to the slide, covering with parafilm and incubating in a humidified atmosphere at 37°C for 30 min. Wash the slides three times for 5 min each in TNT buffer. Apply 100 µL of a secondary antibody (rabbit anti-mouse FITC) and mount preparations in DAPI in Vectorshield and view with fluorescence microscope with image analysis system.
4. It is possible to combine immunolocalisation with FISH. Make slides for the combined technique using the spreading technique up to the end of the incubation with the primary antibodies (*See Section 3.4.1*, step 10). Following washing, apply anti-biotin (raised in rabbit or rat depending on the primary antibody) at a concentration of 1:50 in immunolocalisation blocking buffer. This protects the primary antibody in the subsequent FISH protocol. Incubate for 30 min at 37°C in a moist box. Following washing, take the slides through the alcohol series 70, 85 and 100% for 2 min each. The slides can now be used for the FISH protocol (*See Section 3.2*). Note that you need to use either directly labelled probes or digoxigenin-labelled probes only. Following the last wash in the FISH protocol, apply an anti-biotin FITC or Cy3 to detect the biotinylated primary antibody. Incubate for 30 min at 37°C in a moist box. Wash as before, mount in DAPI /Vectorshield.
5. It may be useful to combine the time course with immunolocalisation, particularly if you would like to assess the relationship between the duration of specific meiotic stages with immunolocalisation. Apply the BrdU pulse as already described in the time course protocol (*See Section 3.3*, up to end of Step 3). Do not fix the buds, but use them for slide

preparation with the immunolocalisation procedure (*See Section 3.4.1*). We detect the antibody first, then follow with detection of BrdU (*See Section 3.3*, Steps 5 and 6).

6. When making slides of fixed material, we use 60% acetic acid to dissociate the cells. Too long on the hot block may cause damage to the cells and loss of material.

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Chapter 10

Electron Microscopic Immunogold Localization of Recombination-Related Proteins in Spreads of Synaptonemal Complexes From Tomato Microsporocytes

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Abstract

Many of the structures involved in meiotic synapsis and recombination such as synaptonemal complexes (SCs) and recombination nodules (RNs) can be resolved only by electron microscopy. Therefore, electron microscopic (EM) immunolocalization using gold-conjugated antibodies is the best way to verify whether certain proteins are components of SCs or RNs. Here, we describe (1) preparing tomato primary microsporocyte protoplasts in leptotene, zygotene, and pachytene stages; (2) hypotonically bursting the protoplasts on glow-discharged glass and plastic-coated slides to make spreads of SCs; (3) immunolabeling proteins in SCs and RNs with colloidal gold; (4) staining SC spreads for EM; and (5) transferring SC spreads on plastic films to grids for EM.

Key words: Immunogold localization, electron microscopy, tomato, synaptonemal complex, recombination nodules, *Solanum lycopersicum*.

1. Introduction

Chromosomes can be prepared for visualization using a variety of methods for different applications. One of the major differences among these techniques is the fixation procedure. If chromosomes are fixed in 1:3 acetic acid:alcohol (typically ethanol or methanol), separation of chromosomes is usually achieved either by physically flattening (squashing) cells that have been made turgid in 45% acetic acid or by exposing the dehydrated cells to water thereby causing them to burst. Chromosomes fixed in acetic acid:alcohol maintain their structure reasonably well and are suitable for non-differential staining (e.g., aceto-carmine, aceto-orcein, crystal

violet, Giemsa staining), differential staining (e.g., Q-banding, G-banding, C-banding, R-banding), and fluorescent *in situ* hybridization (FISH). However, this method often destroys protein epitopes, so immunolocalization usually is not possible with this fixative.

Another popular method utilizes formaldehyde fixation that generally is compatible with antibody localization. However, it is difficult to obtain good spreads of chromosomes after cells have been fixed with formaldehyde because it induces extensive cross-links. To avoid this problem, chromosomes can be spread by exposing living cells to an air–water interface or to a hypotonic solution before or during formaldehyde fixation. Chromosomes separate well during this process because chromatin expands and pushes the chromosomes apart, but in doing so, chromosome structure is distorted. This is an advantage when examining meiotic chromosomes at prophase I because the chromatin expands to reveal axial elements, synaptonemal complexes (SCs), and recombination nodules that are all relatively resistant to these distortion forces (1–3). Such preparations are ideal for detailed examination of synaptic patterns as well as determining the number and distribution of recombination nodules (4).

Here we describe a technique for spreading SCs from the model plant species *Solanum lycopersicum* (tomato) and the use of SC spreads for morphological and immunocytological investigation of synapsis and recombination by both light and electron microscopy (5, 6). These chromosome spreads are also suitable for localization of DNA sequences by fluorescence *in situ* hybridization (FISH) (7).

2. Materials

2.1. Microscope Slide Preparation

1. VWR or Corning 25 × 75 mm glass microscope slides frosted at one end on one side. Other brands may work as well.
2. Kimwipes®. Other comparatively lint-free paper towels may work as well.
3. Plastic box holding 25 slides, e.g., like those available from Carolina Biological Supply Company. The bottom of the box needs to be cut out with a fine saw or a hot needle so there is only a narrow rim on each side of the base to keep slides from falling through.
4. For plastic-coated slides only:
 - a. 0.3 g of plastic (*see Note 1*).
 - b. 50 mL of dichloroethane.

- c. 100 mL bottle with cap.
- d. Ultrasonic cleaner or magnetic stirrer and stir bar.
- e. 100 mL glass graduated cylinder.
- f. Paper clip or binder clip and 50 cm long lint-free string.
- g. Rack that holds 2.5 cm diameter test tubes, used for drying slides. Hereafter termed a “drying rack.”
- h. Fingernail polish.
- i. Glow-discharge apparatus large enough to hold the plastic box for 25 slides. A glow-discharge apparatus can be a commercially available unit (e.g., Electron Microscopy Sciences or Ladd Research), but it will need to be modified to deliver alternating current. Alternatively, a similar apparatus can be assembled from parts. For example, we use a modified Virtis freeze-dryer for the mechanical vacuum pump (**Fig. 10.1**) and a Ladd sputter coater for the vacuum chamber assembly (**Fig. 10.2**). The vacuum pump must be capable of drawing down a four cubic liter chamber to at least 0.1 Torr (mm of mercury). The vacuum chamber assembly should have these characteristics: a clear, circular, heavy (≥ 4 mm thick) glass vacuum chamber that is at least 20 cm in height and 20 cm in diameter with a closable valve connection to the freeze-dryer vacuum pump, a rapid air intake valve for opening the chamber to atmospheric pressure, a vacuum gauge connected to the

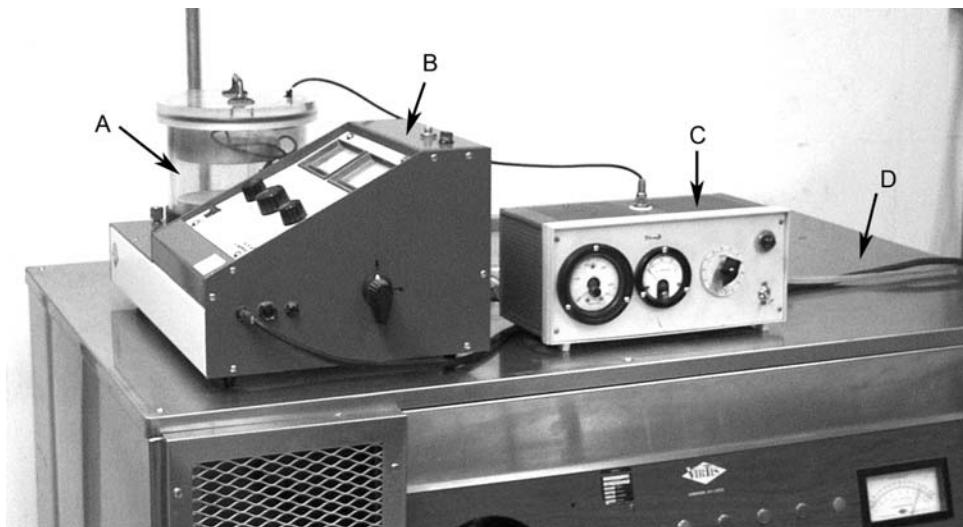


Fig. 10.1. Glow-discharge apparatus. **A.** Vacuum chamber. **B.** Ladd sputter coater. **C.** AC power supply for aluminum electrodes in the vacuum chamber. **D.** Virtis freeze dryer.

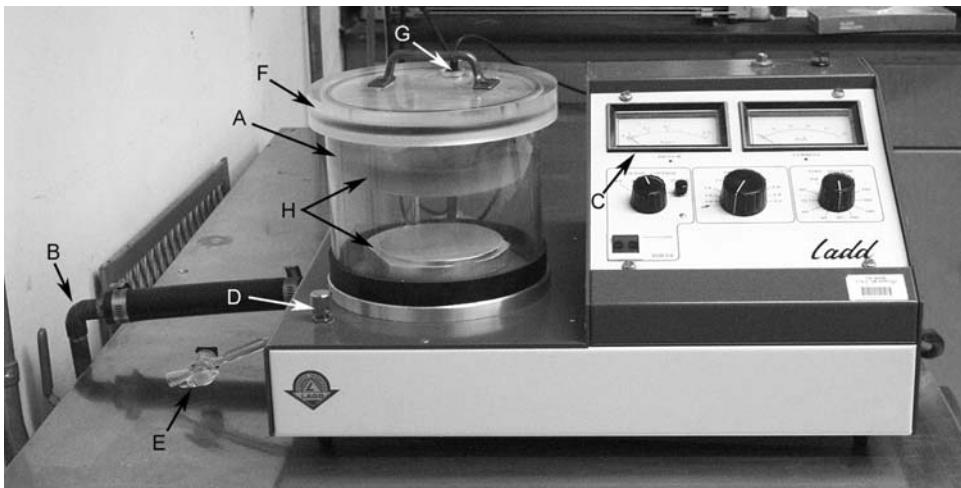


Fig. 10.2. Ladd sputter coater hooked up for AC glow discharge. **A.** Vacuum chamber. **B.** Pipe leading from vacuum pump in the freeze-dryer cabinet to the vacuum chamber. **C.** Vacuum gauge. **D.** Needle valve to bleed air into the vacuum chamber. **E.** Stop cock to let room air into the vacuum chamber. **F.** Lid for vacuum chamber. **G.** Vacuum-tight plug for alternating current in the vacuum chamber to charge the aluminum electrodes (plates). **H.** Aluminum electrodes (plates) between which glow discharge occurs.

chamber, a needle valve for admitting room air to control the vacuum in the chamber, and a vacuum-tight connection for wires to the aluminum plates (electrodes) in the chamber and to an outside power source. Both the power source and lid are customized for the vacuum chamber. The power source was built to deliver up to 200 mA of current at up to 450 V of alternating current. Alternating current is required because direct current that is normally used for glow discharge during sputter coating will deposit metal on the microscope slides from the aluminum electrode plates, whereas alternating current does not deposit much, if any, metal on the slides. The power source was made by the electrical engineer who maintains our electron microscope, and his wiring diagram is available as a supplemental figure on the companion CD for this volume. A 22 cm diameter circular lid for the chamber was cut from a 3 cm thick slab of Lucite (**Fig. 10.3**). A circular groove was cut in the lower surface to hold a rubber O ring and into which the upper rim of the vacuum chamber fits. A hole was also cut in the lid for a vacuum-tight plug to carry current from the power source to the glow-discharge plates. Two wires extend from the plug into the vacuum chamber and connect to the two aluminum discharge plates (**Fig. 10.3**). These electrodes consist of a lower circular flat aluminum plate and an upper circular ring of aluminum, each about 14 cm in diameter and 1.5 cm in

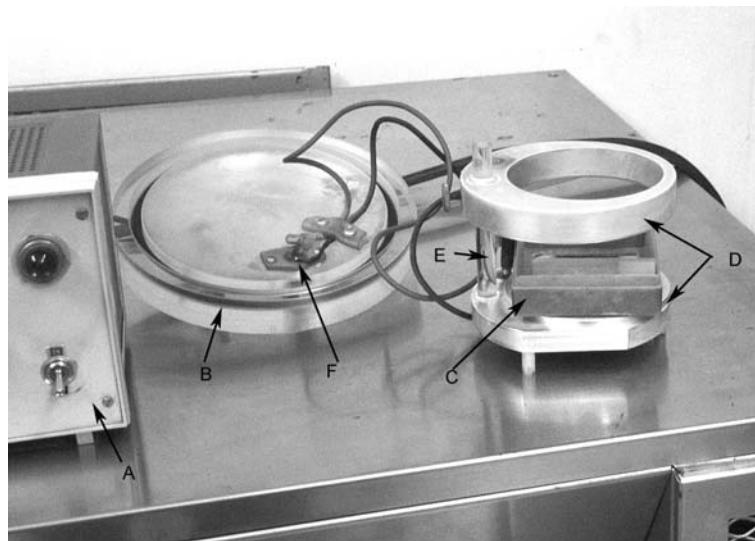


Fig. 10.3. Assembly for the vacuum chamber. **A.** AC power source for glow discharge. **B.** Lucite lid for vacuum chamber. Note deep groove around the edge containing an "O" ring. The top of the vacuum chamber fits into this groove to form a vacuum seal. **C.** Plastic slide box for 25 glass microscope slides located between the aluminum electrodes for glow discharge. **D.** Aluminum electrodes (plates) connected by wires to the vacuum-tight plug in the lid of the vacuum chamber. **E.** Lucite rod on which the aluminum electrodes extend. **F.** Vacuum-tight plug in vacuum chamber lid to transmit current from the AC power source to the wires leading to the aluminum electrodes.

thickness. A hole is drilled to one side in both plates for a Lucite rod, so the aluminum ring extends over the aluminum plate with a space between the plates that is just large enough to accept a plastic box for 25 slides. The lower aluminum plate sits on short plastic legs to insulate it from touching the floor of the vacuum chamber. Each plate is connected by a wire to the plug in the lid of the vacuum chamber. Aluminum plates are used, rather than plates made from some other metal, because they form an oxidized layer over their surface that resists depositing metal by sputter coating.

2.2. Making Squashes to Determine Stages of Meiosis

1. Healthy, young, blooming tomato plants. Plants that are 8–12 wk old are ideal.
2. Sharp forceps for pinching buds from plants.
3. Glass microscope slides and 18 × 18 mm or 22 × 22 mm cover glasses.
4. Kimwipes or other comparatively lint-free paper towels.
5. A compound microscope and a dissecting microscope with an ocular micrometer calibrated in millimeters.

6. Chisel-shaped and pointed steel dissecting needles. These needles are modified stock needles that are available from most biological supply houses. Usually the steel in dissecting needles is too soft to hold a sharp edge and needs to be hardened before sharpening. Flame-hardening can be achieved by heating the distal centimeter of the needle red hot in a Bunsen burner or an alcohol lamp and then immediately cooling the needle in water. Pointed dissecting needles can be prepared by rolling and rubbing the end of a hardened needle at a narrow angle ($\sim 5\text{--}10^\circ$) in a drop of light machine oil (e.g., 3-in One[®] oil) on a sharpening stone until the end is in the form of a gradually beveled point. To create a sharp tip, slightly increase the angle of the needle ($\sim 15^\circ$) during sharpening on the stone. The finished point should look sharp when viewed under a dissecting microscope at $10\times$ magnification. Starting with a rough sharpening stone, and finishing with a fine stone speeds up the operation. To make a chisel-shaped dissecting needle, cut the beveled end off a dissecting needle with wire cutting pliers. Flame-harden the needle, and then rub the cut end first on one side and then on the other on a rough sharpening stone to make a beveled, flat edge at the tip, i.e., an edge that is perpendicular to the long axis of the needle. Using a fine stone, finish the flat edge, which should look sharp when viewed on its side at $10\times$ under a dissecting microscope.
7. Dissecting platform made by breaking off the sides of the top or bottom of a clear plastic Petri dish. This makes a good dissecting platform because it is hard enough to cut against but soft enough not to dull your dissecting needles.
8. A few mL of 2% aceto-orcein. Aceto-orcein is prepared by pouring 2 g of powdered orcein in 98 mL of aqueous 45% acetic acid. Boil this mixture for an hour, preferably in a reflux condenser. If a reflux condenser is not available, then boil the solution in a hood and continually add enough 45% acetic acid to maintain the volume. After the boiling time is completed, cool the dye solution to room temperature, filter the solution through medium-grade filter paper, and then filter the solution again through the same filter paper. The dye solution can be kept indefinitely in a closed dropper bottle at room temperature. When precipitate inevitably forms in the solution over time, simply refilter it the same way.

2.3. Preparing Protoplasts

1. Healthy, young (8–12 wk old) blooming tomato plants.
2. Dissecting microscope with an ocular micrometer calibrated in millimeters.

3. Medium fine-tipped forceps, a scalpel with a small blade (#11, Feather brand, Ted Pella), a sharp chisel-shaped dissecting needle, and a sharp pointed dissecting needle (*see Section 2.2, Step 6*).
4. Dissecting platform made by breaking off the sides of a Falcon-plastic Petri dish.
5. Digestion medium: Two different salt solutions work equally well for preparing digestion media. One is made using 625 micro liters of Carolina Biological Supply Company® potato culture medium [the formula is available in the Carolina Plant Tissue Culture Media Formulation Booklet (Carolina Biological Supply 1986)] plus 1mg of potassium dextran sulfate (Nacalai USA) is 5 mL of distilled water. The other is prepared by mixing one mL of each of the following aqueous components: 2.8 mM KH₂PO₄, 4.0 mM CaCl₂, 0.5 mM acid PIPES, 1 mg/mL aqueous potassium dextran sulfate (freshly prepared), and distilled water. To complete 5 mL of both digestion media, add 0.64 g of mannitol (Sigma or Calbiochem) and 0.1 g of PVP (polyvinylpyrrolidone, ave. MW 10,000, Sigma – plant cell culture tested). Adjust pH to 5.1 using 0.1 N KOH (and 0.1 N HCl, if needed). It is important for the viability of the cells to keep sodium ions to a minimum, therefore do not adjust the pH with NaOH.
6. Desalted, lyophilized cytohelicase (Sigma). Cytohelicase is a complex mixture of enzymes, but mainly β -glucuronidase that is obtained from the gut of the snail *Helix pomatia*. Cytohelicase can be desalted with a Sephadex G25 column. The enzyme is light-sensitive, so do not use a UV-monitored fraction collector during desalting.
7. 25 × 75 mm glass depression slide.
8. Plastic Petri dishes with “V”-shaped glass rods (that are about 5 cm in diameter) in the bottom or Petri dishes with dividers that make three compartments (= Y-plate; Falcon 1004 type) or some other type of incubation dish in which slides can be incubated over water to maintain a humid environment. These will be referred to as incubation Petri dishes hereafter.
9. A scalpel with a #11 blade. The blade can be resharpened using a fine sharpening stone.

2.4. Spreading Synaptonemal Complexes (SCs)

1. Bursting medium: Aqueous 0.05% IGEPAL® CA-630 [Sigma (octylphenoxy) polyethoxyethanol], a nonionic detergent formerly marketed as Nonidet P-40] and 0.1% bovine serum albumin (BSA, Sigma Fraction V). We keep bursting medium as a frozen stock (-20°C) in 500 μ L aliquots in closed 650 μ L microcentrifuge tubes. Just before use,

bursting medium is modified by adding 5 μL of aqueous 0.1 mg/mL potassium dextran sulfate (Calbiochem) and 30 μL of 4% formaldehyde. Mix the solution by inversion.

2. 4% Formaldehyde: Add 4 g of paraformaldehyde powder and 1 mL of 1 N NaOH to 95 mL of deionized or distilled water in a beaker on a stirring hot plate in a hood. The temperature of the solution should not rise above 60°C. Once the solution clears, cool it to room temperature, and then add one mL of 0.05 M sodium borate to buffer the solution. With stirring, titrate the solution to a pH of 8.5–8.7 with 1 N HCl. This 4% solution of formaldehyde can be aliquoted into small bottles and stored indefinitely at –20°C in a freezer.
3. Siliconized Drummond disposable micropipettes with aspirator tube assembly (cat. # 2-000-000 – see Fisher and VWR as distributors). Micropipettes are drawn out in a flame from capillary glass tubing made for collecting blood samples (e.g., Kimax – 51, #34500, size 1.5, 1 \times 100 mm). A small butane torch (e.g., Blazer Products, Inc.) gives an appropriate flame. When the drawn tubing is broken to make micropipettes, the bore of the tips should be about 0.075 mm in diameter, and the glass edges of the tips should not be ragged. Tips can be smoothed with light honing on a fine sharpening stone. The bore of the micropipette tip should be barely smaller than the diameter of a rod of cells from anthers after the rods have been digested. If the tip is the right size, a digested (but still intact) rod of cells will break up into individual protoplasts when drawn into the tip, but the protoplasts will not be damaged. Micropipettes are siliconized by drawing a solution of 5% methylchlorosilane (Aldrich) in chloroform into the micropipette and expelling it. The pipette should be rinsed twice with water after being siliconized. We resilinconize pipettes before each experiment. Siliconizing micropipettes keeps cells from sticking to the sides of the micropipette and being damaged.
4. Medical nebulizer apparatus designed for inhalation therapy (e.g., Devilbiss) and an air compressor for the nebulizer (e.g., Devilbiss).
5. Photoflo 200 (Kodak) diluted with water to 0.4%.

2.5. Immunolabeling and Post-Staining for Electron Microscopy

1. Plastic incubation Petri dishes.
2. Plastic container with a lid that will hold four or more Petri dishes.
3. Plastic autoclave bags that have been cut into $\sim 55 \times 30$ mm strips to completely cover the nonfrosted part of glass microscope slides. There should be some overhang at the three edges of the unfrosted part of a slide to simplify removing plastic cover slips.

4. Tris-buffered saline with Triton (TBST): 10 mM Tris-HCl, 150 mM NaCl, 0.05% Triton (from 20% stock solution stored in the refrigerator), pH 8.
5. Antibody dilution buffer (ADB): 10% normal goat serum, 3% BSA (fraction V, Sigma), 0.05% Triton X-100 (from 20% stock), 0.05% sodium azide (from 10% stock solution) in TBST (8). The solution is used at full concentration for the antibody incubation steps and at 10% concentration (in TBST) for blocking and intermediate wash steps.
6. Ammonium chloride quenching solution: 0.1 M NH₄Cl in TBST.
7. DNase I digestion buffer: 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.5 mM CaCl₂, pH 7.5. We make this solution at 10X concentration and store it at room temperature.
8. DNase I (Fluka # 31135): 1 mg/mL in 50% glycerol. Store at -20°C.
9. Primary antibodies to your protein of interest. Usually we affinity-purify the antibodies from serum to reduce background problems.
10. Secondary antibodies conjugated to 1, 6, or 10 nm gold particles (Electron Microscopy Sciences). 1-nm gold particles are referred to as ultra-small (US).
11. 1% Glutaraldehyde (from 50% stock solution, Sigma) in 150 mM potassium phosphate buffer, pH 7.4. Recheck final pH (using pH paper) because glutaraldehyde converts to the acid form over time. Store at -20°C in 1 mL aliquots.
12. Silver enhancement kit for EM (Electron Microscopy Sciences).
13. Uranyl acetate (UA): 2% (w/v) in distilled water. UA is light-sensitive so it should be stored in an amber bottle in the refrigerator. UA usually does not go completely into solution at this concentration. Either allow the solution to settle and use only the clear solution above the precipitate or centrifuge the solution to remove precipitate before use in staining

2.6. Transferring Plastic to Grids

1. Nickel center-marked 75-mesh “finder” grids (Ted Pella, 1GN75).
2. Grid glue: Put about 20 mL of dichloroethane in a small glass bottle. Use a pair of forceps to swish a 2–3 cm long, clean piece of cellophane tape in the solvent. Swish for about one min to allow some of the adhesive to go into solution. Remove cellophane backing and any large “blobs” of adhesive from the solution. Store grid glue at room temperature. Grid glue is good for several months.

3. Eyelash tools: Sharpen one end of a wooden applicator stick (about 10 cm long) and glue a clean eyelash to the sharp end.
4. Hydrofluoric acid (HF): 1% in water. HF acid dissolves glass, so it must be kept in a plastic bottle. The bottle should be placed in a stable carrier to minimize any chance of spillage. HF acid can be absorbed through the skin and moves into bones where it displaces calcium, so care should always be taken to avoid skin contact and to wash the HF solution off immediately with copious water if there is skin contact.
5. Aqueous 5% acetic acid.
6. Sharp dissecting needle.
7. Nylon screen – preferably 300–400 mesh (Tetko).
8. Bowl of water. We use a culture dish about 13–15 cm in diameter that holds about 200–300 mL of water.

3. Methods

3.1. Preparing Microscope Slides for SC Spreading

1. All slides need to be wiped, even if they are labeled pre-cleaned. Place a glass slide on a table top with the frosted side up. While holding the frosted end down with the fingers of one hand, wipe the clear portion of the frosted side repeatedly with a pad of dry Kimwipe® until resistance is reduced, i.e., the surface seems relatively slick. We think this may remove any remaining foreign substances from the glass to make the surface “clean.” After wiping, the glass surface should not be touched. If it is touched, the slide must be wiped again. There is no reason to wipe the back sides of slides. Wipe as many slides as you anticipate using in one spreading session. This is usually no more than 25. Slides to be used for light microscopy should be wiped immediately before they are glow discharged (*see Section 3.2*) and used for SC spreading. For electron microscopy, slides should be wiped immediately before they are coated with plastic.
2. Dissolve plastic: Place 0.3 g of plastic (*see Note 1*) in a bottle containing 50 mL of dichloroethane and close the bottle with a screw cap. The dichloroethane stock solution should be kept sealed so it does not absorb water. If water contamination occurs, the plastic film on the slide will have holes where water droplets were present. If there are very many and/or very large holes, the plastic will appear cloudy after drying on the slide. Even if the plastic appears clear after drying on the slide, it should be examined by phase microscopy to be sure that

there are few if any holes. Dissolving the plastic may take several hours without stirring, but only a few min are required when magnetic stirring or sonication is used.

3. Apply plastic to slides: Select an area of the lab that is free of air drafts that can cause uneven drying of the plastic film. Pour the plastic solution into a clean 100 mL glass graduated cylinder. Tie a string to a small paper or binder clip, and attach the clip to the frosted end of a freshly wiped slide. Although the back of the slide does not need to be wiped until it is slick, the slide should be free from any particles that could contaminate the plastic solution. Lower the slide into the graduated cylinder until the clear part of the slide is submerged just above the frosting. Do not let the liquid touch the clip or there will be a streak on the plastic film. Pull the slide out of the liquid, and then slowly draw the slide straight up the graduated cylinder. The atmosphere in the cylinder is saturated with dichloroethane. As a result, as long as the slide remains in the cylinder, it will not dry, and the solution will continue to flow down the slide to make an ever thinner plastic coat. Try different speeds of removal, but generally it should take 10–15 s. Hold the slide upright vertically over the cylinder until it is completely dry. Remove the clip and stand the slide upright in the drying rack until all slides have been coated. We usually plastic-coat a whole box of slides (72) at a time. The plastic coating is thin and fragile, so it is important not to touch or scratch the plastic on the frosted side of the slides.
4. Storing plastic-coated slides: After drying, slides can be stored indefinitely in a closed slide box. Beware of some plastic slide boxes because they somehow cause the plastic film not to become sufficiently hydrophilic during glow discharge. In our experience old-fashioned wooden slide boxes work well and some plastic boxes are also all right. Possibly many plastic boxes would do if they have been properly aged (degassed?).

3.2. Making Slides Hydrophilic Using Glow Discharge

Both plain glass and plastic-coated slides need to be glow discharged immediately before use to make the slide surface hydrophilic. This causes the bursting medium to disperse actively over the surface of the slide and helps to spread the SCs.

1. Put the slides in a plastic slide box with the bottom cut out to facilitate glow discharge.
2. Place the open slide box between the two aluminum plates (electrodes) in the vacuum chamber of the glow-discharge apparatus, and close the lid.
3. Close the air inlet valve to the vacuum chamber, and turn on the vacuum pump.

4. When the vacuum reaches about 0.2 mm of mercury (0.2 Torr), adjust the needle valve to let just enough air into the chamber to hold this vacuum.
5. Dim the room lights (so you can see the glow in the chamber). Turn on the power source, and increase the voltage. At around 350 V, glow discharge between the aluminum plates will start. The glow between the plates will be a pinkish violet. The blue component is due to water vapor, and generally, the higher the humidity, the more purple the glow and the better the slides are glow discharged, i.e., negatively charged and hydrophilic. Glow discharge the slides for 5 min.
6. Turn off the current and the power unit (to avoid being shocked when touching the aluminum plates), close the vacuum chamber to the pump, release the vacuum in the vacuum chamber, remove the lid, and remove the slide box. The slides should not be warm to the touch. If so, something is wrong with the set-up.
7. Open the vacuum pump to the vacuum chamber, and quickly turn off the vacuum pump. This will prevent the vacuum pump from shutting down while still under vacuum.
8. The slides should be hydrophilic and usable for chromosome spreading for several hours. When a drop of water is placed on a glow-discharged slide, the drop should not bead up (like a water drop does when placed on a plastic-coated slide before glow discharge), and the drop should leave a wet trail when it is drained off the slide. If unused slides are left over from an experiment, they can be reused later, but glass slides should be rewiped, and both glass and plastic-coated slides need to be glow discharged again.

3.3. Preparing Protoplasts for Spreading SCs

1. Immediately before use, pick five to ten buds from healthy, young plants. Pick them by pinching off their pedicels with forceps to keep from damaging the buds. Float the buds on a small quantity of distilled water in a small beaker. The length of buds with anthers at zygotene/pachytene varies with tomato varieties. For cherry tomato, buds at zygotene/pachytene are often about 3 mm in length. It is important to be gentle in handling buds because bruising the buds and the anthers inside results in cytomixis and cell death of the primary microsporocytes. Buds and anthers become dark at damage sites due to oxidation of polyphenolic compounds.
2. Transfer a bud to a clear plastic dissecting platform on a dissecting microscope stage, i.e., dissect under a dissecting microscope.
3. While holding the apical end of the bud, i.e., the tips of the sepals, in place with the sharp dissecting needle, use the chisel-shaped end of another dissecting needle to make a shallow

lengthwise slice in the bud between two sepals and through the underlying petal from the receptacle to the apex. Cutting here passes between adjacent anthers on the same side of the bud without damaging them.

4. Use the dissecting needles to pry open the bud at the receptacle to reveal the anthers and pistil, but do not touch the anthers.
5. Use the chisel-shaped dissecting needle to cut the short filament at the base of one of the anthers and lift it out. Measure the anther from the base to the tip using the ocular micrometer. Generally, tomato anthers at leptotene are about 1.6 mm long, anthers in zygotene are 1.7–1.8 mm long, and anthers in pachytene are 1.9–2.1 mm long. However, this can vary somewhat depending on the variety and health of the plant as well as the time of year.
6. Check the stage of meiosis by preparing a temporary squash. Wipe a glass slide with a Kimwipe to remove dust from the surface. Place a half drop (~20 µL) of 2% aceto-orcein onto the slide. Transfer a measured anther into the aceto-orcein. Using dissecting needles, cut the anther in half transversely to its long axis. Squeeze out the rods of primary microsporocytes from the four locules in the anther, and remove the anther walls. Warm the slide over an alcohol lamp just long enough for a fog to form and disappear from the underside of the slide. Do *not* let the 2% aceto-orcein boil. Add a cover glass that has been wiped to remove dust, and reheat the slide over an alcohol lamp as described above. Cover the cover glass with a pad of paper towel or Kimwipe, and while holding the edge of the cover glass with the fingers of one hand (so the cover glass does not slip), firmly press the cover glass down with the thumb or finger of the other hand to squeeze out excess aceto-orcein and squash the cells. Extreme pressure is not needed. Transfer the slide to a compound microscope to determine the stage of meiosis. See (9) for illustrations of the stages of meiosis in tomato.
7. If the primary microsporocytes are at the right stage, transfer the remaining (usually) four anthers into 0.2 mL of digestion medium in a depression slide.
8. Add 3 mg of desalted, lyophilized cytohelicase. Using a small, sharp scalpel, slice the anthers transversely near the middle of the anthers.
9. Place the depression slide in an incubation Petri dish with water in the bottom, and cover the Petri dish. Place an opaque cover over the dish to protect the light-sensitive enzyme and wait 5 min for the cells to plasmolyze. Then under a dissecting microscope, press out the rods of cells from all four anthers

using the chisel-shaped dissecting needle, and remove the anther walls. Since there are four rods of cells from each anther, there should be a total of 16 rods of cells in the depression. At two-rods of cells per slide, this will be enough to make eight slides.

10. After 10–15 min at ~21°C, cell walls have been digested, and the protoplasts are ready to be used for spreading SCs.

3.4. Spreading SCs

1. Draw (aspirate) two rods of cells in approximately 0.25 µL of the digestion medium (keep the total volume as low as possible) into a siliconized micropipette. It is easy to get too many cells and too much medium, both of which interfere with making good spreads. The bore of the micropipette tip should be just smaller than the diameter of a rod of cells, so that when a rod is drawn up, it breaks up into individual protoplasts, but the protoplasts are not damaged.
2. Expel the protoplast suspension gently into a 7 µL droplet of modified bursting medium at the end of a mechanical pipette tip, e.g., a P20 Pipetman.
3. Touch the droplet of modified bursting medium/protoplast mixture either onto a 7 µL droplet of water on a glow-discharged slide or directly onto the surface of a glow-discharged slide. If the slides have been properly wiped and glow discharged, the bursting solution with protoplasts will spread over much of the surface of the slide as soon as the bursting medium is added. In both cases, follow this immediately with an additional 7 µL of the modified bursting medium.
4. Take the slide immediately to a hood, and give it 30 sweeps of nebulized aqueous 4% formaldehyde. Nebulizers produce a fine mist that is commonly used as an inhalant to treat respiratory illnesses such as asthma. In this case the mist is blown directly on a slide at approximately 1–2 cm from the mouth of the nebulizer. Nebulizing requires a medical air compressor (e.g., DeVilbis) attached to a nebulizer. The nebulized solution should come out fast enough to visibly moisten the frosted end of the slide during each pass. The rate of delivery should be fast enough that the slide does not dry out during nebulization in spite of the air flow into the hood.
5. Dry slides for at least an hour in the hood. If the slides are not completely dry, some of the spreads can come off the slide in subsequent steps.
6. Paint plastic-coated slides with fingernail polish around the edges of the slide and across the margin between the frosted and unfrosted portions of the slide to prevent the plastic from separating prematurely from the glass. Make the polish thick and ridged on the edges to help protect the plastic during

subsequent procedures. However, try not to cover up much of the slide surface with polish. Allow fingernail polish to dry before proceeding.

7. Wash the slides two times for 10 s each in aqueous 0.4% Photoflo 200. Wipe the back of the slides after the first wash to remove damaged plastic and prevent it from lifting off the slide and contaminating subsequent washes and drying on fronts of slides where the SC spreads are located. Air dry the slides at room temperature. Slides dry faster in the hood.
8. Store slides in a sealed box at -80°C for later use for immunocytology or fluorescence *in situ* hybridization (FISH). While we have not found a limit to the length of time slides can be stored and still be used successfully for FISH, slides for immunolabeling should be used within 6 months.

3.5. Immunolabeling and Post-Staining for Electron Microscopy

1. Remove slides from -80°C freezer and bring them to room temperature. Review **Notes 2-8** before beginning procedure.
2. If the edges of slides have not already been sealed with fingernail polish, do this, and let the polish dry completely before beginning the rest of the procedure. A slide can be divided in half with a vertical line of nail polish if two different treatments (with or without DNase, for example) or two different antibodies will be used. Allow the fingernail polish to air dry.
3. Quench any remaining reactive aldehyde groups with ammonium chloride solution (500 μL per slide) at room temperature for 5 min.
4. Prepare primary antibodies by diluting them in full strength ADB and centrifuging at $2,940g$ (13,000 rpm) in a microcentrifuge at 4°C for 30 min (or more). This will pellet any large antibody aggregates and help reduce background.
5. Pour the quenching solution off, but leave the slide wet, and add 100 μL of DNase I solution (if desired) prepared by diluting DNase I stock 1:1,000 in DNase I digestion buffer. Control slides should be incubated with DNase I digestion buffer alone. Cover with a plastic cover slip, avoiding bubbles under the slip. DNase I digestion removes most chromatin, which helps to improve penetration of the gold-conjugated antibodies. Place each slide in a damp incubator Petri dish, and enclose the Petri dishes in a plastic container that contains a layer of wet paper towels to maintain humidity. Incubate the covered slides at 37°C for 15 min. If a slide has been divided into two parts using fingernail polish, treat one half of the slide with 50 μL DNase I digestion buffer only and the other half with 50 μL buffer plus DNase I. To avoid mixing solutions, use two smaller plastic cover slips that only cover the

parts of the slide on either side of the fingernail polish separation line. This procedure can also be used for two different antibody solutions on the same slide.

6. Remove the slides from the incubator. Carefully remove the cover slips so as not to damage the plastic coating on the surface of the slide. One way to do this is to turn the slide upside down, add TBST to the overhanging edges of the plastic cover slip until the slip loosens from the slide. Then, grasp the end of the plastic slip near the frosted end of the slide with forceps, and gently pull the plastic cover slip off in a movement rather like pulling a lid from a sardine can. Rinse slides with about 1–2 mL of extra TBST by gently squirting the solution from a plastic Pasteur pipette onto the frosted end and letting it drain over the surface of the slide.
7. Block slides by adding 500 µL of 10% ADB solution to each slide. Incubate 15 min in a humid chamber, pour off the 10% ADB solution, add 500 µL of fresh 10% ADB and incubate for another 15 min.
8. Pour off the 10% ADB solution and immediately add 100 µL of primary antibody per slide. Cover the primary antibody solution with a plastic cover slip, and incubate the slides for 1–2 h at 37°C in a damp chamber (*see Step 5 above*).
9. Remove the slides from the incubator, and remove the plastic cover slips (*see Step 6 above*).
10. Wash the slides three times for 15 min using at least 500 µL of 10% ADB per wash. Keep slides level in a humid chamber during each wash.
11. Dilute the gold-conjugated secondary antibody in ADB. We typically use dilutions of 1:20–1:50, as recommended by the manufacturer. Do not centrifuge because this will pellet the gold-labeled antibodies (*see Note 9*.)
12. Add 100 µL of diluted secondary antibody per slide. Cover with a plastic cover slip, and incubate 1 h at 37°C in a damp chamber.
13. Remove the cover slips (*see Step 6 above*), and wash the slides three times for 5 min each using at least 500 µL of TBST per wash. Keep slides level in a humid chamber during these wash steps.
14. Fix with 1% glutaraldehyde for 5 min. This step prevents dissociation of the antibodies from their antigens when exposed to the low pH (~2) of the uranyl acetate staining solution.
15. Quench excess aldehyde groups by incubating with 500 µL of ammonium chloride solution per slide at room temperature for 10 min in a damp incubating Petri dish.

16. Wash slides three times for 3–5 min each with 0.15% Photoflo 200 in tall Coplin jars or 150 mL beakers. The slides should be kept vertical, and the amount of liquid should be enough to cover the slide just up to the frosted end. Move the slides upright into a slide rack, and air dry the slides overnight. Cover the rack with a piece of paper that will prevent dust from settling on the slides yet still allow plenty of air flow. If the fingernail polish has become cracked and/or the plastic has begun to lift up in places, reseal these places with fresh fingernail polish.
17. If the secondary antibody was conjugated to US gold particles, visualization of the particles will be greatly enhanced by enlarging their size using silver enhancement. This step must be done prior to UA post-staining because the procedure will also enhance atoms of uranium. The following steps should be followed for US gold only after slides have been re-sealed with new fingernail polish.
 - a) Wash the slides with five changes of distilled or deionized water in 150 mL beakers by placing each slide into a beaker and incubating for 2–3 min, and then transferring the slide to another beaker of fresh water. Do not agitate slides or solution (for example, by placing containers on a rotator). Before silver enhancement, it is essential to remove all ions that could interact with silver particles to cause background. This requires a number of washes in pure deionized or distilled water (*see Note 10*).
 - b) Air dry slides for 1–2 h at room temperature (*see Step 16*).
 - c) Prepare enhancing solutions as described by the manufacturer, but measure volumes using a mechanical pipetter rather than counting drops.
 - d) Place a total of 100–200 μ L of enhancer solution on each slide using a series of small drops that covers most of the area of interest, and cover with a plastic cover slip (*see Note 11*).
 - e) Place the slides upside down in a rectangular slide staining dish (e.g., Fisher # 08-811) that is resting on its side. Transfer the dish on its side into a plastic container containing a wet paper towel to maintain humidity. Close the container, and incubate at room temperature (20°C) for 20–30 min (*see Note 12*).
 - f) Wash slides as described (*see Step 17a*), and remove cover slips (*see Step 6*), except use fresh deionized or distilled water. Wash in water, then air dry.
 - g) Post-stain with UA. Bring the UA solution to room temperature. Repeat Steps 17d–f except use six beakers of water and 10–20 s per wash. Do not add any Photoflo to

water washes. Air dry slides upright in a test tube rack (*see Step 16*). Once slides have been stained, they can be stored at room temperature for several weeks.

3.6. Scanning Slides for Good Spreads and Picking Plastic Up from Slide

1. Scan slides using phase contrast microscopy to locate good spreads (**Fig. 10.4**). The location of each spread should be recorded either using a vernier scale on the microscope stage or electronically using a motor-driven stage. If there are many spreads, it is good to indicate the priority of each spread using some sort of code to assist in later deciding which spreads to pick up onto grids.

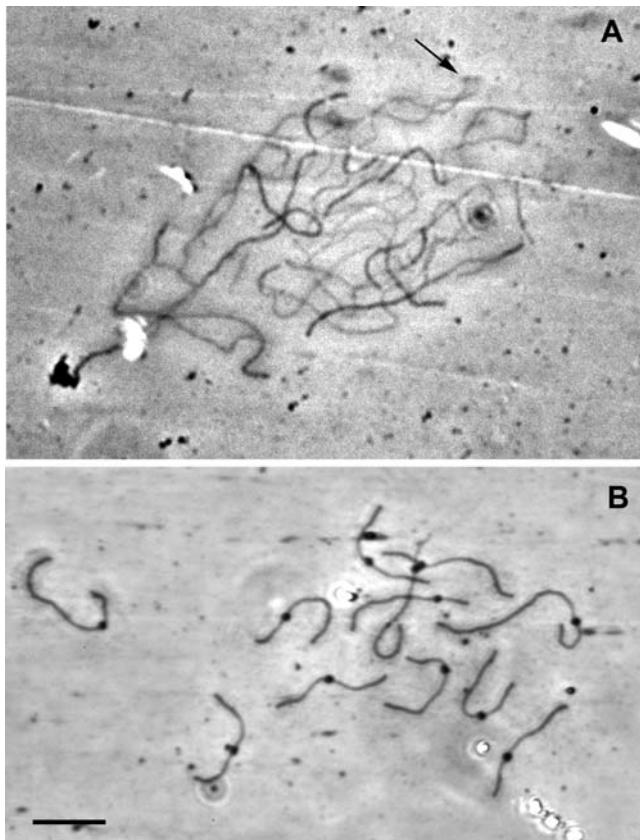


Fig. 10.4. Phase contrast images of **(A)** zygote and **(B)** late pachytene SC spreads from tomato. The image was made with a 40X dry objective with a 2X magnifier. The *dark lines* are SC segments, and the *lighter lines* are single AEs, some of which are aligned (arrow). *Dark spots* on the SCs at pachytene are kinetochores. *Bright flecks and lines* that are visible by phase contrast microscopy are often due to flaws in the glass of the microscope slide. These flaws are usually not apparent in the plastic film once it is lifted off the slide for electron microscopy. Bar = 10 μm .

2. Prepare tacky grids by dipping each one into grid glue using a pair of EM forceps and then placing the grids onto waxed paper to dry. We use 50- or 75-mesh finder grids (Ted Pella Inc.). It probably doesn't matter which side ("shiny" or "dull") that you choose to put down onto the plastic, but whichever side you choose, be consistent. For example, we always put the shiny side down onto the plastic, so after dipping in grid glue, we put the shiny side up to dry. The grids should be slightly tacky so that they don't easily move on the plastic surface, but not so sticky that they cannot be moved at all.
3. Select an SC spread by phase contrast LM, pick up a tacky grid, and gently place the grid onto the slide in the approximate location desired. Look through the oculars and see if the spread is visible within one of the grid openings. If not, move the grid around on the slide with the eyelash tool until it is visible in a grid opening. Continue with other grids. Often it is possible to arrange a grid so that several spreads are visible in different holes of the grid. Also, it may be necessary to move one grid slightly to allow another one to be placed close by.
4. Once all the grids have been positioned, carefully move the slide from the compound microscope stage to a dissecting microscope. It is essential that the slide be held horizontal and not bumped to prevent the grids from moving.
5. Using the sharpened probe, cut the plastic coating by carefully tracing completely around each grid to leave a margin of about 1 mm. If the grids are too close together to do this, then trace around the entire group of grids, leaving the same 1 mm margin on the edge-most grids. Trace around all grids and grid groups on a slide before going to the next step. Adjust the light on the dissecting microscope so that the edges of the newly cut plastic are easy to see.
6. Place a small (~1–2 µL) drop of 1% HF acid near each cut edge. Use the sharpened probe to draw the HF acid to the edge. The HF acid should immediately go under the plastic surface and begin to dissolve the glass so the plastic with the overlying grid begins to lift up from the slide surface. Once this occurs, add small amounts of 5% acetic acid (upto N/mL) to lift plastic and grids until they are floating completely free from the slide. We usually add HF acid to all plastic edges first, and then quickly add the 5% acetic acid. Do not leave the plastic and grids exposed to HF acid for more than a couple of min since a precipitate can form that interferes with EM visualization.
7. When all of the grids are freely floating above the slide, remove any excess plastic that has also come off the slide so that it does not interfere with the grids.

8. Hold the slide level, and move it to an open glass bowl filled with distilled or deionized water. Gently push the slide into the water at a low angle. Once water starts coming over the slide surface, the plastic islands carrying grids will float free on the water surface, and then the slide can be carefully removed from the bowl (*see Note 13*).
9. Use a probe to “herd” the grids together into one part of the culture dish by gently moving the surrounding water and/or lightly touching the edges of the plastic. Do not touch the surface of grids or plastic.
10. Using a nylon screen, make contact with the floating grids, and pick them up with a smooth sweeping motion through the water. If we designate the “front” of the screen as the side with plastic and grids and the back as the side without plastic or grids during the sweep, during the sweeping motion the water should continue to press on the “front” of the screen so that the plastic and grids stay in contact with the nylon. If the motion is not smooth, the water will come through the nylon on the back, and the grids and plastic will not remain in contact with the nylon. The final order of the “sandwich” from the front side is plastic film with SC spreads, grids, nylon screen.
11. Once the grids are picked up onto the nylon, use a Kimwipe to remove excess fluid from the backside of the nylon. Let the grids dry completely (~1 h).
12. Pick grids off the nylon using forceps, and place them onto a clean microscope slide with the side of the grid covered with plastic facing up. Using phase microscopy, examine each grid and record the location of good spreads based on the center mark of the finder grid. Examine in the electron microscope (**Fig. 10.5**).

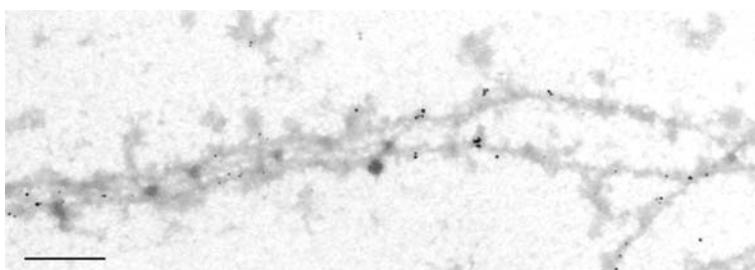


Fig. 10.5. Portion of a zygotene tomato SC immunolabeled with anti-SMC3 (rabbit) and goat anti-rabbit US gold-conjugated antibodies. The US gold particles were enhanced for 30 min at 20°C with silver. The label is present intermittently along the axial and lateral elements, a pattern also seen at the LM level with fluorescence. This preparation was not treated with DNase I prior to immunolabeling. Bar = 0.5 μm.

4. Notes



1. Any one of the following plastics may be used. Poly(styrene-co-acrylonitrile) (PSAN, Aldrich), Formvar [15-95 resin powder (polyvinyl formal powder, Ted Pella, Inc.), or Falcon® plastic (broken from a Petri dish). These plastics have been used successfully to make films, but each has its limitations. Plastic broken from clean Falcon® Petri dishes is probably the most widely used. Falcon plastic film permits silver, phosphotungstic acid (PTA), and uranyl acetate/lead citrate (UP) staining, but the film is brittle and breaks easily. Formvar film is stronger than Falcon plastic film and permits silver, PTA, and UP staining. However, Formvar is more difficult to remove from the slide after making spreads, and it often forms little folds. We prefer film made with PSAN because it is stronger than Falcon plastic without the problems of Formvar. However, PSAN films are incompatible with silver and PTA staining on slides, possibly because there are tiny holes in the plastic that let the stains move between the plastic film and the glass.
2. Wipe back of slides between each antibody incubation and wash step to avoid background.
3. Be careful with plastic surfaces because they are easily damaged during all of the steps, whether wet or dry.
4. Once the immunolabeling procedure has started, never let the surface of the slides dry.
5. All EM solutions need to be made using ultrapure water. Protect from precipitates, dust.
6. It is a good idea to run parallel incubations with SC spreads on plastic-coated slides using fluorescently labeled secondary antibodies to assess labeling success and diagnose problems before spending extra time at the EM level.
7. If there is little or no label using both fluorescent and gold-conjugated antibodies, it is possible that the blocking solution was too concentrated. Here is an optional blocking solution that uses lower concentrations of proteins: 1% BSA, 1% normal goat serum, 0.05% sodium azide in TBS.
8. Remember, anything that is a problem for LM will be unusable for EM.
9. If the number of background gold particles on the plastic surrounding the SC spreads is excessive, then the blocking step may be improved by adding 0.1% acetylated-BSA to the original blocking solution (Electron Microscopy Sciences).

10. In previous immunolabeling steps, detergent was present in each incubation and wash. This helps to keep the plastic film from lifting up from the glass slide. Once the slides are exposed to pure water, the chance that any damaged areas of plastic will lift from the slide is greatly increased. The only way to avoid this is to be careful in all the previous incubation steps and to re-seal the slides with fingernail polish before silver enhancement and UA staining.
11. Because the slides are now very hydrophobic, the solution will not spread easily, and separating the drops helps to cover the area more efficiently. If necessary, you may carefully press on cover slip to spread the solution better, but be careful not to move the cover slip laterally which is likely to damage the plastic.
12. As the manufacturer states, the proper amount of enhancement is dependent on both time and temperature, which must be determined empirically. This is best done using gold-conjugated secondary antibodies dried onto a plastic surface, fixed with glutaraldehyde, washed with water, and enhanced for different times and at different temperatures. In order to reduce the amount of enhancer solution needed and to avoid potential loss of plastic from the slides, these trials can be done on plastic that has already been transferred to nickel grids.
13. We have not had any trouble with HF acid burns using these small volumes of dilute HF, but you may wear gloves during this operation since you may contact the HF containing water at some step. If you wear gloves, be sure that there is no powder residue that could contaminate the water surface and interfere with EM visualization.

Acknowledgments

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Chapter 11

Cytological Analysis of Meiosis in *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* has emerged as an informative experimental system for analysis of meiosis, in large part because of the advantageous physical organization of meiotic nuclei as a gradient of stages within the germline. Here we provide tools for detailed observational studies of cells within the worm gonad, including techniques for light and electron microscopy.

Keywords: *C. elegans*, meiosis, nuclear architecture, chromosomes, meiotic prophase, immunofluorescence, fluorescence in situ hybridization, electron microscopy.

1. Introduction

Over the last several years, *C. elegans* has become one of the preeminent organisms for experimental studies of meiosis. This is largely due to the facility of combining powerful molecular genetics with high-resolution cytological analysis in this system. Many key aspects of meiosis, including meiotic entry and exit, sex determination in the germline, chromosome pairing and synapsis, crossover recombination, meiotic checkpoints, chromosome cohesion, and assembly and function of the segregation apparatus, have been investigated in the worm. Available mutants enable the researcher to manipulate most of these processes in versatile ways, facilitating ongoing and future studies. The goal of this chapter is to provide robust techniques for visualizing meiosis in the nematode. We will cover methods for sample preparation and staining to permit subcellular and subnuclear localization of chromosome loci and proteins through fluorescence microscopy, as well as preparation techniques for transmission electron microscopy.

These techniques have been developed for *C. elegans* but can be easily adapted to related species, such as *C. briggsae* and *C. remanei*, as well as more distantly related organisms with a similar body plan and composition.

In a wild-type population of *C. elegans*, most animals are self-fertilizing hermaphrodites, which contain a bilobed gonad. The ovotestes sequentially produce sperm and then oocytes. Males, which arise spontaneously in wild-type populations at frequencies of $\sim 1/500$, can be propagated by deliberate matings with hermaphrodites. Oocytes (in hermaphrodites) and spermatocytes (in both males and hermaphrodites) display similar meiotic progression through mid-prophase, then diverge in their chromosome condensation and segregation behavior in that oocytes undergo the classically defined stages of diplonema and diakinesis, while spermatocytes condense their chromosomes and divide twice without any obvious individualization of homologs or bivalents. As in most metazoans, oocytes divide asymmetrically to produce a single haploid female pronucleus that can be incorporated into a new zygote, eliminating three quarters of their genetic material into the polar bodies. By contrast, spermatocytes undergo two symmetrical divisions to generate four potentially functional spermatids.

C. elegans offers the great advantage that all stages of meiosis are present in a convenient arrangement within the gonads of individual adult animals (**Fig. 11.1A and B**). The gonad contains a monolayer of germ cells arranged around the periphery of a “rachis” comprised largely of extracellular proteins, much as kernels are arranged on an ear of maize. There are no nurse cells, meaning that all of the germline nuclei within the gonad are destined to undergo meiosis. However, a substantial fraction of

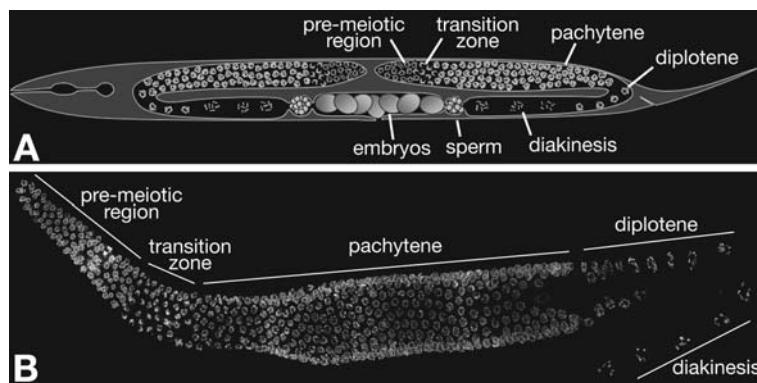


Fig. 11.1. Meiotic progression in *C. elegans*. **(A)** Diagram of a *C. elegans* hermaphrodite. The bi-lobed gonad contains a temporal progression of meiotic nuclei. **(B)** Whole gonad dissected out of a wild-type worm and stained with DAPI. The stages of meiotic prophase can be clearly identified by the chromatin morphology.

oocytes will normally exit this pathway at late pachytene and undergo apoptosis, rather than condensing and executing meiotic divisions. Nuclei with defects in chromosome synapsis or recombination are preferentially targeted for apoptosis, but many apparently normal nuclei are also eliminated in wild-type animals.

The plasma membranes of meiotic cells are discontinuous, and the gonad is therefore regarded as a syncytium. This organization permits experimental introduction of pharmacological agents, DNA, or fluorescent tracers into the cells by microinjection into the distal region of the gonad. However, membrane-impermeant molecules do not readily enter the nuclei other than during nuclear envelope disassembly during the premeiotic mitoses and meiotic divisions. As nuclei enter and progress through the stages of meiosis, they also advance spatially from the distal to the proximal end of the gonad (defined with respect to the vulva, where embryos emerge, or the spermatheca in males). The gonad therefore contains a gradient of premeiotic and meiotic stages, greatly facilitating analysis of the effects of mutations or other perturbations.

The stages of meiosis can be clearly identified by the morphology of the chromatin within meiotic nuclei, as well as their locations within the gonad (**Fig. 11.1B**). As nuclei enter meiosis, the chromosomes, which are normally dispersed around the periphery of the nucleus, become somewhat polarized, resulting in a crescent-shaped appearance of nuclei following DNA staining. This region, referred to as the “transition zone” in *C. elegans*, represents the leptotene and zygotene stages of meiosis, during which homologous chromosomes undergo pairing and synapsis, and recombination initiates through formation of double-strand breaks (DSBs). The cause of the polarization is not fully understood, although it likely involves interactions between the chromosomes and the nuclear envelope. As the chromosomes complete synapsis, they redistribute around the nuclear periphery, and recombination events are completed. This pachytene stage is followed by diplotene, during which the synaptonemal complex begins to disassemble, revealing the physical linkages produced by recombination, or chiasmata. The final stage of prophase is diakinesis, where the chromosomes condense dramatically in preparation for meiotic division. These events are readily observed in oocytes, due to a dramatic expansion of nuclear volume during late prophase, but not in spermatocytes, where both the chromosomes and the nuclei compact prior to segregation.

Oocytes paused at diakinesis are ovulated through the spermatheca, where sperm entry triggers the first meiotic division, followed closely by MII. These division events can be observed in the newly fertilized embryos, which are retained in the uterus of the adult hermaphrodite until after several rounds of mitotic cell division have occurred.

The gonad is optically transparent, as is the entire body of the animal, making the worm amenable to whole-mount immunofluorescence and *in situ* hybridization techniques for localization of meiotic proteins and DNA sequences. Germline nuclei can also be dissociated prior to staining, but this eliminates the spatial information available within the context of the intact tissue, and we will not detail such techniques here.

In other model systems, fluorescent tagging of proteins is commonly used in cytological observation in fixed or live cells. However, in *C. elegans*, expression of transgenes in the germline is problematic due to the phenomenon of cosuppression, in which high-copy transgenes not only fail to be expressed but also silence homologous genomic sequences (1). This problem has been circumvented in some cases by introduction of transgenes at low copy number through microparticle bombardment, a.k.a. ballistic or biolistic transformation (2). However, bombardment is an inefficient procedure that has not yet routinely recapitulated the normal expression of genes required during meiosis. For these reasons, we focus here on cytological methods that do not rely on transgene expression.

The following sections outline methods to perform immunofluorescence and fluorescence *in situ* hybridization (FISH) to localize proteins and DNA sequences in meiotic nuclei, and preparation methods for electron microscopic analysis of the worm gonad. Where appropriate, we have also suggested variations that may help to optimize these protocols for particular experimental goals. Complementary methods for analysis of recombination in *C. elegans* are presented elsewhere in this volume (Chapter 7).

2. Materials

2.1. Light Microscopy

2.1.1. Immunofluorescence

1. Egg Buffer (10x): 250 mM HEPES-NaOH, pH 7.4, 1.18 M NaCl, 480 mM KCl, 20 mM EDTA, 5 mM EGTA. Sterilize by filtration and store at room temperature (*see Note 1*).
2. NPG-Glycerol mounting medium: 2 g N-propyl gallate, 50 g optical-grade glycerol. Dissolve by mixing slowly on nutator or rotary mixer overnight, and store at room temperature. Dissolution of NPG may also be expedited by heating the solution gently in a waterbath or microwave oven.
3. Flat aluminum block, such as those typically supplied with dry heat blocks, chilled on a bed of dry ice.
4. Egg Buffer + Tween-20 + azide (EBTA) (1 mL): 100 µL 10x Egg Buffer, 10 µL 10% Tween-20 (make a 10% (v/v) solution in H₂O, sterile filter and store at room temperature), 40 µL 0.5 M sodium azide (toxic), 850 µL H₂O. Make fresh on the day of use.

5. Fixative solution (for 3.7% formaldehyde final): 100 μ L 10x Egg Buffer, 200 μ L 37% formaldehyde, 700 μ L H₂O. Make fresh on the day of use (*see Note 2*).
6. Methanol. Stored in Coplin jar at -20°C (*see Note 3*).
7. 10x PBS (Phosphate-Buffered Saline): Per liter, add 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, 2.4 g KH₂PO₄. Adjust pH to 7.4 with HCl.
8. PBST (1 L): 100 mL 10x PBS, 890 mL H₂O, 10 mL 10% Tween-20.
9. Blocking agents: normal serum from the species used for secondary antibody production or bovine serum albumin (BSA).
10. Primary antibodies (*see Note 4*).
11. Secondary antibodies.
12. DAPI or Hoechst dyes for fluorescent counterstaining of DNA.
13. Mounting Medium: 35 μ L 2 M Tris base (not pH-adjusted), 450 μ L NPG-Glycerol. Make fresh on the day of use. Mix this solution using a P-1000 tip that has been cut to a larger bore with a razor blade, vortex to mix further, then spin for 1–2 min at top speed to remove bubbles. Optional: Using a finely pulled glass capillary, sparge the solution with nitrogen to reduce oxygen content, then spin to remove bubbles.
14. 18 × 18 mm coverslips No. 1.
15. 22 × 22 mm coverslips No. 1½.
16. No. 11 Scalpel blades. We prefer Feather brand (available from Electron Microscopy Sciences), which are more consistently sharp than other commercial brands.
17. Polylysine-subbed slides, or commercial charged slides such as Histobond (Marienfeld) or Superfrost Plus (Erie Scientific) (75 × 25 × 1 mm).
18. Humid Chamber: a flat, waterproof container (e.g., Tupperware box) with moist paper towels in the bottom covered with Parafilm.
19. Nail polish (we prefer to use clear Sally Hansen Hard-as-Nails, but other brands/colors are also acceptable).

2.1.2. Fluorescence In Situ Hybridization (FISH)

1. Egg Buffer (10x): (*see Section 2.1.1*)
2. NPG-Glycerol mounting medium: (*see Section 2.1.1*)
3. Hybridization solution: 5 mL formamide, 1.5 mL 20x SSC, 1.0 g dextran sulfate, H₂O added to a total vol of 10.0 mL. Prepare in advance (dextran sulfate takes a long time to dissolve). Store at 4°C. Stable for several months. 20x SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.

4. Flat aluminum block chilled on a bed of dry ice.
5. Egg Buffer + Tween-20 + azide (EBTA) (1 mL): (*see Section 2.1.1*)
6. 1.25x EB (1 mL): 100 µL 10x Egg Buffer, 700 µL H₂O. Aliquot 32 µL of 1.25x EB into each of several 0.5-mL Eppendorf tubes, one tube per sample that will be fixed. Make fresh on the day of use.
7. 4% (w/v) Ethylene glycol bis[succinimidylsuccinate] (EGS) in dimethyl formamide (DMF): Weigh by difference into a 1.5-mL screw-cap conical tube approximately 2–4 mg EGS (Pierce), stored at room temperature over Drierite dessicant. This cross-linking agent is not soluble in aqueous medium, so it is dissolved in DMF and then added to buffer just before use. Add DMF (toxic/mutagenic) as required to yield a 4.0% w/v solution – i.e., 25 µL DMF per mg of EGS (use plain old bottled DMF, not special-grade dry DMF in ampules, since it will not dissolve EGS as well). 8 µL of this solution is used per sample. Make fresh on the day of use.
8. Methanol. Stored in Coplin jar at –20° (*see Note 3*).
9. 2x SSCT (per liter): 100 mL 20x SSC, 1 mL Tween-20. The pH does not usually require adjusting, but should be ~7.
10. Egg Buffer + 3.7% formaldehyde (EBF) (50 mL): 5 mL 10x Egg Buffer, 5 mL 37% formaldehyde, 40 mL H₂O. Make fresh on the day of use.
11. Formamide (Fluka 47670; substitute other brands at your own risk). Toxic. Store at 4°C.
12. DAPI or Hoechst dyes for fluorescent counterstaining of DNA.
13. Mounting Medium: (*see Section 2.1.1*).
14. 18 × 18 mm coverslips No. 1.
15. 22 × 22 mm coverslips No. 1½.
16. No. 11 Scalpel blades (Feather brand).
17. Histobond slides (75 × 25 × 1 mm).
18. Humid Chamber: a large Tupperware container with moist paper towels in the bottom covered with Parafilm.
19. Heatblock set at 95°C with flat surface facing up.
20. Nail polish (Sally Hansen Hard-as-Nails, clear).

2.1.3. FISH Probe Labeling

1. Restriction Enzymes (New England Biolabs): AluI, HaeIII, MboI, MseI, MspI, RsaI.
2. Glycogen (molecular biology grade). Glycogen is added to ethanol precipitation reactions to promote precipitation of small DNA fragments and to produce a visible pellet.

3. 3 M sodium acetate (for DNA precipitation).
4. Ethanol (95–100%).
5. 3.3 μ L 1 mM aminoallyl-dUTP (aa-dUTP). This amine-modified nucleotide is available in powder form from Sigma, or from Ambion as a 50 mM stock solution (nucleotides are more stable in solution than as lyophilized solids, so this may be preferable). If you buy the solid form, dissolve 1 mg in 164 μ L water to make a 10 mM stock, aliquot 20 μ L per tube, and store –80°C. Keep a working aliquot at –20°C and dilute 1:10 to make a 1 mM working stock when needed.
6. 6.6 μ L 1 mM unlabeled dTTP (freshly diluted from concentrated stock solution).
7. Recombinant terminal deoxynucleotidyl transferase (TdT). Roche supplies this enzyme at high concentration (400 U/ μ L) with a 5x TdT reaction buffer (contains Tris pH 7.2, potassium cacodylate, and BSA) and 25 mM CoCl₂ solution.
8. 1 M bicarbonate/carbonate buffer: dissolve 0.1 g sodium carbonate and 0.75 g sodium bicarbonate in 10 mL of water. Sterilize this solution using a syringe filter. This will have a pH of ~9. This can be stored frozen in aliquots, but if stored at room temperature the pH will become more acidic with time due to reaction with CO₂ in the air.
9. Fluorescent NHS-esters (reactive dyes for probe labeling) (*see Note 5*).
10. 1 M glycine-HCl pH 8.

2.2. Electron Microscopy

2.2.1. High-Pressure Freezing (HPF)

1. High-pressure freezer (*see Note 6*).
2. Specimen carriers (*see Note 7*).
3. 20% BSA in M9 buffer. M9 buffer (per liter) contains 3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 mL 1 M MgSO₄ (*see Note 8*).
4. Paper points (Ted Pella).
5. 1-hexadecene.
6. 60 mm Petri dish lined with filter paper.
7. 0.5–10 μ L Pipetman with tips.
8. Fine forceps.
9. Worm plates with thick *E. coli* lawn.
10. 4-liter Dewar filled with liquid nitrogen.

2.2.2. Freeze Substitution

1. Automated freeze-substitution device or a freeze-substitution device using dry ice (*see Note 9*).
2. Freeze substitution fixatives (*see Note 10*).

2.2.3. Embedding

2.2.3.1. Epoxy Resins

1. An epoxy resin of your choice such as Epon, or Epon-Araldite. We do not recommend Spurr's resin unless mixed 1:1 with Epon.
2. Microscope slides. We prefer those with frosted or white ends so they can easily be labeled with a pencil. Do not use ink, as it can be dissolved if resin contacts the label.
3. Parafilm.
4. Teflon release agent (Miller-Stephenson).
5. Dry acetone, preferably in 100 mL unopened bottles (Electron Microscopy Sciences).
6. A piece of cardboard about 5 × 7 in. with two 6-in. wooden sticks (Electron Microscopy Sciences) taped about 2 in. apart oriented parallel to the long direction of the cardboard.

2.2.3.2. Methacrylate Resins

1. LR White resin (hard grade) (*see Note 11*).
2. Flat-bottomed embedding capsules (Ted Pella).
3. Aclar plastic (Ted Pella, Inc., Redding, CA; cat. no. 10501-10).
4. Office paper punch.
5. Fine forceps or needles.
6. Parafilm.
7. Vacuum embedding oven set at 60°C.

2.2.4. Remounting Worms for Sectioning

1. Epoxy 907 Adhesive System (Miller-Stephenson).
2. No. 11 scalpel blades.
3. Light microscope with DIC or phase optics.
4. Blank epoxy blocks for remounting. Both BEEM capsule and flat-embed mold geometries are recommended.
5. Jeweler's saw with fine blade.
6. Table-top vise.
7. Double-edged razor blades.

3. Methods

3.1. Light Microscopy

3.1.1. Immunofluorescence

1. Before you start: make EBT and fixative solutions and place flat aluminum block on dry ice so it starts cooling.
2. Pick 15–25 worms into a 30-µL drop of EBT on an 18 × 18 mm coverslip on top of a glass slide (*see Note 12*). Use a scalpel blade to nick each worm immediately behind the pharynx and/or just before the tail to release the gonad arms.

3. Pipette 30 μ L of fixative solution into the drop of dissected worms. Pipette up and down a few times to mix and extrude more gonads. Let worms fix for 4–5 min total (*see Note 2*).
4. Pipette off all but 15 μ L, being careful to leave the tissue behind. Pick up the coverslip by touching the drop with the center of a HistoBond slide. Invert slide and wick away excess liquid from the edges of the coverslip using a torn piece of absorbent paper. The more liquid you remove, the better the worms will stick to the slide.
5. Freeze-crack the samples. Freeze the sample by placing it on the aluminum block in dry ice for >5 min. Carefully flick off the cover slip by catching the edge with a fresh razor blade.
6. Place the slide immediately in methanol (prechilled to –20°C) for 1 min, then move to a Coplin jar of PBST at room temperature (*see Note 3*).
7. Repeat Steps 2 through 6 until all samples are dissected.
8. Wash slides 3x (10 min/wash) by moving slides to fresh Coplin jars of PBST.
9. Block slides in a 1:10 dilution of normal serum proteins (typically supplied at 6% w/vol following reconstitution) in PBST or 0.5% BSA in PBST. Pipette 50–100 μ L of block onto the slide near the worms and cover with a Parafilm coverslip (just a piece of Parafilm cut to size). Place slides in a humid chamber for >30 min at room temp.
10. Primary Antibody. Remove the Parafilm coverslip by placing each slide in a Coplin jar of PBST and letting it float off. Apply 50–100 μ L of primary antibody (*see Note 4*) in PBST (or PBST plus block), cover with a Parafilm coverslip, and incubate in a humid chamber for 2 h at room temperature or overnight at 4°C.
11. Wash slides 3x (10 min/wash) by moving slides to fresh Coplin jars of PBST.
12. Secondary Antibody. Remove the Parafilm coverslip as in Step 10. Apply 50–100 μ L of secondary antibody in PBST (or PBST plus block), cover with a Parafilm coverslip, and incubate in a humid chamber for 2 h at room temperature or overnight at 4°C.
13. Wash and DAPI stain samples (*see Note 13*). Remove the Parafilm coverslip as in Step 10. Wash slides 10 min in a fresh Coplin jar of PBST, then move to Coplin jar of PBST plus 0.5 μ g/mL DAPI (add 5 μ L of 5 mg/mL stock to 50 mL PBST in a Coplin jar) and incubate for >10 min. Finally, wash slides >30 min in a fresh Coplin jar of PBST.

14. Mount slides. Pipette 10–12 μL of mounting medium onto a 22 \times 22 mm coverslip. Remove the slide from buffer, remove as much buffer as possible without desiccating the tissue sample by wiping with a tissue. Be careful not to wipe off your worms. Invert the slide and touch it to the mounting medium. Invert the slide again, and carefully wick off any excess mounting medium. Seal with nail polish.
15. Observe slides (*see Note 14*).

3.1.2. Fluorescence In Situ Hybridization (FISH)

1. Before you begin dissections: prepare EBTA, 1.25x EB, and 4% (w/vol) EGS in DMF. Also, place aluminum block on dry ice, flat side up, for freezing slides.
2. Pick 15–25 worms into a 30- μL drop of EBTA on a 18 \times 18 mm coverslip on top of a glass slide (*see Note 12*). Use a scalpel blade to nick each worm immediately behind the pharynx and/or just before the tail to release the gonad arms.
3. To a 32- μL aliquot of 1.25x EB, add 8.0 μL 4% EGS in DMF. Transfer 30 μL of this solution to the drop with the dissected worms. Pipette up and down a few times to mix and extrude more gonads.
4. Using a P-20 Pipetman, remove all but \sim 15 μL , being careful to leave the tissue behind. Pick up the coverslip by touching the drop with the center of a HistoBond slide. Invert slide and wick away excess liquid from the edges of the coverslip using a torn piece of absorbent paper. The more liquid you remove, the better the worms will stick to the slide.
5. Incubate in a humid chamber for 30 min at room temperature.
6. Freeze-crack the samples. Freeze the sample by placing it on the aluminum block in dry ice for >1 min. Carefully flick off the cover slip by catching the edge with a fresh razor blade.
7. Place the slide immediately in -20°C methanol for 1 min, then move to a Coplin jar of 2x SSCT at room temperature (*see Note 3*).
8. Repeat Steps 2 through 7 until all samples are dissected.
9. Remove each slide from 2x SSCT and drain. Wearing gloves, place in EBF in Coplin jar. Wait 5 min.
10. Carefully dip slides into a beaker filled with 2xSSCT to remove fixative. Transfer slides to Coplin jar filled with 2xSSCT and wash for 5 min. Wash with one change of 2xSSCT for 5 min.
11. Prepare 100 mL of 50% formamide in 2xSSCT and divide equally between two Coplin jars. Label jars No. 1 and No. 2.
12. Transfer slides to jar No. 1. Wait 5–10 min and move slides to jar No. 2. Place jar No. 2 at 37°C overnight.

13. Remove jar with slides from 37°C while preparing probe solution.
14. Make up probe solution [per slide: 20–500 ng of each probe (*see Section 3.1.3, Step 9*)] diluted into a total vol of 15 µL hybridization solution.
15. For each sample: pipette 15 µL probe solution onto a 22 × 22 mm coverslip. Wearing GLOVES: Drain slides and wick away as much liquid as possible without damaging or drying out sample. Touch the worms to the drop of probe solution on the coverslip, allow the liquid to spread out, and invert the slides. Seal coverslips with nail polish.
16. Denature slides (two at a time) on a 95°C heat block for 3 min.
17. Place in a humid chamber at 37°C overnight (*see Note 15*).
18. Carefully remove coverslip by using a sharp razor blade to cut off nail polish.
19. Wash slides in 50% formamide in 2xSSCT at 37°C for at least an hour, first in jar No. 1 then in jar No. 2 (*see Note 15*).
20. Wash away formamide and DAPI stain samples (*see Note 13*). Wash slides 10 min in a fresh Coplin jar of 2x SSCT, then move to Coplin jar of 2x SSCT plus 0.5 µg/mL DAPI (add 5 µL of 5 mg/mL DAPI stock to 50 mL 2x SSCT in a Coplin jar and mix). Incubate for >10 min. Finally, wash slides >30 min in a fresh Coplin jar of 2x SSCT.
21. Mount slides. Pipette 10–12 µL of Mounting Medium onto a 22 × 22 mm coverslip. Remove the slide from buffer, remove as much buffer as possible without desiccating the tissue sample by wiping with a tissue. Be careful not to wipe off your worms. Invert the slide and touch it to the mounting medium. Invert the slide again, and carefully wick off any excess mounting medium. Seal with nail polish.
22. Make a sacrifice to the FISH gods, skip around the room, or perform other rituals as desired.
23. Observe slides (*see Note 14*).

3.1.3. FISH Probe Labeling

1. Generating DNA for probe synthesis: probes can be generated from most DNA sources (PCR products, plasmids, cosmids, oligonucleotides, etc) (*see Note 16*). For probes consisting of DNA fragments longer than ~100 bp, the DNA must be digested into small fragments. This is most reliably and economically achieved using multiple restriction endonucleases. To digest 30 µg DNA (scale up or down as appropriate, do not exceed ~0.1 µg/mL DNA in digest): Dilute 30 µg probe DNA to a vol of 250 µL with H₂O, add 30 µL NEB Buffer 2 (10x), 3 µL NEB 10 mg/mL BSA (100x), and 30–60 units each of the following six enzymes:

AluI, HaeIII, MboI, MseI, MspI, and RsaI (note: you may choose to substitute Tsp509 I for this enzyme mixture to digest the *C. elegans* 5S rDNA repeat). Add water to a final vol of 300 μ L. Mix and place at 37°C for ≥ 2 h (overnight digestion is fine).

2. Check digest by loading 0.25–5 μ g DNA onto a 2% gel and compare to low MW markers. The average fragment size should be ≤ 150 bp.
3. Ethanol precipitate digested DNA: Add 1 μ L 20 mg/mL glycogen, 30 μ L 3 M sodium acetate (don't use ammonium acetate, since ammonium ions will compete for dye in the conjugation reaction), and 750 μ L 95–100% ethanol. Chill at –20°C or colder for 10 min (or longer). Spin down 15 min at maximum speed, wash pellet carefully with cold 70% ethanol, and air-dry pellet or remove residual ethanol in a centrifugal evaporator. Resuspend DNA in 30 μ L H₂O.
4. If DNA concentration was not accurately known prior to digestion, you should now measure OD₂₆₀ of a 1:200 dilution to calculate actual DNA concentration.
5. Tailing 10 μ g of DNA with aa-dUTP (can be scaled up or down as desired): Dilute 10 μ g fragmented DNA or oligonucleotide with H₂O to a total vol of 50 μ L. If DNA is double-stranded: Cap tube and place tube in a 95°C water bath for 2 min, then chill immediately on ice; spin briefly to collect condensed water from sides of tube (single-stranded DNA is a preferred substrate for TdT). Add at room temperature: 20 μ L Roche 5x TdT reaction buffer (contains Tris-HCl pH 7.2, potassium cacodylate, and BSA), 20 μ L Roche 25 mM CoCl₂ solution (or other CoCl₂ stock to 5 mM final), 3.3 μ L 1 mM aa-dUTP, 6.6 μ L 1 mM unlabeled dTTP, 2 μ L (800 units) recombinant Terminal deoxynucleotidyl Transferase (TdT). Incubate for 1 h at 37°C.
6. Ethanol precipitate digested DNA: Add EDTA to 5 mM final to chelate the cobalt. Add 1 μ L 20 mg/mL glycogen, 10 μ L 3 M sodium acetate (don't use ammonium acetate), and 500 μ L 95–100% ethanol. Chill at –20°C or colder for 10 min (or longer). Spin down 15 min at maximum speed, wash pellet carefully with cold 70% ethanol, and air-dry pellet (or remove residual ethanol in a centrifugal evaporator) (*see Note 17*).
7. Conjugation with dye: resuspend 10 μ g of aa-labeled DNA in 10 μ L H₂O. To one aliquot of dry dye (*see Note 5*), add 5 μ L of DNA (5 μ g) in water and 3 μ L of 1 M bicarbonate/carbonate buffer. If using Alexa dyes, also add 1.5 μ L high-quality DMSO to increase the solubility of the dye. Mix thoroughly by tapping, spin the tube briefly, and incubate the reaction shielded from light for 1 h at room temperature (longer is fine

but probably doesn't improve incorporation). Add 1 μ L 1 M glycine-HCl pH 8 to quench the remaining reactive dye. Incubate a further 15 min at room temperature.

8. Ethanol precipitate digested DNA: Dilute labeling reaction to 100 μ L with H₂O, add 1 μ L 20 mg/mL glycogen, 100 μ L 4 M ammonium acetate, and 500 μ L 95–100% ethanol. Chill at –20°C or colder for 10 min (or longer). Spin down 15 min at maximum speed to pellet labeled DNA, wash pellet carefully with cold 75% ethanol (labeled DNA may be slightly soluble in 70% ethanol due to hydrophobic properties of the dyes), and air-dry pellet (or remove residual ethanol in a centrifugal evaporator) (*see Note 18*).
9. Resuspend the labeled probe DNA in hybridization buffer (*see Note 19*). It is convenient to dissolve probes at a concentration such that a standard volume (typically 0.5 μ L) is used for each sample to be hybridized. Complex, single-copy probes (such as cosmid mixtures or BACs) should be stored at concentrations as high as 1 μ g/ μ L, while high-copy probes (targeting rDNA or other repetitive elements) can be made to 40 ng/ μ L, since less is needed per hybridization. Store probes in nondefrosting –20°C or –80°C freezer.

3.2. Electron Microscopy

3.2.1. High-Pressure Freezing

1. Start up the high-pressure freezer according to instructions.
2. Place a piece of filter paper in a small Petri dish and saturate the filter paper with 1-hexadecene. Place specimen planchettes on the filter paper, coating both sides if you are using a BAL-TEC or Wohlwend system, just the cup side if you are using the Leica system. Wick or wipe off the excess 1-hexadecene prior to loading the worms and/or filler solution. 1-Hexadecene acts as a release agent so that your sample will come out of the metal planchette easily.
3. Select a type of specimen planchette that is appropriate for the stage of worm you are freezing. In general, the depth of the planchette should be about the same as the thickness of the worm. For gravid adults, use a 100 μ m deep well.
4. If using 20% BSA as a filler, add just enough to fill the cavity of the specimen planchette. Try to make sure that the BSA doesn't touch the rim of the planchette because that will allow the worms to crawl out of the center of the cup.
5. Pick 10–15 worms of the appropriate stage and/or phenotype and transfer them to the BSA in the cup.
6. If necessary, use paper point wicks or a micropipette to adjust the level in the cup so that it is just barely overfilled. Under no circumstances should you underfill the cup and trap air because the air will interfere with the heat transfer during freezing.

7. Depending on the type of freezer you are using, either (1) place a flat-bottomed top planchette on the cup with worms (BAL-TEC and Wohlwend machines), or (2) secure the planchette in a freezing pod (Leica).
8. Freeze.
9. Remove planchette from specimen holder tip under liquid nitrogen and transfer to storage vial or into frozen fixative vial for freeze substitution (*see Note 20*).

3.2.2. Freeze Substitution

- 1a. Fill the automatic freeze substitution (AFS) device with liquid nitrogen, set the program (*see Note 10*), and cool to -90°C .
- 1b. If you don't have an AFS, pre-cool a dry block heater (with 13-mm holes) on dry ice in a box until a dummy vial with acetone cools to -78°C .
2. Transfer the cryovials with samples and fixative to the AFS at -90°C , or the dry ice box at -78°C .
3. When the program ends, or the dry ice device warms to room temperature, proceed to the embedding process.

3.2.3. Embedding

3.2.3.1. Epoxy Resins

1. Inspect the tubes to see if the worms have separated from the specimen planchettes or not. If using *E. coli* as a filler, there is a good chance that the worms may be loose in the bottom of the tube. To ensure that the worms aren't lost during subsequent solution changes, transfer to a 1.5 mL Eppendorf tube so the samples can be concentrated by either gravity or centrifugation prior to solution transfers.
2. Rinse the samples a minimum of three times (5 min each) in pure acetone.
3. Infiltrate in a graded series of resins. We usually start with 25% resin in acetone for 1 h. At this step, it is convenient to remove the worms from the carriers. This can be done by gently nudging the disk of BSA/worms in the cup with fine forceps or a needle. In most cases, the BSA will be fixed around the worms and you will have a solid, disk-shaped object to handle. If you choose to freeze worms surrounded by *E. coli*, then the worms will often separate from the bacteria at this step and you should transfer them to an Eppendorf tube for further processing so they can be spun down between solution changes.
4. Continue the infiltration by incubating in 50% resin-acetone mix for 2 h, 75% for 4 h, then a brief rinse in pure resin followed by incubation overnight in pure resin. All steps should be done on a rocker or rotator to facilitate fluid exchange.
5. Carry out one more exchange of pure resin before the final embedding steps.

6. For each worm sample to be prepared, prepare two Teflon-coated slides. This is done by dipping slides in a solution of Miller-Stephenson MS-143 V TFE release agent, letting them dry until they are an opaque white, then wiping them clean with paper or cloth.
7. Label two slides for each sample and on one of the set place a double thickness of Parafilm (about 5 × 10 mm in size) at each end of the clear part of the slide.
8. Put about 300 µL of resin in a strip between the two pieces of Parafilm.
9. Using the needle tool, or a sharpened toothpick, transfer the worms to the strip of resin. If the worms were frozen in BSA, they may all be stuck together in a disk of fixed BSA. Using a pair of No. 11 scalpel tips it is possible, with practice, to cut out individual worms from the disk. Worms frozen in *E. coli* will separate easily, if they did not spontaneously separate during earlier stages of resin infiltration. In either case, spread out individual worms so they are well-separated but not at the edge of the slide. Let them settle to the surface of the slide.
10. Carefully place the other slide of this set on top of the slide with resin and worms so that only the clear portions of the slide overlap. This is best done by resting one end of the top slide on the piece of Parafilm nearest the labeled end and lowering it slowly so the resin spreads out to the edges of the slides. If the worms were resting on the bottom slide, they should not move much toward the edges.
11. Place the slide astride the two sticks on the cardboard. When all slides are done, put into an embedding oven to cure. Propping the slides on the sticks makes it easy to remove the slides in case excess resin spills onto the cardboard.

3.2.3.2. Methacrylate Resins

1. Check the tubes to see if worms have separated from the planchettes and/or filler (*see Section 3.4.3.1*, Step 1).
2. Rinse as above for epoxy resins, but after the final acetone rinse, rinse for 5 min in a 1:1 mixture of pure ethanol and pure acetone, then two rinses for 5 min each in pure ethanol (*see Note 21*).
3. Infiltrate for 1 hr or more in a 1:1 solution of pure ethanol and LR White Hard Grade.
4. Rinse twice in pure LR White for 5 min, then pure LR White overnight on a rotator.
5. Prepare flat-bottomed resin capsules by first punching out disks of Aclar with the hole punch and inserting one Aclar disk in the bottom of each capsule. You can use one capsule per worm, or group three to four worms in one capsule.

6. Label each capsule with the identifying information.
7. Transfer the worms to a watch glass or glass spot plate or some small dish that you can see through. Regular plastic Petri dishes may react with LR White and are not recommended. Because the worms are colorless, you will need some dish that you can use with transmitted illumination.
8. Separate the worms as best you can from each other using needles or No. 11 scalpel blades.
9. Fill the capsule about half full with LR White.
10. Transfer one to four worms to each capsule, separating them from each other as best you can and making sure they are on the Aclar disk.
11. Fill the capsule and put a small square of Parafilm over the top before putting on the top. This will help seal the capsule from oxygen during polymerization.
12. Polymerize in a 60°C oven for 2 days, preferably a vacuum oven or one that can be filled with a gas such as argon or nitrogen.

3.2.4. Remounting Worms for Sectioning

3.2.4.1. Epoxy Resin

1. Remove slides with worms from the embedding oven and let cool to room temperature.
2. Remove excess resin from the edges of the slides with a razor blade or scalpel.
3. Place a single-edged razor blade between the slides and gently push so that one of the slides separates from the polymerized resin. Remove that slide completely. The remaining slide will have a thin layer of polymerized resin containing the worms.
4. Make a small mark near one of the worms with a marker pen or scalpel tip.
5. Find that mark and the nearby worm using bright-field illumination on a compound light microscope. Using the fine focus knob, determine whether the worms are on the top side of the resin, or on the bottom (i.e., nearest the slide). Mark the slide accordingly, e.g., as “top” or “bottom.” This will be extremely helpful when remounting for sectioning.
6. Scan the slide on the microscope to check the worms and to identify those that have a favorable orientation for your purposes – for example, if you plan to access a particular region of the gonad.
7. Cut out selected worms from the slide using a No. 11 scalpel blade, and remount on blank epoxy stubs for sectioning. Because you know which side of the thin layer of epoxy the worm is on (“top” or “bottom”), be sure to remount it so that the worm is away from the remount glue and at the surface of the remounted chip. This will enable you to cut

sections of worm as soon as you begin sectioning. If the remount glue is not going to be sectioned, then any two-part epoxy, or even superglue may work. However, if remounting requires that some of the glue be sectioned (e.g., to cut cross-sections through the worm) then we recommend using Miller-Stephenson 907 two-part epoxy because it sections well and is stable under the electron beam.

8. Because worms processed at low temperature tend to retain more cytoplasm, we recommend cutting fairly thin sections, perhaps 50–60 nm. Thicker sections may yield images that are difficult to interpret; however, they may be satisfactory for certain cytoplasmic features.
9. To find particular features such as the transition zone of the gonad, it may be necessary to screen serial EM sections, or to screen blocks with semi-thick sections in the light microscope, until the right region is located and thin sections can be taken.

3.2.4.2. Methacrylate Resin

1. Remove the polymerized resin from the flat-bottomed capsule. The capsule plastic is very hard and is best removed by placing the capsule in a vise and sawing along the sides with a jewelers saw. Using razor blades is not advised, since it is both more difficult and there is a risk of injury.
2. Remove the resin around the edges of the Aclar disk and peel off the Aclar.
3. Worms can be observed in a light microscope by propping up the block on a slide and focusing on the worm at the surface. An inverted microscope is handy because you can simply place the flat top surface of the block with the worm directly on the slide.
4. If a polymerized block contains only a single worm and you want longitudinal sections, simply trim and section. If you want cross sections, or if there is more than one worm on the Aclar disk, you will need to cut off the tip (1–2 mm) of the block with the jeweler's saw, divide the disk with a razor blade so that there is one worm per section, and (if you want cross sections) remount on the side of a flat-embedding mold blank block with remount glue.

4. Notes



1. Egg buffer was developed by Lois Edgar for isolation and manipulation of blastomeres from living *C. elegans* embryos. We adapted this buffer for gonad dissection on the assumption that it recapitulates the internal environment of the

gonad fairly well, and have found that it yields good chromosome and nuclear morphology. Other buffers we have tested, including "Buffer A" or sperm salts, have produced unsatisfactory morphological preservation based on comparisons between fixed and living tissue. Azide is included in the dissection buffer to rapidly paralyze worms, making them easier to dissect; levamisole or other agents may be substituted. Nonionic detergent (Tween-20 or Triton X-100) is included in the dissection buffer to reduce surface tension and minimize sticking of the tissue to the scalpel blade, as well as facilitating membrane permeabilization. Detergents are also included throughout staining protocols to reduce surface tension of the staining solutions, which enhances retention of the tissue on slides.

2. *C. elegans* germline tissue is unusually difficult to fix consistently so as to preserve fine structure while permitting antibody diffusion and epitope accessibility. We do not know why this is the case, but plausible explanations include a high internal protein concentration and/or the composition of the muscular gonad sheath, which may be easily crosslinked by aldehyde fixatives. Many sensitive antibodies will not show specific staining following even brief fixation in 3.7% formaldehyde. We therefore routinely fix at a final concentration of 1% formaldehyde. We have not observed any reproducible differences in fixation using commercial formalin (37% aqueous formaldehyde stabilized with methanol) versus formaldehyde freshly prepared from paraformaldehyde. In some cases, it may be useful to eliminate formaldehyde fixation entirely; following dissection and freeze-cracking, the tissue can be simply fixed by immersion in cold ethanol, methanol, or dimethylformamide (DMF). Sample morphology is less stable and more variable when the formaldehyde fixation step is eliminated, but specific antibodies (e.g., anti-H3meK9 (Upstate, Lake Placid NY)) and cellular structures (e.g. microtubules) are particularly sensitive to formaldehyde fixation. An alternative or adjunct to reducing fixative concentration is to permeabilize the gonad after fixation by treatment with collagenase (we prefer Type 3 from Worthington Biochemical Corporation). However, since collagenase treatment adds several additional variables to the procedure, we do not do this routinely.
3. Methanol can be substituted with 95% or absolute ethanol or with dimethylformamide (toxic/mutagenic). We have not seen dramatic differences among these three solvents, and we prefer to use methanol or ethanol because of the toxicity of DMF.
4. Primary antibodies can be preadsorbed against whole worms to remove some background staining. This procedure follows the immunofluorescence protocol closely except that the

fixation steps are performed in a microcentrifuge tube. This can be done using mutant worms lacking the protein of interest, but preadsorption against wild-type animals is frequently an effective way to suppress background staining without reducing the signal.

Wash worms off of at least one 60-mm plate of gravid adult worms using EBT. Transfer to a 1.5-mL microfuge tube.

Spin at maximum speed in a microcentrifuge for 30 s. Discard supernatant. Add 1 mL fresh EBT and mix.

Spin down and discard supernatant. Wash once with 50% EBT / 50% fixative solution for 5 min.

Spin down worms and discard supernatant. Freeze tube on dry ice.

Add 500 µL methanol prechilled to -20°C, mix, and leave on ice for 1 min.

Spin down and remove supernatant. Add 500 µL 1x PBST. Leave on nutator for 30 min at room temperature.

Spin down and remove supernatant. Add 500 µL 1x PBST containing blocking agent (0.5% w/vol serum proteins or BSA). Leave on nutator for 30 min at room temperature.

Spin down and remove supernatant. Add 500 µL 1x PBST containing blocking agent, then 50 µL primary antibody. Leave on nutator overnight at 4°C.

Spin at maximum speed in microfuge. Transfer supernatant to a fresh tube and discard worms. Since the antibody is already diluted 1:10 in this preadsorption step, adjust subsequent dilutions accordingly.

5. In choosing dyes, a major consideration should be the wavelength of the excitation lasers and/or filters on your microscope system. Fluorescent NHS-esters are sold by Invitrogen (the Alexa dyes) and GE Biosciences (Cy3 and Cy5). They are sold in convenient multipacks marketed for labeling of FISH or microarray probes. It's cheaper to buy in bulk quantities (e.g. 5 mg of dye, which is sufficient for about 100 5-µg DNA labelings), but some loss of activity is expected during aliquoting. Follow manufacturers' recommendations for aliquoting and storage. Alternatively, aliquots can be prepared by dissolving dyes in dry DMSO, aliquoting appropriate quantities to individual tubes, and evaporating the DMSO under vacuum in a centrifugal evaporator or lyophilizer (this takes a while, since DMSO is not very volatile). Store lyophilized dyes protected from light and desiccated, preferably in a vacuum desiccator, at 4°C; they must be scrupulously free of water and solvents to minimize degradation.

6. Although it is possible to prepare EM samples of worms by methods other than high-pressure freezing, the quality of preservation is so much better by HPF that the method should be used if at all possible. For nonHPF methods, see the article in *Methods in Cell Biology* by Shai Shaham on the online Wormbook at: http://www.wormbook.org/toc_wormmethods.html. There are three types of high-pressure freezers currently available: the Bal-Tec HPM 010 (Liechtenstein; www.bal-tec.com), the Wohlwend Compact HPM 01 (Sennwald, Switzerland; www.technotradeinc.com), and the Leica EM PACT systems (Vienna, Austria; www.leica-microsystems.com). Each of these machines has proven itself capable of excellent freezing of *C. elegans* worms. To see if there is a machine near you, contact the vendors through their respective websites. The necessary details of how to use these machines to prepare worms for EM is beyond the scope of this article. To fill in the gaps of the overview presented here, see the following articles regarding the Bal-Tec HPM 010, with a section specifically for worms (3), the Leica EM PACT system (4), and more detailed instructions specifically for worms (5–7). Bal-Tec, Ag was recently sold to Leica Microsystems, Vienna. The Bal-Tec HPM 010 is no longer available, though the leica HPM 100 may serve as a replacement.
7. There are numerous options for specimen carriers for each type of high-pressure freezer. The general rule is to use the shallowest holder that will contain your material without compressing or crushing it. For adult worms we usually use 100 µm deep membrane carriers for the Leica HPF machines, and 100 µm deep aluminum carriers for the BAL-TEC and Wohlwend HPF machines. For smaller worm stages, there are custom carriers available for the Bal-Tec/Wohlwend machines, or, one can use EM slot grids of known thickness to form variable-depth spacers (4).
8. BSA is a good filler for HPF work, but it has the drawback that it will form a solid meshwork around the worms when they are fixed during freeze substitution. One can also use *E. coli* from worm plates as a filler and it has the advantage that it will separate from individual worms after fixation, making it easy to screen them by light microscopy. The disadvantage of *E. coli* is that it is not as effective a cryoprotectant as BSA. It is best used with very shallow wells such as those formed when using slot grids as variable spacers.
9. Leica Microsystems makes an automated freeze-substitution device, as does Boeckeler Instruments. A description of how to use dry ice for freeze substitution can be found in Ref. (8) and http://www.wormbook.org/toc_wormmethods.html.

10. There are many different freeze-substitution mixtures in the literature, but the most popular use a combination of acetone and osmium tetroxide for morphological studies, and acetone and glutaraldehyde for immunological work. We use a 1% solution of osmium tetroxide in pure acetone plus 0.1% uranyl acetate and 5% water. The water helps to visualize membranes (9). For immunoEM, we use 0.2% glutaraldehyde plus 0.1% uranyl acetate in acetone. Detailed instructions for how to make up these fixatives can be found in Ref. (3).
11. We prefer to use LR White Hard Grade because it is convenient to work at room temperature where one can see the samples under a stereomicroscope if necessary. Other methacrylate resins include LR Gold and the family of Lowicryl low-temperature resins. The Lowicryls can give quite excellent preservation of ultrastructure and antigenicity, but they are quite volatile and can cause contact dermatitis if adequate protection is not used during handling. For these reasons we prefer to use the LR White resins which were originally designed as a less hazardous alternative to the epoxy resin chemistry.
12. Age-matched adult worms are important for many types of quantitative or temporal analysis or because the representation of different stages of meiosis within the gonad changes as a function of the age of the animal: pachytene nuclei tend to accumulate over time relative to premeiotic or leptotene/zygotene-stage nuclei. We generally analyze young adults, which can be synchronized to within a few hours of development by picking late L4 larvae 12–24 h prior to dissection. Older adults (2–3 days post-L4) are useful for scoring nuclei at diakinesis, since this stage persists longer as sperm are depleted.
13. Sometimes it can be advantageous to combine immunofluorescence with FISH. The order in which these steps should be performed will depend on the sensitivity of the antibody to fixation conditions and the demands of the experiment. For best morphological preservation, FISH is performed prior to immunofluorescence, moving from the final wash steps of the FISH protocol (*see Section 3.1.2*, Step 20) to the blocking step of the immunofluorescence protocol (*see Section 3.1.1*, Step 9). Because the FISH procedure is more damaging to the tissue, stabilization of the tissue with EGS offers advantages for sample preservation, but is not compatible with all antibodies (*see also Note 2*). For this reason, we often fix the samples optimally for immunofluorescence, carry out immunofluorescent detection, and then follow with FISH. In this case, after the final washes of the immunofluorescence protocol (*see Section 3.1.1*, Step 13), the samples are post-fixed

with a higher concentration of formaldehyde, and the FISH protocol is then executed (*see Section 3.1.2*, Step 9). In either case, samples can be counterstained with DAPI or other dyes during the final washes before mounting.

14. Because of the convenient organization of the gonad, temporal analysis of meiotic nuclei is a common technique used in meiosis in *C. elegans*. These pseudo-time course experiments are typically executed by dividing each gonad into a number of sections, moving from the proximal (early) to the distal (late) portion of the gonad. Nuclei within each section are scored separately. If the animals are age-matched and the sections are specified in a systematic way, data from corresponding sections in multiple animals can be pooled. We generally divide the gonad into five sections of equal physical length, starting in the premeiotic/mitotic region through to the end of pachytene, because the zones correlate fairly well with the mitotic region in zone 1, the transition zone in zone 2, and early, mid and late pachytene in zones 3–5, respectively (10, 11).
15. We have adapted our FISH protocol to incorporate microwave irradiation during the prehybridization and hybridization procedure. Microwaves are thought to accelerate diffusion and chemical reactions in a partially heat-independent fashion, although this remains controversial. The rationale for applying this to FISH is that the body of a worm is fairly impermeable to both fixatives and to probes, and microwaves may enable the large probe molecules to gain access to target DNA in the limited time window before chromosomes re-anneal. Empirically, this version of the protocol has given better results to date than any other with respect to both structural preservation of worm nuclei and reproducible, strong FISH signals with low fluorescent background. The procedure is designed around the capabilities of the Ted Pella BioWave microwave oven, which is equipped with variable wattage settings, restrictive-temperature control, and a “ColdSpot” circulating water bath with a flat glass surface that sits on the floor of the microwave oven and is controlled by an external heater/chiller/pump. According to the manufacturer, this circulating water eliminates standing waves and thus “hotspots” within the volume of the oven, resulting in even irradiation throughout. It also allows the temperature of a slide placed directly on the surface of the glass to be controlled fairly precisely and semi-independently of irradiation. The following changes are made to the FISH protocol to utilize the BioWave:

The overnight hybridization step (*see Section 3.1.2*, Step 17) is replaced by approximately 1.5 h in the BioWave. Prior to denaturation (*see Section 3.1.2*, Step 16), place the slides

on the surface of the ColdSpot, preequilibrated to 30°C. Irradiate slides (10 min ON – 5 min OFF – 10 min ON) at setting No.6 with the temperature probe inserted into the ColdSpot port and a restrictive temperature of 37°C. Denature slides as in **Section 3.1.2**, Step 16. Return slides to surface of ColdSpot in BioWave. Irradiate slides (10 min ON – 5 min OFF – 10 min ON) at setting No.6 with restrictive temperature at 37°C. Repeat this cycle again for a total of ~1 h of hybridization. Continue with coverslip removal and washing steps (*see* **Section 3.1.2**, Step 18).

The final hour of washes in 50% formamide in 2xSSCT (*see* **Section 3.1.2**, Step 19) is replaced by incubation with microwave irradiation as follows: Place slides in jar No. 1. Insert temperature probe to the same depth as samples and irradiate with restrictive temperature of 37°C (2 min ON – 2 min OFF – 2 min ON) at setting No. 2. Transfer to jar No. 2 containing fresh 50% formamide/2xSSCT and repeat irradiation. Continue washing and DAPI staining steps (*see* **Section 3.1.2**, Step 20).

16. FISH probes can be generated from repetitive or single-copy regions. For nonrepetitive probes, a probe targeting approximately 5 kb of genomic sequence is (empirically) about the minimal length required to detect a signal above background fluorescence using protocols outlined here. However, probes generated to repetitive regions work more robustly. Two repetitive regions on the *X* chromosome make very good FISH probes. The sequence 5'-TTTCGCTTAGAGCGATTCCCT-TACCCTTAAATGGGCGCCGG-3' is highly enriched near the center of the *X* chromosome, on cosmid C07D8, and 5'-GACTCCATCCACCAAGCACTGCTTCGAGTACGACAGA AAGCACTTC-3' is highly concentrated in a short (~6-kb) region near the right end of the *X* chromosome (12). Probes to short, repetitive sequences, including these can be generated as synthetic oligonucleotides and end-labeled with aa-dUTP without digestion (*see* **Section 3.1.3**, Step 5). A probe to the *C. elegans* 5S rDNA repeat is a robust tool to label this region on the right arm of chromosome V. Such a probe can be synthesized by amplification of the 1-kb repeated sequence using the following primers: 5'-TACTTGGATCGGAGAC GGCC-3' and 5'-CTAACTGGACTAACGTTGC-3'. This product can be digested to appropriately small fragment sizes using a single enzyme, Tsp509 I.
17. This same tailing protocol (omitting the dye conjugation step) may be used to incorporate other nucleotide analogs, including digoxigenin-dUTP or fluorescent dNTPs (biotinylated probes tend to be unsatisfactory in *C. elegans* due to high background staining). Digoxigenin-labeled FISH probes work more robustly than those labeled directly with

fluorophores because of the secondary amplification of the signal by an anti-dig antibody. However, use of haptene-labeled probes adds extra steps to the FISH procedure (anti-dig immunofluorescence after the final washes in 50% formamide in 2xSSCT) and multiple digoxigenin probes cannot be used together. When using fluorescent dNTPs, we mix the modified nucleotide with a two-fold excess of unlabeled nucleotide, for two reasons: (1) The enzyme will be unhappy if it only has the modified substrate to incorporate, and (2) incorporation of fluorophores at too high a density will cause quenching of the fluorescence.

18. If the precipitated DNA is not obviously colored (particularly for Cy3- and Cy5-labeled probes), the labeling has probably not gone well. The terminal transferase reaction is more finicky than the dye conjugation step, so when poor dye incorporation is observed, DNA should probably be re-purified and/or relabeled with aa-dUTP.
19. Storing probes in hybridization buffer is convenient because an arbitrary amount can be mixed with additional hybridization solution to titrate the optimal amount of probe. In addition, hybridization solution does not freeze at -20°C due to the presence of 10% dextran sulfate. This may minimize probe degradation over long-term storage by eliminating freeze-thaw cycles. Probes may also be reconstituted in water or buffer, in which case the hybridization stock solution should be made more concentrated to accommodate dilution by probe.
20. Frozen material can be stored in liquid nitrogen for indefinite periods of time. We prefer to transfer the samples to vials with frozen fixative and use these for storage. This eliminates one transfer step when one is ready to begin freeze substitution. For longer-term storage, we use empty (no fixative) cryovials with small holes in the top so that they will stay filled with liquid nitrogen.
21. In our opinion, this is an optional step, as samples infiltrated with acetone:LR White mixtures work perfectly well for immunolabeling. However, the makers of LR White recommend ethanol, so it can also be done this way. In our experience, the use of ethanol for freeze substitution gives less satisfactory results than acetone.

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Chapter 12

Cytological Analysis of Meiosis in Fixed *Drosophila* Ovaries

Kim S. McKim, Eric F. Joyce, and Janet K. Jang

Abstract

Methods are described to analyze two different parts of the *Drosophila* ovary, which correspond to early stages (pachytene) and late stages (metaphase I and beyond) of meiosis. In addition to taking into account morphology, the techniques differ by fixation conditions and the method to isolate the tissue. Most of these methods are whole mounts, which preserve the three-dimensional structure.

Keywords: Meiosis, recombination, double-strand break, nondisjunction, chromosome, *Drosophila* females, methods, oocyte, synaptonemal complex, pachytene.

1. Introduction

Meiosis in *Drosophila* females can be studied at two distinct stages of oocyte development. Each oocyte develops within a cyst which includes 15 nurse cells. Within each of two ovaries are strings of developing oocytes, or ovarioles. Each ovariole is divided into the germarium, where early prophase occurs, and the vitellarium, where oocyte growth and differentiation occurs (Fig. 12.1) (1–3). The germarium is where meiotic prophase initiates and pachytene stages are found with such hallmarks for meiosis as the synaptonemal complex (SC), recombination nodules (4) as well as other indicators that this is where meiotic recombination initiates (5). Most studies on meiotic recombination and synapsis focus on the germarium.

The vitellarium is primarily devoted to growth and differentiation of the oocyte. This phase of development is divided into 14 stages, based on morphology of the oocyte and its accompanying 15 nurse cells (6, 7). Stage 1 is actually the last stage of the germarium, stage 2 is the first stage of the vitellarium, the SC

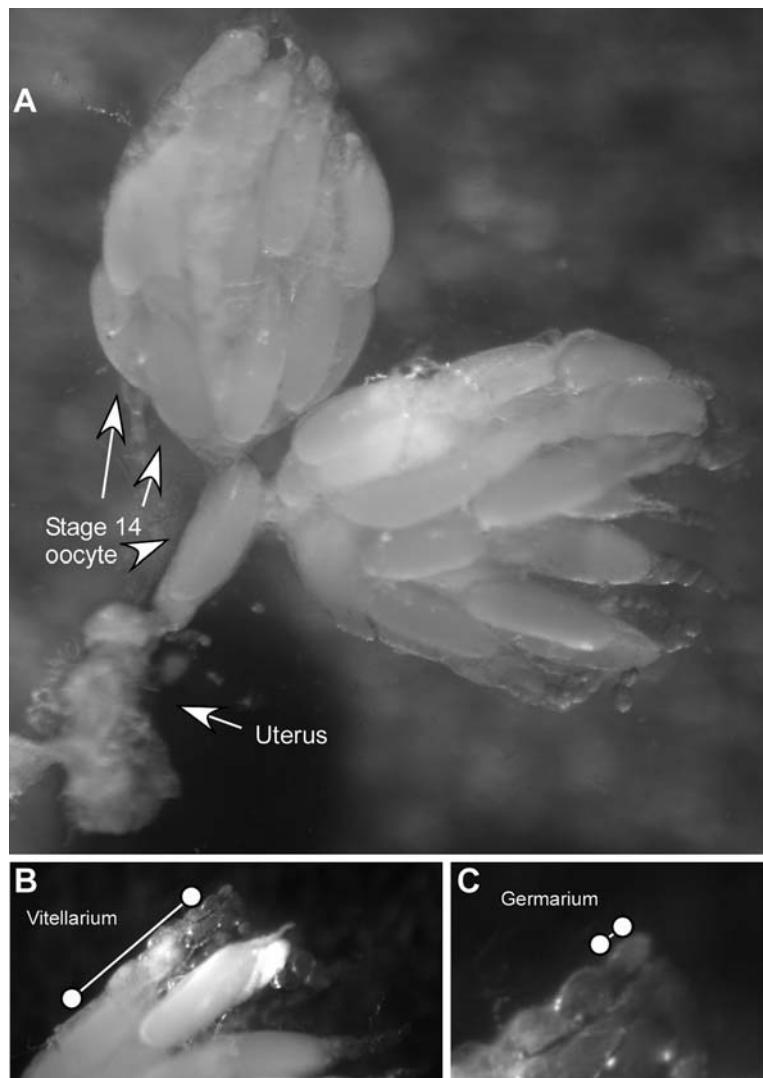


Fig. 12.1. The *Drosophila* ovary. (A) Within the female ovary are two sacs of ovarioles, which are strings of developing oocytes. The *right* ovary has been opened up slightly to show the ovarioles. At the anterior tip of each ovariole is the germarium, where each oocyte is born as part of a 16-cell cyst and in which pachytene occurs. Individual 16-cell cysts, containing one oocyte, bud off from the germarium and enter the vitellarium. Within the vitellarium the oocyte grows in size. Approximately four days elapse between the completion of recombination (in the germarium) and oocyte maturation (stage 14) when the metaphase spindle forms. One mature oocyte has left the ovary and is moving down the reproductive tract towards the organs containing the stored sperm and the uterus. (B, C) Higher magnification images showing the location of the germarium at the anterior end of the ovariole.

disappears around stage 5 or 6, and the oocyte grows in size at the expense of the nurse cells until stages 13–14. At this stage, the nurse cells have degenerated. The transition between 13 and 14 is marked by breakdown of the nuclear envelope and it is in stage 14

oocytes that the metaphase I spindle forms and then progression arrests. In addition to the references given above, a major compilation of *Drosophila* biology, genetics, and methods can be found in Ashburner et al. (8) and Sullivan et al. (9).

Germarium and stage 13/14 oocytes are convenient to isolate by cytological methods. Progression past metaphase I does not occur until the oocyte passes down the oviduct. It is apparently the change in environment that activates anaphase since fertilization is not required. As the oocyte moves down the oviduct and is laid, the stages of anaphase I, metaphase II and anaphase II occur. While more challenging to examine than metaphase I, these later stages can be captured with the appropriate collection methods. In addition, and in common to some mammalian oocytes, the meiotic divisions occur without centrosomes. Thus, this is an attractive system for the analysis of acentrosomal spindle assembly. There are at least three important differences between the various methods described below: fixation conditions, the dissection and incubation buffer, and how the tissue is obtained, for example hand dissection versus mass isolation by physical disruption (i.e., a blender). Most of these procedures involve whole mounted tissue, although one “spread” procedure is described.

1.1. A Brief Comment on Genetics

This article will focus on the cytological methods used to analyze meiosis in *Drosophila* females. However, it is pertinent to also review important genetic methods, since the greatest power comes from a combination of genetic and cytological approaches.

Meiotic nondisjunction is most easily assayed on the X-chromosome. Females are crossed to males carrying a dominant marker on the X or Y chromosome (Fig. 12.2) (see Note 1). It is also usually advisable to have at least one recessive marker on the female X-chromosome. For example, when *y/y* females are crossed to *y w/B^SY* males, the regular progeny are phenotypically yellow-bodied females with normal eyes (*y/y w*) and yellow-bodied males with Bar eyes (*y/B^SY*). Female nondisjunction, to generate gametes with two X-chromosomes (diplo-X) or none (nullo-X), generate yellow-bodied Bar-eyed females (*y/y /B^SY*) and yellow-bodied, white-eyed males (*y w/O*) in addition to an equal number of dead embryos due to aneuploidy. The advantage of this method is that the frequency of nondisjunctional gametes can be calculated since both the normal and exceptional progeny are recovered.

Autosomal nondisjunction can also be detected but the cross recovers only the nondisjunctional gametes since autosomal monosomy or trisomy generates inviable zygotes. Compound autosomes are used, in which two arms or two whole chromosomes are joined to a single centromere. The result is that the only male gametes to produce viable diploid zygotes carry two or zero copies of an autosome and fertilize oocytes generated from

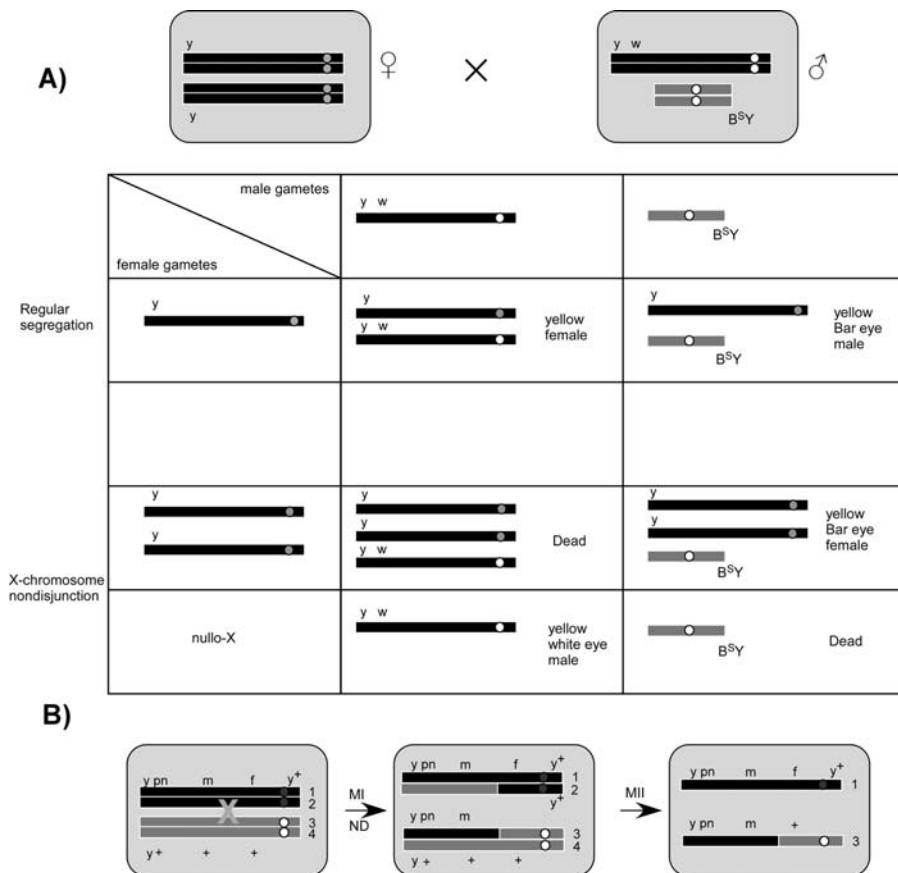


Fig. 12.2. **Genetic analysis of nondisjunction.** (A) In this example, *y/y* females are crossed to *y w/B^SY* males. The regular progeny are yellow-bodied females with normal eyes and yellow-bodied males with Bar eyes. Nondisjunction in the female germline generates gametes with two X-chromosomes (diplo-X) or none (nullo-X). The progeny that indicate these events are yellow-bodied Bar-eyed females and yellow-bodied, white-eyed males. The frequency of nondisjunction gametes can be calculated as $2(\text{exceptional progeny})/[2(\text{exceptional progeny})+(\text{regular progeny})]$. (B) If the females are heterozygous for multiple X-chromosome markers, a crossover followed by meiosis I nondisjunction can result in zygotes (if fertilized by a Y-bearing sperm) homozygous for distal markers.

nondisjunction with no copies or two copies, respectively, of a particular autosome. Since progeny from regular disjunction in the female do not survive, a frequency of nondisjunction is more difficult to calculate.

Crossing over can be measured on any chromosome except the tiny 4th. For example, the cross of *st cu e ca/++++* to *st cu e ca/st cu e ca* males (the textbook *test cross*) measures crossing over along the entire right arm of chromosome 3, including the region spanning the centric heterochromatin (*st - cu*). This is important since some mutants affect the distribution of crossing over. Measuring crossing over on the X-chromosome has the advantage that it is possible to simultaneously measure nondisjunction. For example, when *y pn cv m f•y⁺/y* females are crossed to *y w/B^SY* males

(see Note 1), crossing over along the entire X-chromosome, including an interval containing the centromere (f to y^+), can be scored among the male progeny. In addition, female progeny with Bar eyes and male progeny with normal eyes are the result of nondisjunction. These crosses can also detect when chiasmate bivalents nondisjoin (Fig. 12.2B) (10).

2. Materials

2.1. Buffer A Fixation

1. It is usually safest to use high-grade reagents, particularly formaldehyde (Ted Pella), formamide (Fluka cat. # 47670), Tween-20 and Triton X-100 (Pierce “Surfact-Amps”) and BSA (Sigma A4161).
2. $10 \times$ Buffer A: 150 mM PIPES (Sodium Salt), pH 7.4, 0.8 M KCl, 200 mM NaCl, 20 mM EDTA, 5 mM EGTA with final pH adjusted to 7.0.
3. 37% Formaldehyde solution: 0.37 g paraformaldehyde in 0.7 mL ddH₂O, heat in boiling water bath for 60 s and add 18 μ L of 1 N NaOH. Shake the tube constantly while immersed in boiling water for about 60 s, immediately cool solution by shaking tube under stream of cold tap water.
4. Buffer A fix (1 mL): 100 μ L $10 \times$ Buffer A, 108 μ L 37% formaldehyde, 1 μ L 1.0 M dithiothreitol (DTT), 1 μ L 0.5 M spermidine, 1 μ L 0.15 M spermine, 789 μ L ddH₂O.
5. Buffer A with 0.1% Triton X-100 (BAT): Combine 0.5 mL $10 \times$ Buffer A, 50 μ L 10% Triton X-100, 5 μ L 1.0 M DTT, 5 μ L 0.5 M spermidine, 5 μ L 0.15 M spermine, ddH₂O to 5 mL.
6. BAT with 10% normal goat serum (BAT-NGS): Combine 1 mL $10 \times$ Buffer A, 1 mL NGS, 100 μ L 10% Triton X-100, 10 μ L 1.0 M DTT, 10 μ L 0.5 M spermidine, 10 μ L 0.15 M spermine, ddH₂O to 10 mL.
7. BAT-BSA 0.2%: Combine 2 mL $10 \times$ Buffer A, 200 μ L 10% Triton X-100, 0.04 g bovine serum albumin (BSA), 20 μ L 1.0 M DTT, 20 μ L 0.5 M spermidine, 20 μ L 0.15 M spermine, ddH₂O to 20 mL.
8. Dissecting materials: It is useful to have fine-tipped tweezers [e.g., 5622 Dumont tweezers high precision grade (style 5 from Ted Pella)] and a tungsten needle. Eppendorf tubes graduated at 100 μ L increments are also needed. For a Tungsten needle, you will need a sharpened wire needle made from a 2–3 cm long piece of tungsten wire (0.13 mm diam.; Ted Pella Inc. Cat No. 27-11). Put a kink near one end of the wire and insert this end into the beveled opening of a 26 G syringe

needle. The kink will create enough friction so that the base of the wire will remain securely in place within the syringe needle. The wire can then be sharpened electrically using a 1 N solution of NaOH and a direct current power supply (e.g., one that supplies 6.3 amp at an adjustable voltage between 2 and 6 V). Wear face and hand protection to avoid being splashed by droplets of NaOH that might bubble up from the solution. Place one electrode in a small beaker of 1 N NaOH and clip the other onto the syringe needle. Dipping the needle in and out of the solution will cause the needle to taper to a point. Start at a setting of 4–5 V to taper the end of the wire to a blunt point, and then switch to 2–3 V to put a sharp point just at the end of the needle. Avoid creating a long, thin end as this will be bent too easily when you use it to dissect embryos or oocytes.

2.2. PBS Fixation

1. PBS – In 1 L, combine 10 mL of 1 M sodium phosphate buffer pH 7.4 (from a mix of 1 M Na₂HPO₄ and 1 M NaH₂PO₄ to make a solution of pH 7.4) and 7.6 g NaCl. It is often convenient to make a 10× PBS pH 7.4 stock solution.
2. PBST (PBS + 0.2% Tween-20): For 20 mL use 2 mL 10× PBS, 400 µL 10% Tween-20 and 17.6 mL ddH₂O.
3. PBST + 1% BSA: for 1 mL use 100 µL 10× PBS, 0.01 g BSA, 20 µL 10% Tween-20, ddH₂O to 1 mL.
4. PBS fixative: For 200 µL use 25 µL 16% formaldehyde, 1 µL 100% NP-40, 20 µL 10× PBS, and 154 µL ddH₂O. In some cases, formaldehyde can be made from paraformaldehyde. Alternatively, a high-grade source such as the EM grade 16% solution from Ted Pella should be used.

2.3. Chromosome Spread of Germarium Oocytes

1. Hypo buffer: 17 mM trisodium citrate dihydrate, 5 mM EDTA, 0.5 mM DTT, 30 mM Tris-HCl pH 8.2, 0.5 mM Pefabloc (protease inhibitor).
2. Fixative: 0.25 g paraformaldehyde, 22.5 mL water, one drop 1 N NaOH, subsequently adjusted to pH 9.2 using 50 mM boric acid supplemented with 350 µL Triton X-100.
3. PBS wash buffer: PBS plus 5% donkey serum (goat serum can probably be substituted), 2% BSA, 0.1% Triton X-100, 0.1% sodium azide.
4. PBS incubation buffer: PBS with 0.1% BSA and 0.1% Triton X-100.

2.4. Fluorescence In Situ Hybridization (FISH)

1. 20× SSC: 3 M NaCl, 0.3 M sodium citrate.
2. 2× SSCT: 0.3 M NaCl, 0.03 M sodium citrate, 0.1% Tween-20. For a 50 mL solution, mix 5 mL of 20× SSC and 0.5 mL of 10% Tween-20 and fill final volume to 50 mL with ddH₂O.

3. $2 \times$ SSCT/20% formamide: For 1 mL solution add 10 μ L of 10% Tween-20, 200 μ L of formamide, 100 μ L of $2 \times$ SSC and 690 μ L of ddH₂O.
4. $2 \times$ SSCT/40% formamide: For 1 mL of solution, add 10 μ L of 10% Tween-20, 400 μ L of formamide, 100 μ L of $2 \times$ SSC, and 490 μ L of ddH₂O.
5. $2 \times$ SSCT/50% formamide: For 6 mL solution add 60 μ L of 10% Tween-20, 3 mL of formamide, 600 μ L of $2 \times$ SSC, and 2.34 mL of ddH₂O.
6. Cacodylate Fixative: 100 mM potassium cacodylate with pH adjusted to 7.2 with 10 N KOH, 100 mM sucrose, 40 mM potassium acetate, 10 mM sodium acetate, 10 mM EGTA, and 5% formaldehyde. A 1 mL fix solution can be made by mixing 0.1 mL of 1 M potassium cacodylate, 66.8 μ L of 1.5 M sucrose, 20.0 μ L of 2 M potassium acetate, 5.0 μ L of 2 M sodium acetate, 20.0 μ L of 0.5 M EGTA, 0.313 mL of 16% formaldehyde, and 0.475 mL of ddH₂O.
7. PBST: 0.2% Tween-20 in PBS.
8. PBSTB: 0.2% Tween-20 and 1% BSA in PBS.
9. Hybridization solution: $2 \times$ SSC, 50% formamide, 10% (w/v) dextran sulfate.
10. A variety of fluorescent dNTPs (Cy3, Cy5, or Alexa-labeled) are available from GE Healthcare and Molecular Probes. We have used Terminal Deoxynucleotidyl Transferase (TdT) from Invitrogen, which comes with a 5 \times buffer.

2.5. Fixation of Stage

14 Oocytes

1. Modified Robb's Buffer: 55 mM sodium acetate, 40 mM potassium acetate, 100 mM sucrose, 10 mM glucose, 1.2 mM MgCl₂, 1.0 mM CaCl₂, 100 mM HEPES (free acid), pH 7.4.
2. Cacodylate Fixative: 100 mM potassium cacodylate with pH adjusted to 7.2 with 10 N KOH, 100 mM sucrose, 40 mM potassium acetate, 10 mM sodium acetate, 10 mM EDTA, 8% formaldehyde (from 16% EM grade methanol-free formaldehyde from Ted Pella Inc.). Paraformaldehyde may not work as well. See also **Section 2.4**, step 6.
3. PTB: PBS + 0.1% Tween-20, 0.5% BSA.

2.6. Fixation of

Embryos (Recently Fertilized Oocytes)

1. Grape juice plates: 500 mL will pour about three sleeves of 60 \times 15 mm plates. For 500 mL add the following ingredients while stirring: 15 g bacto agar, 29 g dextrose, 14.5 g sucrose, 90 mL grape juice, 409 mL ddH₂O, 1.25 mL 10 N NaOH. Boil using a microwave, being careful not to let the solution boil over, until the agar is dissolved, then cool slightly and add 5.6 mL acid mix while stirring (acid mix is 20.9 mL propionic

acid plus 2.075 mL phosphoric acid filled to 50 mL with ddH₂O). Autoclaving instead of using a microwave can cause the agar not to solidify.

2. PBS, *see Section 2.2.*

2.7. Activation of Stage 14 Oocytes

1. Isolation Buffer: 55 mM sodium acetate, 40 mM potassium acetate, 110 mM sucrose, 1.2 mM MgCl₂, 1 mM CaCl₂, 100 mM Hepes (free acid), final pH 7.4.
2. Activation buffer: 3.3 mM NaH₂PO₄, 16.6 mM KH₂PO₄, 10 mM NaCl, 50 mM KCl, 5% polyethylene glycol 8000, 2 mM CaCl₂, pH to 6.4 with 1:5 mixture of 10 N NaOH : 10 N KOH.
3. Zalokar's Buffer: 9 mM MgCl₂, 10 mM MgSO₄, 2.9 mM NaH₂PO₄, 0.22 mM sodium acetate, 5 mM glucose, 27 mM glutamic acid, 33 mM glycine, 2 mM malic acid, 7 mM CaCl₂, pH to 6.8 with 1:1 mixture of 10 N NaOH: 10 N KOH.

3. Methods

3.1. Whole Mount Preparation of Early Meiotic Stages – Buffer A

1. Prior to dissection of ovaries for immunostaining of germaria, feed females with yeast for 16 h at 25°C (*see Note 2*). They are not fed yeast for too long because the germarium is relatively small while the later and larger oocyte stages can get in the way. This procedure for preparation of germarium tissue is more complicated than that described in the next section (based on PBS) but it is reputed to preserve chromatin structure well (*11*), a conclusion we agree with. The ovaries from 15 to 20 flies should be dissected in 1 × Robb's media and moved to a clean well containing fresh media (*see Note 3*). A tungsten needle can then be used to remove the ovariolar sheath and to tease the ovaries apart. This procedure should take no more than 20 min from the first dissection. Move the separated ovaries to the cap of a graduated 1.5 mL Eppendorf tube containing 500 µL of Buffer A Fix solution. Leave the ovaries rotating in Fix solution for 9 min at room temperature. Let them settle for 1 min and then promptly aspirate as much liquid as possible without removing the ovaries.
2. All washes should be performed at room temperature in 1 mL of solution unless otherwise noted. Wash the fixed ovaries twice for 15 min each with 1 mL of BAT solution. Block with two washes of BAT-NGS for 15 min each, making sure to aspirate the liquid after each wash. After the liquid from the second BAT-NGS wash is removed, replace with 100 µL of fresh BAT-NGS solution. Add primary antibodies diluted for a

volume of 300 µL. The dilution of each primary antibody has to be determined experimentally. Fill to 300 µL mark on graduated tube with BAT-NGS. Incubate rotating overnight at 4°C.

3. Let ovaries settle and aspirate supernatant containing primary antibody in BAT-NGS. Proceed with four washes of BAT-BSA at room temperature for 30 min each. This should be followed by one wash with BAT-NGS for 30 min. While the ovaries are in the final BAT-NGS wash, centrifuge the secondary antibodies/embryos for 10 min at full speed in a microfuge (*see Note 4*). After the wash, allow the ovaries to settle and remove the liquid. Add the secondary antibody supernatant without any embryos to the ovaries and fill to the appropriate final volume (usually 300–500 µL) with BAT-NGS. Incubate by rotating the tube for 2–4 h at room temperature in the dark. At this point, the ovaries should be kept in the dark during all subsequent washes.
4. After incubation, remove the liquid and wash the ovaries one time in BAT solution for 30 min. To stain for DNA, let the ovaries settle, remove liquid and replace with DNA dye in fresh BAT solution (*see Note 5*). If using Hoechst, a 1:5,000 dilution (i.e., 0.2 µL 10 mg/mL Hoechst in 1 mL BAT) should be used and left rotating for 7 min. Proceed with one final BAT wash for 15 min and one quick wash in 1X Buffer A. Remove liquid and store ovaries in 1 mL of 1X Buffer A at 4°C in the dark until ready to mount. Ovaries should not be stored longer than a week. Mount the ovaries in fluorescence mounting media such as Vectashield or Slowfade gold (*see Note 6*). The ovarioles should be completely separated using a tungsten needle before being secured with a coverslip and sealed with nail polish.

3.2. Whole Mount Preparation of Early Meiotic Stages – PBS

1. Mix 200 µL PBS fix with 600 µL heptane. Shake vigorously for a few minutes and then leave on a rotator until it turns milky white (actually really tiny bubbles resulting from emulsion of the organic and aqueous phases).
2. Age the females and yeast-feed as with the Buffer A procedure (*see Section 3.1*). Dissect the ovaries in 1X PBS with gentle teasing to open them up a little. Fix for 20 min in PBS fix plus heptane.
3. Rinse three times in 1 mL of PBST quickly. It may be necessary to add directly to the fix in order to get the ovaries to settle and be able to see them. Remove the fix in the first wash. Follow with three more washes of 5 min in PBST.
4. Block for 1 h at room temperature in 1 mL PBST + 1% BSA. At this time preabsorption of the secondary antibody can be started (*see Note 4*). Add the primary antibody(ies) in PBST for 1 h at room temperature or overnight at 4°C.

5. Remove the primary antibody and wash three times for 20 min in 1 mL PBST. Add the secondary antibodies in PBST for 1 h at room temperature or overnight at 4°C.
6. Remove the secondary antibody and wash twice for 20 min in 1 mL PBST. Stain the DNA in PBS (e.g. 0.2 µL 10 mg/mL Hoechst in 1 mL PBS for 5 min, *see Note 5*) at room temperature followed by a wash for 20 min in 1 mL PBS.
7. Mount immediately in suitable medium (*see Note 6*) or store in 1X PBS overnight at 4°C. Once mounted, the slides should be examined immediately.

3.3. Squash Preparation of Early Meiotic Stages

The previous two preparations are whole mounts, with the objective of maintaining the structure of the germarium. Webber et al. (12) described a method to spread oocyte chromosomes. Newly eclosed females are used so that the ovaries are small (the more mature stages are larger and can take up the majority of the tissue volume).

1. Feed yeast paste to approximately seven newly eclosed females overnight and dissect their ovaries in PBS (*see Notes 2 and 3*). Rinse the ovaries in freshly made hypo buffer, incubate in hypo buffer for 20–30 min and transfer to a drop of hypo buffer.
2. Separate the germaria (the tips of the ovaries) from the later yolk stages (vitellarium) with a tungsten needle. Transfer the germaria to a drop of 100 mM sucrose and mince with the tungsten needle to separate the cells. Finally, pipette the tissue up and down using a P-2 tip coated with BSA.
3. Prepare slides by dipping for ~15 s into fixative. While holding the slide at an angle, apply 10 µL of cells to the slide and spread. A second slide can be used for the remaining 10–15 µL. Keep the slides in a humidified chamber for ~14 h and then allow them to dry, which takes ~6 h. Place the dry slides in a 0.4% solution (in water) of photoflo (Kodak) for 2 min. Dry slides around the edges with a Kim-Wipe and then air dry in a Coplin jar (~2 h) and store overnight (not longer) at –20°C.
4. Rehydrate the tissue in PBS for 15 min and block for 1 h in a humidified chamber at room temperature in PBS wash buffer. Dilute antibodies in PBS incubation buffer. Apply 100–200 µL of antibody solution to the slide, cover it with a parafilm coverslip and incubate in a humidified chamber for 1 h. Rinses slide quickly three times and then wash three times for 10 min each with PBS incubation buffer. Repeat for the secondary antibodies and then stain tissue with DAPI or Hoechst at 1 µg/mL in the second 10 min wash (*see Note 5*). Mount the tissue with suitable medium such as Slowfade or Vectashield (*see Note 6*).

3.4. FISH to Ovaries (Germarium or Vitellarium Oocytes)

Although originally developed and optimized for the analysis of stage 14 oocytes (13), this protocol can be applied to all stages (14). Thus, it has not yet been determined if the use of the cacodylate-based fixative can be replaced with a Buffer A or PBS-based fixation procedure.

For unknown reasons, probe hybridization to germarium nuclei is stronger in nurse cells than oocytes. Indeed, some oocytes do not show any signal. An alternative to FISH is to use the LacI-GFP/LacO system, which has been used effectively to examine homolog pairing in *Drosophila* female meiosis (15). However, the same problem that some oocytes lack a GFP signal has been found.

- Prior to dissection of ovaries for immunostaining of germaria, age females for 16 h (or longer, 3–4 d for stage 14 oocytes) at 25°C. Dissect the ovaries from 15 to 20 flies in 1 × Robb's media or PBS and move to a clean well containing fresh media (*see Note 3* and **Section 3.1**). If stage 14 oocytes are the target tissue, the mass isolation procedure (with a blender, *see Section 3.5*) can be used. It can be a problem if one is trying to get both early (pre-stage 14) and late (stage 14) oocytes in the same dissection since the latter need to have their membranes removed, for example by rolling, which is a procedure that can damage the early stages. The dissection procedure should take no more than 20 min. Once complete, transfer all ovaries to a well containing the Fix solution for 4 min. During this time a tungsten needle can be used to remove the ovarian-lolar sheath and to tease the ovaries apart. It is normal for the ovaries to swell and become rigid during this step.
- Move the separated ovaries to the cap of a graduated 0.5 mL Eppendorf tube and do two quick washes with 500 µL of 2 × SSCT each. Then, step into 2 × SSCT with 50% formamide by adding and removing sequentially for 10 min each while rotating at room temperature the following washes: one wash with 2 × SSCT/20% formamide, one wash with 2 × SSCT/40% formamide, and one wash with 2 × SSCT/50% formamide. Add fresh 2 × SSCT/50% formamide and incubate at 37°C for 1–2 h.
- Use a terminal transferase reaction to generate the probe. The following protocol is to label 10 µg of DNA (such as a BAC clone) or oligonucleotide for simple sequence probes, but the reaction can be scaled down. BAC DNA must be fragmented first so the probe can penetrate the tissue. Digest the DNA with a combination of four-cutter restriction enzymes (e.g. AluI, HaeIII, MseI, MspI, RsaI, Sau3AI, all of which can work in the same buffer, NEB 2).
- Heat the fragmented DNA or oligonucleotide to 95°C for 2–5 min, place on ice for 5 min and then add to the reaction mix. Reaction mix is 6.75 µL 1 mM fluorescent-dUTP or

dCTP (Alexa-labeled from Molecular Probes or Cy3-labeled from GE Health Care), 13.5 μ L 1 mM cold dTTP or dCTP, 20 μ L 5 \times TdT buffer, 4 μ L 15 U/ μ L TdT (Invitrogen), and water to 100 μ L. Incubate at 37°C for 1 h to overnight. Precipitate by adding 2 μ L 20 mg/mL glycogen, 10 μ L 4 M ammonium acetate, 250 μ L 100% ethanol and chill at -20°C (for oligonucleotides, the temperature can be decreased to -80°C). Spin for 15 min at full speed in a microfuge, dry the pellet and dissolve in TE. Store probes at a concentration so that 1–2 μ L per sample is used in a hybridization experiment. For simple-sequence probes (oligonucleotides), a concentration of about 50–100 ng/ μ L can be used, while for complex, single-copy BAC probes, it can be as high as 200–500 ng/ μ L.

5. Let the ovaries settle and aspirate as much as possible without removing any ovaries. Add desired amount of probe (2 μ L for each probe or one probe plus water) to 36 μ L of the hybridization solution and fill volume to 40 μ L with water. Add this 40 μ L solution to the ovaries and mix by pipetting up and down or gently flicking the tube with a finger. The solution will be very viscous. To denature the probe and the chromosomal DNA, heat the solution to 91°C for two min in a PCR machine. Mix again, which is easier when the solution is warmed. For most complex sequences, hybridize at 37°C overnight. For AT-rich sequences, hybridize at 30°C overnight.
6. Warm 2 \times SSCT/50% formamide solution to 37°C or 30°C depending on what hybridization temperature was used. Because of the thickness of the hybridization solution, the ovaries might not settle. Therefore, add 500 μ L of the pre-warmed 2 \times SSCT/50% formamide to the ovaries in hybridization solution. Mix by inverting a couple times and let the ovaries settle. Continue to wash the sample with the pre-warmed 2 \times SSCT/50% formamide three times at 37°C (or 30°C) for 20 min each. Next, exchange the ovaries back into 2 \times SSCT without formamide at room temperature by adding and removing two washes sequentially for 10 min each while rotating. The first wash should be done with 2X SSCT/40% formamide and the second with 2X SSCT/20% formamide. Then, wash the sample three times in 2X SSCT for 10 min each at room temperature.
7. At this point the ovaries can be stored at 4°C or mounted to image the annealed probe (*see Note 6*). If antibody staining is desired, the subsequent washes can be performed with a PBS-based buffer or continue with 2 \times SSCT. As mentioned above, it is also possible that Buffer A could be used. If using a PBS-based buffer, rinse the sample twice in PBST and then block the sample in PBSTB for 1–4 h at room

temperature (if in 2 × SSCT, blocking could be done in normal goat serum). The ovaries are then ready to be immunostained with primary antibody overnight at 4°C in PBST at the appropriate dilution in 300 µL followed by the preabsorbed secondary antibodies diluted in a volume of 300–500 µL (*see Note 4*).

8. Wash the ovaries three times with PBST for 20 min each at room temperature. At this time centrifuge the secondary antibodies/embryos (*see Note 4*) for 15 min at full speed in a microfuge. After the final PBST wash, allow the ovaries to settle and remove the liquid.
9. Add the preabsorbed secondary antibody supernatant without any embryos to the ovaries and fill to the appropriate final volume (usually 300–500 µL) with PBST. Incubate by rotating the tube for 4 h at room temperature in the dark. The ovaries should be kept in the dark during all subsequent washes. Wash once in PBST for 20 min. To stain for DNA, let the ovaries settle, remove the liquid and replace with DNA dye in fresh PBS solution (a 1:5,000 dilution, i.e., 0.2 µL 10 mg/mL Hoechst in 1 mL PBS) and mix by rotating for 7 min (*see Note 5*). Follow this step with one more 20 min wash in PBST.
10. Remove the liquid and store ovaries in 500 µL of PBST at 4°C in the dark until ready to mount. Ovaries should not be stored longer than a week. Mount the ovaries in suitable fluorescence mounting media (*see Note 6*). When mounting, the ovarioles should be completely separated using a tungsten needle before being secured with a coverslip.

3.5. Whole Mount of Metaphase-Arrested Mature Oocytes

This procedure is as described in (16) and modified from (17). It is important to use well-fed, properly aged females. Use of old, unhealthy, or mutant flies will affect the quality of the preparation. This is a mass isolation procedure that does not involve a dissection. This procedure also tends to isolate only the latest oocyte stages. An alternative is to hand dissect the females (*see Note 3*), which has the advantages that fewer females are needed and a wider variety of stages can be obtained. The dissection would be done in the same buffer (Robb's) and then fixed as described below for the mass isolation method. The process of actually grinding the flies in order to extract the stage 14 oocytes takes practice.

1. Add enough 1X Robb's media to cover the blades of the blender. Females should be collected and aged in vials supplemented with a yeast paste food source for 5–7 days. Yeast-feed and collect about 200–250 females, although as few as 50 can be used. Add anesthetized females to the blender and cover. Pulse the blender three times using the grind button. Each pulse should bring the blender blades just up to full speed and then be released (*see Note 7*).

2. Once grinding is complete, swirl the liquid within the blender and then pass it through a 1–2 mm mesh into a 250 mL beaker. Allow the oocytes to settle to the bottom of the beaker (approx. 1–2 min) and then aspirate off the supernatant being careful not to suck out the oocytes. Wipe the walls of the beaker with a Kim-wipe to remove any body parts. Give the remaining liquid and oocytes a swirl and transfer to a new 250 mL beaker while passing through a fine 250–300 μm mesh (e.g., from Spectrum Laboratories). Allow the oocytes to settle and aspirate off the supernatant. Swirl and transfer to a 15 mL screw cap tube that has been coated with either a 10% BSA solution or PTB (add 1 mL of PTB to the 15 mL tube and rotate until coated and then remove). All Pasteur pipettes are also coated with PTB since this helps prevent the oocytes from sticking to the sides. Oocytes should not be in Robb's media for longer than 20 min in order to prevent artificial activation. Once oocytes have settled, remove the supernatant and add 5 mL of fixative. Fix for 6 min while rotating, and then settle for 2 min and then remove for a total fix time of between 8 and 9 min. Remove fix and rinse once with 1 \times PBS for at least 5 min on a rotator. Allow oocytes to settle and remove all but 2 mL of PBS. Oocytes are now ready to have their membranes removed.
3. Much like the grinding technique, the rolling process requires a bit of finesse and practice. Using a PTB coated $5\frac{3}{4}''$ disposable Pasteur pipette, suck up enough oocytes in PBS to fill the slender neck of the pipette and place this on the frosted side of a glass slide (the part you would normally write on). Using fine-tipped forceps, gently spread the oocytes around and remove excess buffer with a Kim-wipe that has been twisted to a fine tip (do not let the oocytes dry out completely, they must remain moist). Remove any non-oocyte body parts with the forceps. Once cleaned of debris, use the edge of a cover slip to gather the oocytes together. Place the cover slip over the oocytes and roll it around while applying gentle pressure (the number of oocytes and the amount of pressure and rolling is the part of the process requiring a bit of practice). Once rolled, you should be able to see the membranes come off (adding a little PBS can help visualize the removed membranes) (*see Note 7*). Once rolled, rinse the oocytes into a new un-coated 15 mL screw cap tube using PBS/1% Triton X-100. Depending on the number of oocytes, rolling should take 15–25 min.
4. Once rolling is complete, fill tube to the 15 mL mark with PBS/1% Triton X-100 and rotate at room temperature until a total of 2 h has elapsed. Start timing when you add the first rolled oocytes to PBS/1% Triton X-100. After 2 h incubation,

allow oocytes to settle and remove supernatant. Rinse in PBS/0.05% Triton X-100 for about 5 min while rotating. Allow the oocytes to settle, remove the supernatant and transfer the oocytes to a 1.5 mL Eppendorf tube and allow them to settle. Remove the supernatant, add 1 mL PTB and then rotate for 1 h. Remove the supernatant and then add primary antibodies in a minimum of 300 μ L PTB. Incubate overnight at 4°C. The next day, wash four times in PTB for 15 min each and then add secondary antibodies in 300 μ L PTB. Rotate 4 h at room temperature or overnight. Then wash four times in PTB for 15 min each. DNA stain such as Hoescht (2 μ L of 10 mg/mL in 1 mL PTB) can be added to the second or third wash for 5–10 min. Then mount the oocytes in a suitable medium (*see Note 6*).

3.6. Analysis of Living Oocytes

Two methods have been used to image living oocytes: the injection of labeled proteins and vital dyes or expression of GFP fusion proteins. A GFP fusion of the kinesin-like protein NCD was the first to be imaged (18, 19) but more recently GFP-labeled tubulin (20) has been used. Because of the lack of the H2A variant His2Av in the oocyte (J.K. Jang and K. McKim, unpublished), a His2Av-GFP fusion protein commonly used in mitotic cells (21) can not be used. Injection is currently limited to imaging DNA with a vital dye such as oligreen and rhodamine-conjugated tubulin (Molecular Probes) (22, 23). While there is the potential of imaging a variety of GFP fusion proteins, the yolk egg can make the GFP signal weak and it may suffer from photo-bleaching. In either case, the ovaries are dissected into Sigma Halocarbon Oil number 700, which allows for the free exchange of oxygen. Imaging can begin with stage 11–13 oocytes just before nuclear envelope breakdown.

Most live female meiosis studies have been done on metaphase-arrested stage 14 oocytes. However, oocytes can be activated *in vitro* (e.g. **Section 3.7**) and later stages of meiosis have been characterized (18, 24).

3.7. Past Metaphase I – Late Meiotic Stages in Activated Oocytes

Meiosis arrests at metaphase I until the oocyte leaves the ovary and passes down the oviduct, which makes it more difficult to isolate anaphase I and meiosis II stages. The most difficult but intriguing approach is to extract oocytes directly from the female reproductive tract. Oocytes in anaphase I, metaphase II, and anaphase II can be isolated from the oviduct and uterus (25). Embryos undergoing the meiotic divisions can be isolated by collecting shortly after egg lay, although it is possible to squeeze them out of the female's abdomen (26, 27). Once collected, the embryos must be prepared using techniques which remove the egg shell and other membranes.

1. Spindle and chromosome morphology can be observed in post metaphase I oocytes by collecting laid embryos at 15–30 min intervals. This time point collection provides a varied array of stages of naturally activated oocytes although most are in meiosis II. To prepare for optimal egg laying, the females are aged in vials or bottles with yeast paste for ~3 days. With males present to stimulate egg laying, the females are placed in egg-laying chambers, which consist of an inverted beaker fitted over a 35 mm Petri plate containing grape juice agar with a dab of yeast paste. Because of the short collection time, a large number of females may be required (200–300).
2. At 15–30 minute intervals, collect embryos and wash in ddH₂O or 0.7% NaCl. Remove chorion membranes with 50% bleach for 1.5 min and then thoroughly wash the embryos in ddH₂O. Transfer the embryos to 50% heptane, 50% methanol in an Eppendorf tube and then shake for 5 min. Once the embryos settle, remove the top heptane layer and add 1 mL of methanol. Invert for 5 min and then allow the embryos to settle. Repeat two times and then store at –20°C or rehydrate and use immediately. Rehydrate in PBS/0.1% Tween-20 by slow dilution (750 μL methanol/250 μL PBS/0.1% Tween-20, 500 μL/500 μL, 250 μL/750 μL, 1,000 μL PBS/0.1% Tween-20 three times). After blocking in PTB, antibody incubations can then be done in PBS/0.1% Tween-20 or PTB.
3. There are a number of variations on fixation of *Drosophila* embryos (28, 29). One alternative is to fix the embryos in a cold 1:1 mixture of heptane and methanol to remove the vitelline envelope (30). Eggs are then fixed for 10 min in cold methanol, washed in PBS and incubated for 1 h in PBS containing 0.1% BSA. The eggs can then be incubated with antibodies in PBS-BSA.
4. Oocytes can be activated in vitro (17, 31). Stage 13 and 14 oocytes can be isolated using methods similar to that described in **Section 3.5**. Stage 13 and 14 oocytes are isolated in hypertonic Isolation Buffer (IB), which does not activate oocytes. The time in IB (since entering the blender) should not exceed 15 min although an additional 10 min can be tried (31). Oocytes in IB are then washed in several changes of hypotonic Activation Buffer (AB) for 5 min each. AB is washed away with Zalokar's buffer, which is supposed to support growth of embryos with permeabilized vitelline membranes. The length of time since first addition of AB can be varied to optimize for particular stages. Activated oocytes can be selected by the addition of 50% commercial bleach, which destroys unactivated oocytes.

5. Another procedure for activation of oocytes involves dissection and incubation in “*Drosophila* PBS” (18, 32).

4. Notes



1. The nomenclature “*f*•*y*” indicates that the centromere is between *f* and *y*. The *y* allele is a copy of the wild-type *yellow* gene transposed to the right end of the acrocentric X-chromosome. Nondisjunction crosses are also commonly performed using a male of the genotype *C*(1;Y), *vfb*. In this male, the X and Y chromosomes are attached and the dominant *Bar* marker is on the X chromosome instead of the Y. The phenotypes of the normal and nondisjunction progeny are opposite to that shown with the *y w/B^SY* cross (**Fig. 12.2**).
2. Prior to dissection of ovaries for immunostaining the females are yeast-fed at 25°C to promote oocyte production. The yeast paste can be made by mixing yeast and water to a creamy peanut butter consistency and smearing a small amount (approximately a fingernail size) along the side of the vial. The time of feeding can be varied to optimize the size of the ovaries. Longer feeding (5–7 d) produces fatter ovaries, which is good for stage 14 preparations but not for the germarium. Thus, when dissecting for the germarium, yeast-feed overnight.
3. To extract the ovaries, grasp the thorax with one tweezers and the posterior of the abdomen with the other. The gentle separation results in a small opening at the posterior of the abdomen. One tweezers is placed perpendicular to the abdomen and the ovaries are gently forced out of the opening. The fly remains can be discarded. Each ovary contains 16–20 ovarioles that have egg chambers in various stages of development (**Fig. 12.1**). The egg chambers are then teased apart gently with a tungsten needle, forceps, micro-hook, or other suitable tool.
4. We routinely preabsorb secondary antibodies to reduce non-specific binding. Using embryos collected and fixed as described in **Section 3.7**, rehydrate with serial dilutions of the appropriate buffer used for antibody incubations. Let embryos settle, remove 200 µL of the methanol and replace with 200 µL buffer. Invert three to five times and repeat for 500 µL, 750 µL, and finally all liquid in tube. Rotate in the final buffer wash for 15–20 min. Let the embryos settle and remove as much liquid as possible. Block with 1 mL Buffer-NGS or PTB and rotate for 30–60 min. Let the embryos settle

and remove liquid. Replace with 100 µL fresh BAT-NGS or PTB. Add secondary antibodies diluted for a volume of 300–500 µL and multiplied for the number of preparations. Fill final volume to 200–300 µL with BAT-NGS and preabsorb overnight in the dark at 4°C.

5. There are a large variety of DNA dyes which can be used, although DAPI or one of the Hoechst dies are convenient because they don't stain RNA. If using Hoechst or DAPI, a 1:5,000 dilution (e.g. 0.2 µL 10 mg/mL Hoechst in 1 mL BAT) should be used and left rotating for 7 min, although higher dilutions are possible.
6. How the sample is mounted is critical for good imaging. Tissues can be mounted in a suitable medium such as Vectashield (Vector Labs) or Slowfade Gold (Molecular Probes) although it is advisable to check compatibility with the flourophors. After removing as much liquid as possible, add one drop of Vectashield to the ovaries and mix thoroughly. Add this mixture to the slide and cover with a cover slip. It is important to wick with a Kim-wipe to remove excess liquid but do not dry it out. Seal with nail polish (quick drying from Avon). In order to prevent movement of the sample, the tissues can be mounted in polyacrylamide (33) or a hardening agent like Prolong Gold. After removing as much buffer as possible, ~15–20 µL of polyacrylamide solution (10% polyacrylamide from a 30% 29:1 acrylamide:-bis acrylamide solution, 0.68% ammonium persulfate, and 0.08% sodium sulfite in buffer) is added to the ovarioles and covered with a silanized coverslip. The silanized coverslip is removed after 30 min and the resulting polyacrylamide film is equilibrated with glycerol containing antifade and mounted on a glass slide. With the exception of **Section 3.3**, these procedures involve whole mounted tissues. Therefore, the best images are collected by confocal or deconvolution microscopy. This is especially true for the large and yolk stage 14 oocytes, which present with a large amount of out-of-focus light.
7. Some of the techniques used in preparation of whole mount oocytes may require some practice. For example, when initially breaking open the females, if you grind in the blender for too short a time, many of the abdomens will remain unopened. If you grind too long in the blender, you will create a big mess of various body parts that will require “cleaning up” during the membrane rolling process. Later, if the oocytes are not rolled enough, the membranes will not come off and the antibodies will not properly penetrate, so imaging will be difficult or impossible. If you roll them too much, the oocytes will be squashed.

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Chapter 13

Analysis of Chromosome Dynamics and Chromosomal Proteins in *Drosophila* Spermatocytes

Sharon E. Thomas and Bruce D. McKee

Abstract

A wide variety of techniques have been utilized to determine the localization of various proteins from premeiotic through meiotic stages in *Drosophila* males. Live imaging has been instrumental in monitoring chromosome pairing and the localization of fusion proteins. Immunofluorescence has been a widely utilized technique to examine the localization and colocalization of the many proteins involved in meiosis. Recently, an immuno-FISH protocol was developed to observe the co-localization of DNA probes and proteins. In this chapter, detailed protocols outlining these three types of experiments are presented.

Key words: Meiosis, spermatocytes, *Drosophila*, immunofluorescence, FISH, chromosome pairing.

1. Introduction

Drosophila spermatogenesis begins at the apical tip of the testes in a region known as the germinal proliferation center. The germinal proliferation center consists of 5–9 germ-line stem cells and 9–17 cyst progenitor cells. Asymmetric cell divisions of the germ-line stem cells give rise to primary spermatogonia as well as regenerating the stem cells. Primary spermatogonia undergo four mitotic divisions to generate cysts of 16 primary spermatocytes interconnected by ring canals (1–4). Meiotic DNA replication takes place immediately after the completion of the last mitotic division (5) and is followed by a four-day growth phase in which the spermatocytes undergo a 25-fold increase in volume (2). Throughout this period the chromosomes can be visualized by staining with fluorescent dyes such as DAPI or Hoechst 33258, but appear relatively

formless and do not exhibit the morphological features normally associated with meiotic prophase chromosomes such as axes and synaptonemal complexes. In very young spermatocytes, the chromatin fills most of the nucleus and exhibits no obvious organization. However, during the early stages of meiotic prophase I (stages S1-S2), the chromatin gradually resolves into three distinct masses that correspond to the three large pairs of homologs. These three bivalents become fully separate by stage S3 (mid-prophase I) and subsequently occupy separate “territories” associated with the inner nuclear envelope (5). Shortly before prometaphase I, the chromatin in these territories and that of the small fourth chromosome pair condense to form tight masses that subsequently congress to a compact metaphase plate. Condensed chromosomes in the prophase I-metaphase I stages are optimal for assessing homolog conjunction. In males carrying mutations in the conjunction genes *snm* or *mnm*, up to eight condensed chromatin masses, representing unpaired homologs, are evident during these stages instead of the normal four (Fig. 13.1) (6). The meiosis I and II divisions occur relatively rapidly over the subsequent 4–5 h to give rise to cysts of 64 spermatids which then differentiate into spermatozoa over the ensuing four days before being released into the sperm ducts (2–5). Nondisjunction and other meiotic errors can

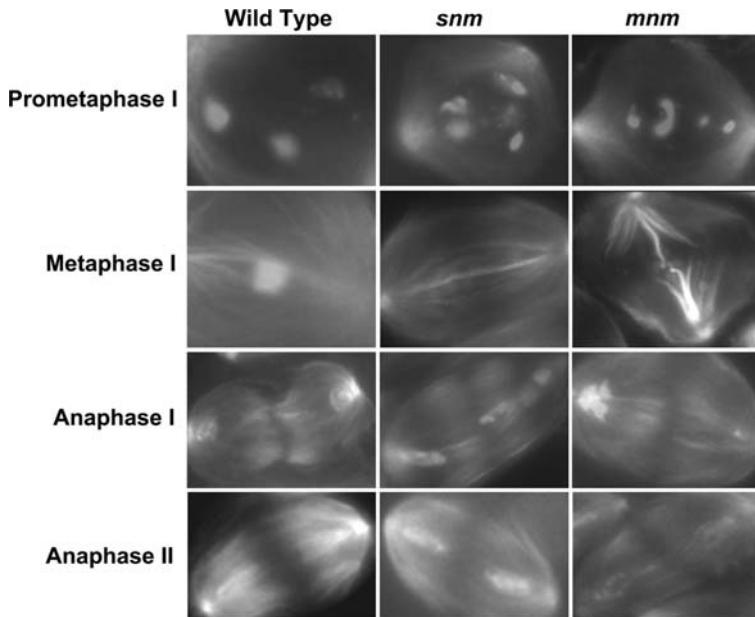


Fig. 13.1. Analysis of meiotic chromosome behavior by DAPI/tubulin staining. Chromosomes from wild-type males and from *snm* and *mnm* mutant males prepared by the method in **Section 3.1.2** and stained with DAPI. Spindle was stained with anti-tubulin monoclonal antibody (6). A color version of this figure is provided on the companion CD for this volume.

be detected at anaphase of both divisions in cells stained with fluorescent dyes such as DAPI (*see Fig. 13.1*) or nonfluorescent dyes such as orcein or Giemsa (5, 7). In addition, cysts of stained or unstained early spermatid nuclei can be rapidly screened for intra-cyst variation in nuclear diameter. Nuclear diameter is proportional to chromosome content and therefore provides a sensitive indicator for nondisjunction or chromosome loss. Adult testes contain cells in all of these stages in proportion to the length of the stage. However, in late 3rd-instar larval testes, the most advanced cysts are usually in late stages of meiosis and spermatids and spermatozoa are absent. Consequently, late larval testes are optimal for examining meiotic and pre-meiotic chromosomes.

1.1. Immunofluorescence

Immunofluorescence is a cytological method for identifying the presence and subcellular localization of proteins *in situ* by taking advantage of antigen–antibody interactions (7, 8). An explosion of commercial as well as in-house generated antibodies has made it possible to visualize a wide range of proteins in several cell types. In addition to the many antibodies now available, secondary antibodies conjugated with fluorophores of different emission spectra have made it possible to detect several different antigens in the same cell. Since most immunocytochemistry experiments cannot be performed using live tissues, tissues must be fixed and permeabilized. Ideally, fixation techniques should not greatly alter the cellular structure or chemical composition of the tissue (5). Unfortunately, it is nearly impossible to anticipate in advance which fixation or permeabilization protocol will work for each antibody. Commonly used fixatives and permeabilization agents and how they function are reviewed in detail in Ref. (9). All of the following immunofluorescence protocols begin with the same first nine steps. All but one of these protocols have been published previously and used with a wide variety of antibodies (5, 10, 11). In **Fig. 13.1**, an anti-tubulin antibody was used to stain meiotic spindles to aid in rapid identification and staging of meiotic cells. **Figure 13.2** illustrates an

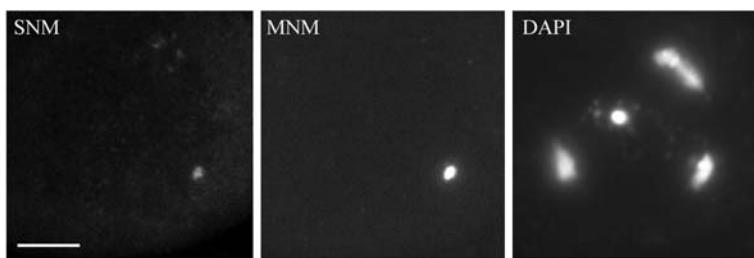


Fig. 13.2. Co-localization of SNM and MNM on the X-Y bivalent at prometaphase I by immunocytochemistry. Spermatocytes from wild-type testes processed by the method in **Section 3.1.2** were stained with anti-SNM and anti-MNM antibodies (6) and with DAPI to visualize the DNA. Scale bar = 5 μ m. *A color version of this figure is provided on the companion CD for this volume.*

immunolocalization experiment involving dual staining of wild-type primary spermatocytes with antibodies against the homolog conjunction proteins SNM and MNM which co-localize to a dense focus associated with the X-Y bivalent at prometaphase I (6).

1.2. ImmunoFISH

Fluorescent in situ hybridization (FISH) was first introduced as a method for mapping sequences on polytene chromosomes (12). This technique has been modified to visualize sequences on chromosomes from a wide variety of cell types, including meiotic cells. FISH can be combined with immunostaining to observe colocalization of DNA sequences with chromosomal proteins. The immuno-FISH protocol is a combination of two previously published protocols (10, 13). Only a limited number of antibodies have been attempted with this protocol and these experiments suggest that the FISH steps need to precede the immunolocalization steps of the protocol. **Figure 13.3** illustrates an immuno-FISH experiment in which the homolog conjunction protein SNM was tested for co-localization with a 240 bp tandem repeat located in the intergenic spacers of the rDNA loci on the X and Y chromosomes, which have been shown to function as the X-Y pairing sites (6, 14).

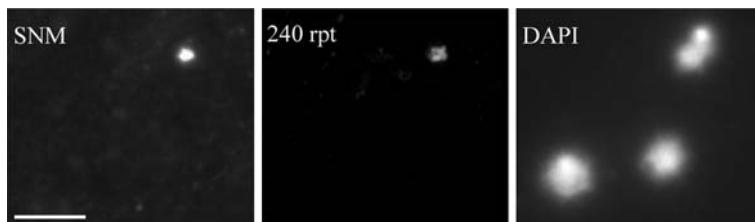


Fig. 13.3. Immuno-FISH analysis to test for co-localization of SNM and 240 bp rDNA repeats. Wild-type spermatocytes processed according to **Section 3.2.** were hybridized with a FISH probe for the 240 bp rDNA repeats (6) and stained with an antibody against SNM. DNA was stained by DAPI. Scale bar = 5 μ m. A color version of this figure is provided on the companion CD for this volume.

1.3. Live Cell Imaging

Live cell imaging is useful for analysis of the dynamics of fluorescently tagged proteins (6, 15). An additional advantage of live imaging is that without fixation and permeabilization of the tissue, cellular structures remain intact. We have also found substantially less background fluorescence using live imaging than using antibody staining protocols, which allows for extended exposure times. This can be helpful for tagged proteins expressed at very low levels. **Figure 13.4** illustrates imaging of a GFP-LacI chimeric protein in late prophase I spermatocyte nuclei from a male homozygous for a P-element transgene carrying a 256mer array of *lacO* repeats inserted on chromosome arm 2R. The four GFP spots

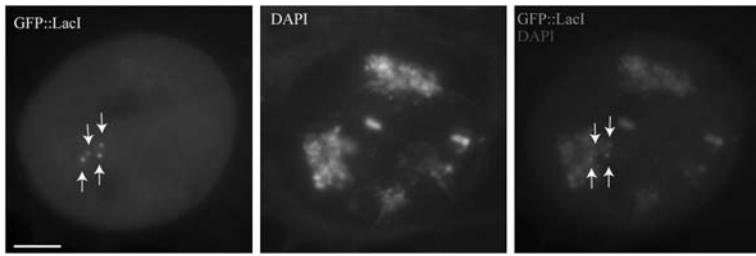


Fig. 13.4. Detection of GFP-LacI foci on primary spermatocyte chromosomes by live cell imaging. Testes from males homozygous for a P element-borne *lacO* array on chromosome arm 2R and heterozygous for a P element carrying the *hsp83::GFP-LacI* construct (15) were processed according to **Section 3.3(6)**. Arrows point to four allelic GFP-LacI foci that are separate but that co-localize within one of the DAPI-positive autosomal territories at this stage (S5). A color version of this figure is provided on the companion CD for this volume.

represent the four chromatids (two pairs of sister chromatids) in the chromosome 2 bivalent (6). This assay is useful for determining whether allelic sites on sister or homologous chromatids are unpaired, as in the image in **Fig. 13.4**, or paired, in which case the spots would be fused (15). In wild-type spermatocytes, both homologs and sister chromatids are usually paired during early prophase I but become unpaired at mid-prophase I (15).

2. Materials

2.1. Immunofluorescence

1. 22 × 40 mm microscope coverslips.
2. Coverglass staining jars (Electron Microscopy Sciences).
3. Fine forceps (Fine Science Tools).
4. 1 cc; 28G × ½ in. insulin syringe.
5. Liquid nitrogen.
6. Coverslips treated with Poly-L-lysine solution (Sigma).
7. Coverslips treated with Sigmacote™ (Sigma): These coverslips can be cleaned with 95% ethanol and reused.
8. Testes Buffer: 183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, pH 6.8, sterilize by filtration.
9. 10 × PBS: For 1 l, add 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄·7H₂O, and 2 g KH₂PO₄. Working solution, pH 7.3: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄.
10. 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI). Make stock solution of 1 mg/mL using water and store at -20°C. Dilute in 1 × PBS for a final concentration of 1 µg/mL and store at 4°C protected from light.

11. Humidity chamber: 150 × 15 mm plates with toothpicks taped to bottom about 3 cm apart and a dampened Kimwipe on the bottom of the plate.
12. Vectashield (H-1000, Vector Laboratories).
13. Microscope slides.
14. Clear nail polish.

2.1.1. Ethanol/Formaldehyde

Fixation (10)

1. 200 proof ethanol.
2. 4% Formaldehyde: dilute 16% Formaldehyde (Ted Pella) with 1 × PBS. This solution should be made fresh daily.
3. 10% Triton X-100: dilute 100% Triton X-100 with water.
4. PBT: 1 × PBS with 0.1% Triton X-100. Store at room temperature.
5. PBS with BSA: 1 × PBS with 1.0% bovine serum albumin (BSA). This solution should be made fresh daily.

2.1.2. Methanol/Acetone

Fixation (5)

1. Methanol.
2. Acetone.
3. 10% Triton X-100: dilute 100% Triton X-100 with water.
4. 1.0% Triton X-100, 0.5% acetic acid in 1 × PBS. Store at room temperature.
5. PBT: 1 × PBS with 0.1% Triton X-100. Store at room temperature.
6. PBS with BSA: 1 × PBS with 1.0% bovine serum albumin (BSA). This solution should be made fresh daily.

2.1.3. Ethanol/Formaldehyde

Fixation with DOC (11)

1. 200 proof ethanol.
2. 4% Formaldehyde: dilute 16% formaldehyde (Ted Pella) with 1 × PBS. This solution should be made fresh daily.
3. 10% Triton X-100: dilute 100% Triton X-100 with water.
4. PBT + DOC: PBS, 0.3% Triton X-100, 0.3% sodium deoxycholate.
5. BBT: PBS, 0.1% Triton X-100, 3% BSA. This solution should be made fresh daily.

2.1.4. Ethanol/Acetone/

Formaldehyde Fixation

1. 200 proof ethanol.
2. Acetone.
3. 4% Formaldehyde: dilute 16% formaldehyde (Ted Pella) with 1 × PBS. This solution should be made fresh daily.
3. 10% Triton X-100: dilute 100% Triton X-100 with water.

4. PBT: 1 × PBS with 0.1% Triton X-100. Store at room temperature.
5. PBS with BSA: 1 × PBS with 1.0% BSA. This solution should be made fresh daily.

2.2. Immuno-FISH (6)

1. 22 × 40 mm microscope coverslips.
2. Microscope slides.
3. Fine forceps (Fine Science Tools).
4. 1 cc; 28G × ½ in. insulin syringe.
5. Coverglass staining jars (Electron Microscopy Sciences).
6. Liquid nitrogen.
7. Coverslips treated with SigmacoteTM (Sigma): These coverslips can be cleaned with 95% ethanol and reused.
8. 0.7% NaCl.
9. 0.5% sodium citrate.
10. 45% acetic acid, 2% formaldehyde: dilute 16% formaldehyde (Ted Pella) in water with appropriate amount of glacial acetic acid.
11. 70% Ethanol.
12. 2 × SSC, 0.1% Tween-20 (SSCT): Make 20 × SSC stock, dilute to 2 × and add appropriate amount of Tween-20.
13. 25% Formamide, 2 × SSCT.
14. 50% Formamide, 2 × SSCT.
15. Probe (10 ng/µL) in hybridization buffer: 3 × SSC, 50% formamide, 10% dextran sulfate.
16. Microscope slides treated with SigmacoteTM (Sigma). These slides can be cleaned with 95% ethanol and reused.
17. 10 × PBS: For 1 l, add 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄·7H₂O, and 2 g KH₂PO₄. Working solution, pH 7.3: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄.
18. 10% Triton X-100: dilute 100% Triton X-100 with water.
19. PBT: 1 × PBS with 0.1% Triton X-100. Store at room temperature.
20. DAPI solution: *see Section 2.1*, step 10.
21. Humidity chamber: 150 × 15 mm plates with toothpicks taped to bottom about 3 cm apart and a dampened Kimwipe on the bottom of the plate.
22. Rubber cement.

23. Clear nail polish.
24. Vectashield (Vector Laboratories).

2.3. Live Cell Imaging of Fluorescently Tagged Proteins (6)

1. 22 × 40 mm microscope coverslips.
2. Fine forceps (Fine Science Tools).
3. 1 cc; 28G × ½ in. insulin syringe.
4. DAPI solution: *see Section 2.1.*, step 10.
5. Microscope slides.

3. Methods

3.1. Antibody Staining of Proteins

3.1.1. Prefixation Steps

All of the immunofluorescence protocols begin with these same nine steps.

1. Dissect four to five pairs of adult or third instar larvae testes in testes buffer on a microscope slide; for adult testes, remove accessory glands leaving only the testes (*see Fig. 13.5*).
2. Transfer testes to a clean, labeled poly-L-lysine-treated coverslip with testes buffer (*see Notes 1 and 2*).
3. Remove excess testes buffer and dice adult testes with the needle of a syringe into several small pieces. Larval testes require just one slice (*see Note 3*).
4. Place Sigmacote-treated coverslip onto testes that were dissected on the poly-L-lysine-treated coverslip, so that the coverslips are perpendicular to each other and the sample is towards the bottom of the poly-L-lysine-treated coverslip (*see Fig. 13.6*).
5. Gently squash testes with Sigmacote-treated coverslip: With nonbrush end of small paintbrush or blunt pencil press the coverslip and roll the end around the area where the sample is located with enough pressure to release the cells from the testes sheath (*see Note 4*).
6. Freeze in liquid nitrogen until bubbling sound stops and remove the coverslips from liquid nitrogen.
7. Wait until coverslips defrost around the edges, but not where the sample is located (about 4–5 s). The area where the sample is located should remain frozen and “frosty” in appearance (*see Note 5*).
8. Very quickly remove Sigmacote-treated coverslip. This should be done in a very sudden movement. The poly-L-lysine coverslip should retain the squashed testes and the

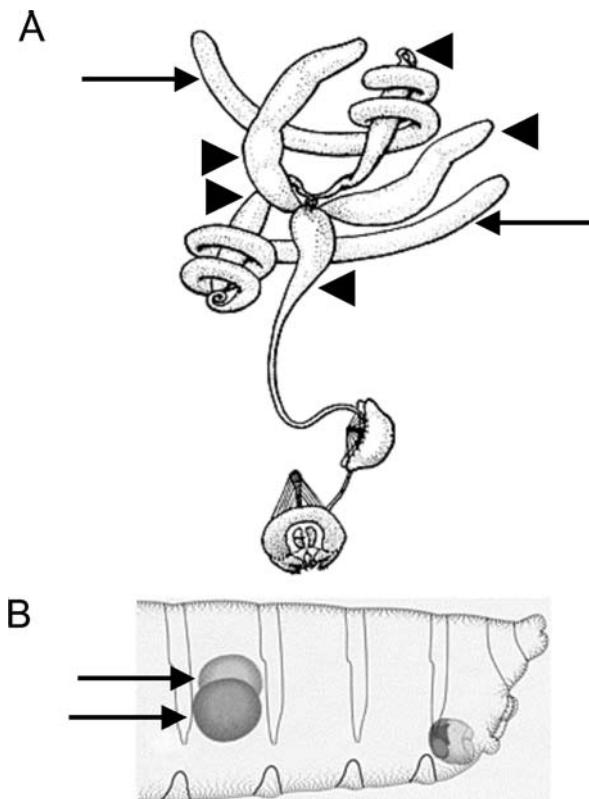


Fig. 13.5. Images of adult and larval male reproductive systems. (A) Adult system. Arrows point to testes. Spermatogenesis begins at the apical (uncoiled end) and proceeds toward the basal (coiled) end. Most spermatocyte cysts are found in the apical 1/3 of the testis. Basal ends of testes connect to and coil around the accessory glands (arrowheads). Image reprinted from (16) by kind permission of Flybase and Cold Spring Harbor Laboratory Press. Original image from (17, p. 508, Fig. 38A). (B) 3rd instar larval testes (arrows). Image reprinted from (16) by kind permission of Flybase and Cold Spring Harbor Laboratory Press. Original image from (18, p. 50).

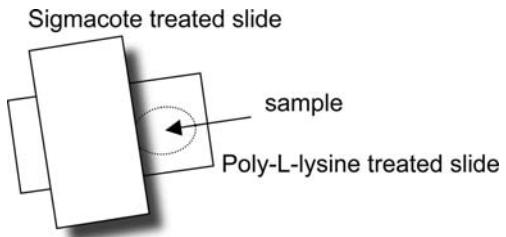


Fig. 13.6. Arrangement of coverslips for squashing of testes. Place Sigma-cote-treated slide onto testes that were dissected on the poly-L-lysine-treated coverslip, so that the coverslips are perpendicular to each other and the sample is towards the bottom of the poly-L-lysine-treated coverslip.

sample should still be visible as a smudge on the poly-L-lysine-treated coverslip after a dip in the first reagent (*see Notes 6 and 7*).

9. Continue with one of the following fixation and staining protocols (**Sections 3.1.2–3.1.5**).

<i>3.1.2. Ethanol/Formaldehyde Fixation (10)</i>	Do not allow coverslips to dry completely between solutions. Drain coverslip by dabbing on a Kimwipe in between solutions. All steps are carried out at room temperature and in coverglass staining jars except where otherwise noted. After performing above fixation steps: <ol style="list-style-type: none">1. Place coverslip in ethanol for 10 min at –20°C (<i>see Note 8</i>).2. Remove coverslip from ethanol, verify presence of sample by observing smudge on coverslip.3. Place coverslip in 4% formaldehyde for 7 min.4. Wash coverslip in 1 × PBS for 5 min twice.5. Place coverslip in PBT for 30 min.6. Wash coverslip in 1 × PBS for 5 min twice.7. Block in 1 × PBS with 1.0% BSA for at least 45 min. Coverslips can be left in block longer (2 h or longer) if necessary.8. Dilute primary antibody in 200 µL 1 × PBS with 1.0% BSA, 0.1% Triton X-100. Place solution directly on coverslip, place coverslip in humidity chamber and place at 4°C overnight. Make sure that the chamber is on a level surface (<i>see Notes 9, 10, and 11</i>).9. Discard primary antibody or antibodies.10. Rinse coverslip in PBT for 5 min twice.11. Wash coverslip in 1 × PBS for 5 min.12. Place coverslip in humidity chamber and add 200 µL of conjugated secondary antibody diluted in 1 × PBS. Incubate for 1 h protected from light (<i>see Note 9</i>).13. Wash coverslip in 1 × PBS for 5 min twice.14. Place coverslip in humidity chamber and add 200 µL of DAPI to coverslip for 5 min.15. Wash coverslip in 1 × PBS for 5 min.16. Mount using Vectashield and seal using clear nail polish: Place one to two drops of Vectashield on sample and place microscope slide on top of coverslip avoiding bubbles. Turn slide over and seal with nail polish.17. Analyze slides (<i>see Note 12</i>).
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3.1.3. Methanol/Acetone Fixation (5)

Do not allow coverslips to dry completely between solutions. Drain coverslip by dabbing on a Kimwipe in between solutions. All steps are carried out at room temperature and in coverglass staining jars except where otherwise noted. After performing above fixation steps:

1. Fix coverslips in methanol for 5 min at -20°C (*see Note 8*).
2. Remove coverslip from methanol, verify presence of sample by observing smudge on coverslip.
3. Place coverslips in acetone 1–2 min at -20°C.
4. Place coverslips in 1% Triton X-100, 0.5% acetic acid in 1 × PBS for 10 min.
5. Wash with 1 × PBS for 5 min twice.
6. Block in 1 × PBS with 1.0% BSA for 45 min.
7. Dilute primary antibody in 200 µL 1 × PBS with 1.0% BSA and 0.1% Triton X-100. Place coverslip in humidity chamber, add antibody directly onto coverslip and incubate at 4°C overnight. Make sure that the chamber is on a level surface (*see Notes 9, 10, and 11*).
8. Wash in PBT 5 min twice.
9. Wash in 1 × PBS 5 min.
10. Place coverslip in humidity chamber and add 200 µL of conjugated secondary antibody diluted in 1 × PBS. Incubate for 1 h protected from the light (*see Note 9*).
11. Wash coverslip in 1 × PBS 5 min twice.
12. Place coverslip in humidity chamber and add 200 µL DAPI for 5 min.
13. Wash coverslip in 1 × PBS 5 min.
14. Mount using Vectashield and seal using clear nail polish: place one to two drops of Vectashield on sample and place microscope slide on top of coverslip. Turn slide over and seal with nail polish.
15. Analyze slides (*see Note 12*).

3.1.4. Ethanol/Formaldehyde Fixation with DOC (11)

Do not allow coverslips to dry completely between solutions. Drain coverslip by dabbing on a Kimwipe. All steps are carried out at room temperature except where otherwise noted. After performing above fixation steps:

1. Place coverslip in ethanol for 10 min at -20°C (*see Note 8*).
2. Remove coverslip from ethanol, verify presence of sample by observing smudge on coverslip.
3. Place coverslip in 4% formaldehyde for 7–10 min.

4. Wash and permeabilize in PBT with DOC 15 min twice.
5. Wash coverslip in PBT for 10 min.
6. Block with BBT 30 min.
7. Dilute primary antibody in BBT. Add about 200 μ L to coverslip and place in humidity chamber at 4°C overnight. Make sure that the chamber is on a level surface (*see Notes 9, 10, and 11*).
8. Discard primary antibody.
9. Wash coverslips four times in BBT for 15 min each.
10. Dilute secondary antibody in BBT, and add about 200 μ L to coverslip and place in humidity chamber protected from light for 1 h at 37°C (*see Note 9*).
11. Wash coverslip twice in BBT for 15 min each.
12. Add 200 μ L DAPI to coverslip and place in humidity chamber protected from light for 5 min.
13. Wash coverslips twice in BBT, 15 min each.
14. Mount using Vectashield and seal using clear nail polish: Place one to two drops of Vectashield on sample and place microscope slide on top of coverslip avoiding bubbles. Turn slide over and seal with nail polish.
15. Analyze slides (*see Note 12*).

3.1.5. Ethanol/Acetone/Formaldehyde Fixation

Do not allow coverslips to dry completely between solutions. Drain coverslip by dabbing on a Kimwipe in between solutions. All steps are carried out at room temperature and in coverglass staining jars except where otherwise noted.

After performing above fixation steps:

1. Place coverslip in ethanol for 10 min at -20°C (*see Note 8*).
2. Remove coverslip from ethanol, verify presence of sample by observing smudge on coverslip.
3. Place coverslip in acetone 1–2 min at -20°C.
4. Place coverslip in 4% formaldehyde for 7 min.
5. Wash coverslip twice in 1 × PBS for 5 min each.
6. Place coverslip in PBT for 30 min.
7. Wash coverslip twice in 1 × PBS for 5 min each.
8. Block in 1 × PBS with 1.0% BSA for at least 45 min. Coverslips can be left in block longer (2 h or longer) if necessary.
9. Dilute primary antibody in 200 μ L of 1 × PBS with 1.0% BSA, 0.1% Triton X-100. Place solution directly on coverslip, place coverslip in humidity chamber and place at 4°C overnight. Make sure that the chamber is on a level surface (*see Notes 9, 10, and 11*).
10. Discard primary antibody or antibodies.

11. Rinse coverslip twice in PBT for 5 min each.
12. Wash coverslip in 1 × PBS for 5 min.
13. Add 200 µL of conjugated secondary antibody diluted in 1 × PBS: Place coverslip in humidity chamber, add secondary antibody directly onto coverslip and incubate for 1 h protected from the light (*see Note 9*).
14. Wash coverslip twice in 1 × PBS for 5 min each.
15. Add 200 µL of DAPI to coverslip for 5 min.
16. Wash coverslip in 1 × PBS for 5 min.
17. Mount using Vectashield and seal using clear nail polish: Place one to two drops of Vectashield on sample and place microscope slide on top of coverslip avoiding bubbles. Turn slide over and seal with nail polish.
18. Analyze slides (*see Note 12*).

3.2. Immuno-FISH

Protocol (For FISH Only,
see Note 13)

Do not allow slides or coverslips to dry completely between solutions except where noted. Drain slides and coverslips by dabbing on a Kimwipe in between solutions. All steps are carried out at room temperature and in coverglass staining jars except where otherwise noted (6).

1. Dissect testes in 0.7% NaCl on a microscope slide.
2. Place whole testes in 0.5% sodium citrate for 10 min. Remove testes from NaCl and place on a clean microscope slide with a few drops of sodium citrate.
3. Put testes in 5 µL of 45% acetic acid, 2% formaldehyde on a coverslip treated with poly-L-lysine for 3 min. Remove sodium citrate with a syringe and add acetic acid/formaldehyde solution.
4. Dice testes into five to six pieces during the incubation.
5. Gently squash testes with a Sigmacote-treated coverslip. You should be able to see the sample spread out from the original diced up pieces.
6. Freeze in liquid nitrogen until bubbling sound stops and remove the coverslips from liquid nitrogen.
7. Wait until coverslips begin to defrost, but not where the sample is located (about 4–5 s). The area where the sample is located should remain frozen and “frosty” in appearance.
8. Very quickly remove Sigmacote-treated coverslip. Sample is located on the poly-L-lysine-treated coverslip.
9. Place coverslip twice in 70% ethanol for 10 min each (*see Note 1*).
10. Place coverslip in 100% ethanol for 10 min.
11. Air dry sample.

12. Place coverslip three times in SSCT for 10 min each.
13. Place coverslip in 25% formamide, 2 × SSCT for 10 min.
14. Place coverslip in 50% formamide, 2 × SSCT for 10 min.
15. Place coverslip in 50% formamide, 2 × SSCT, 37°C, for 3 h.
16. Add FISH probe to sample and cover with Sigmacote-treated microscope slide. Seal with rubber cement (*see Note 14*).
17. Denature sample at 94°C for 2 min.
18. Incubate at 37°C overnight.
19. Remove rubber cement, remove coverslip and place in 50% formamide, 2 × SSCT at 37°C, 1 h.
20. Place coverslip twice in 50% formamide, 2 × SSCT at 37°C, for 1 h each.
21. Place coverslip in 25% formamide, 2 × SSCT for 10 min.
22. Place coverslip three times in SSCT for 10 min each.
23. Wash coverslip twice in 1 × PBS for 5 min each.
24. Place coverslip in PBT for 30 min.
25. Wash coverslip twice in 1 × PBS for 5 min each.
26. Block in 1 × PBS with 1.0% BSA for at least 45 min. Coverslips can be left in block longer (2 h or longer) if necessary.
27. Dilute primary antibody in 200 µL of 1 × PBS with 1.0% BSA, 0.1% Triton X-100. Place solution directly on coverslip, place coverslip in humidity chamber and place at 4°C overnight. Make sure that the chamber is on a level surface (*see Notes 9, 10, and 11*).
28. Discard primary antibody or antibodies.
29. Rinse coverslip twice in PBT for 5 min each.
30. Wash coverslip in 1 × PBS for 5 min.
31. Add 200 µL of conjugated secondary antibody diluted in 1 × PBS for 1 h, protected from the light (*see Note 9*).
32. Wash coverslip twice in 1 × PBS for 5 min each.
33. Add 200 µL of DAPI to coverslip for 5 min.
34. Wash coverslip in 1 × PBS for 5 min.
35. Mount using Vectashield and seal using clear nail polish: Place one to two drops of Vectashield on sample and place microscope slide on top of coverslip avoiding bubbles. Turn slide over and seal with nail polish.
36. Analyze slides (*see Note 12*).

3.3. Live Cell Imaging of Fluorescently Tagged Proteins (5)

1. Dissect four to five pairs of adult or third instar larvae testes in DAPI on a microscope slide; remove accessory glands from adult testes leaving only the testes.

2. Transfer testes to a clean microscope slide with a few drops of DAPI.
3. Remove excess DAPI and dice adult testes with the needle of a syringe into several small pieces. Larval testes require just one slice. The amount of liquid left on the slide depends on the amount of time it takes for dicing. There should be enough to just barely cover testes. Testes should never dry out or be floating at this stage of the procedure (*see Note 15*).
4. Squash testes using an untreated coverslip.
5. Immediately analyze under fluorescent microscope.

4. Notes



1. Poly-L-lysine-treated coverslips may not be necessary after one becomes proficient at the squashing.
2. Labeling the poly-L-lysine-treated coverslip is helpful to determine the side of the coverslip in which your sample is located.
3. The amount of liquid left on the coverslip during dicing depends on the amount of time it takes for dicing. There should be enough to just barely cover testes. Testes should never dry out or be floating during the dicing steps of the procedure.
4. You should be able to see the sample spread out from the original diced up pieces. If the liquid spreads to the edge of the coverslip, there may be too much liquid on the coverslip.
5. If coverslips fall apart unaided after freezing in liquid nitrogen, there may not have been enough liquid on the coverslip.
6. One of the most prevalent problems is very little tissue remaining on the coverslip after the Sigmacote-treated coverslip is removed. There are several possible reasons for this result: (1) coverslips thawed too long after removal from liquid nitrogen; (2) squash was too hard; or (3) the Sigmacote-treated coverslip was removed too slowly. The squash and the freezing steps are the most difficult portion of the protocol. To practice squashing, freezing, and separation of the coverslips, perform the common steps for all the protocols and **Section 3.2.3**, steps 1–4, skip steps 5–10, start again at step 11 and finish the protocol. This will allow the investigator to monitor squashing in a relatively short period of time and save antibody.

7. If the cells do not appear very flat, the investigator may not have squashed hard enough. Practice with the method proposed above.
8. Work station setup: Since an investigator is often performing experiments with a number of coverslips, it is helpful to place the coverglass staining jars with all the solutions in a row with the first jar to the left and the last jar to the right. Use tape to label the contents and the time that the coverslip is to be in each jar. Label your timers to coincide with the labels on your coverslips and simply move your coverslips from one jar to the next when the time is up.
9. If using multiple antibodies made in different animals, add all of them at the same time and incubate them all together overnight. Use secondary antibodies at the same time as well.
10. Antibody dilutions can vary from undiluted to 1:10,000. Unfortunately, it is hard to determine where to start. When a new antibody is being tested, start at 1:500 and adjust based on signal-to-background ratio.
11. Immunolocalization experiments can be challenging. The success of such an experiment depends on the quality and concentration of your antibody, the location of the antigen, the concentration of the antigen, and the behavior of the antigen upon fixation. The protocols presented were developed by leading scientists in the field or combined and/or modified and found to work using antibodies in our laboratory.
12. Meiosis occurs very quickly and so there will be relatively few meiotic cells per slide. Using an anti-tubulin antibody can help identify meiotic cells and can serve as a positive control for staining experiments involving new antibodies. To identify the meiotic cells, scan the slide using a 10X objective and then focus in on cells that are undergoing meiosis as identified by the prominent asters which are visible at 10X.
13. If you are only doing FISH, skip steps 23–32.
14. Flourescein-High Prime (11585622910, Roche) and ChromaTideTM (Invitrogen) have been used in the laboratory to label DNA probes for FISH experiments. Both kits come with detailed protocols and can be used with a wide variety of probes. ChromaTide is very flexible with several different fluorophores available as well as several protocols available for incorporating the ChromaTide dUTPs. The Flourescein-High Prime kit contains everything needed for random-primed labeling of DNA in about one hour.

15. Only a few problems arise using the live imaging protocol. It is important to get the correct amount of DAPI on the slide. If there is too much liquid, the cells will float around while you are trying to observe them under the microscope. Too little liquid will result in altered morphology. Since the DAPI doesn't always permeabilize the tissue, it may be necessary to use brightfield imaging to identify cell types of interest.

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Chapter 14

Methods for Meiotic Chromosome Preparation, Immunofluorescence, and Fluorescence *in situ* Hybridization in *Daphnia pulex*

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Abstract

The genus *Daphnia* has an intriguing reproductive mode of cyclical parthenogenesis. This reproductive mode has been studied for centuries, but cytogenetic information is lacking due to technical limitations of classical methods. We have developed methods for the preparation and examination of meiotic chromosomes of *Daphnia pulex* from oocytes and spermatocytes. Oocyte chromosome preparations are obtained by isolating individual oocytes after the release of yolk granules from the ovary using pressure and capillary action. Spermatocyte chromosomes are prepared using a conventional squash method. Cryosectioning is an easy and fast way to prepare sections. We also illustrate the application of immunofluorescence staining against α tubulin, as well as fluorescence *in situ* hybridization (FISH) using the intergenic spacer of ribosomal DNA or single-copy cosmid clones.

Key words: Chromosomes, meiosis, oocyte, spermatocyte, *Daphnia*, FISH, immunofluorescence staining, cryosection, *D. pulex*.

1. Introduction

The freshwater crustacean genus *Daphnia* has historically been an important organism in ecological and evolutionary research, and is emerging as an important model in genomics. In large part, this is due to the recent *Daphnia* genome project (1) and associated efforts to understand functional and evolutionary aspects of *Daphnia*'s genome structure. For example, one aspect of *Daphnia* biology that has been studied for centuries is its reproductive mode of cyclical parthenogenesis, in which females alternate between sexual and clonal reproduction. Females normally

produce diploid eggs clonally, and subsequently release a number of direct-developing embryos in a dorsal brood pouch (2). However, stressful conditions such as crowding or altered temperature can induce the clonal production of male embryos (by environmental sex determination), or of haploid resting eggs which require fertilization and are laid in a protective casing called an ephippium. Despite voluminous work describing ecological and evolutionary aspects of cyclical parthenogenesis, little is known about the mechanisms responsible for these reproductive pathways. For this reason, we are studying *Daphnia* meiosis and parthenogenesis at the cytogenetic level.

Although *Daphnia*'s reproductive strategy is an intriguing model for chromosome research, its cytogenetics has been under-developed compared with other animal models, such as *Drosophila melanogaster* (3, 4) and *Caenorhabditis elegans* (5, 6). The relatively small size of *Daphnia* chromosomes and tissues (adults are typically 1–3 mm in length), and in particular the opacity of vitellogenic ovaries containing accumulated yolk granules, have proven a major impediment to the examination of *Daphnia* oocyte nuclei. Cytological observation of mitotic and meiotic chromosomes has been done using classical methods such as hematoxilin and eosin staining, giemsa staining, and the aceto-orcein squash method (2, 7–8). However, these classical studies suffered from technical limitations such as morphological artifacts, and were especially hampered by the lack of an appropriate yolk-clearing method to allow direct observation of chromosomes.

In this chapter, methods for preparation and examination of meiotic chromosomes in *Daphnia* are described. In addition, procedures for immunofluorescence staining and fluorescence in situ hybridization (FISH) are presented. We detail a manual yolk-clearing method for observation of meiotic oocyte chromosomes, and squash and cryosectioning methods for observation of spermatocyte chromosomes. We also illustrate the application of immunofluorescence staining against α tubulin, as well as FISH using the intergenic spacer (IGS) of ribosomal DNA (rDNA) and single-copy cosmid clones.

2. Materials

2.1. Preparation of Oocyte and Spermatocyte Chromosomes

1. Positively charged microscope slides ($25 \times 75 \times 1.0$ mm) (see Note 1) and cover slips ($18 \times 32 \times 0.15$ mm) (see Note 2).
2. Super fine tip forceps (Dumont #5SF) (see Note 3).
3. Phosphate-buffered saline (PBS) ($10 \times$ stock): 1.37 MNaCl ,

100 mM Na₂HPO₄, 18 mM KH₂PO₄. Filter (0.22 µm) before storage at room temperature. Prepare working solution by dilution with water.

4. Paraformaldehyde fixative: 4% (w/v) paraformaldehyde in water. To prepare, heat to 40°C with stirring, add 18 drops of 10 N NaOH, cool down to room temperature, adjust pH to 6.5–7.0 with HCl, then adjust to pH 8.2 with 0.02 M borate buffer (add 0.02 M H₃BO₃ to 0.02 M Na₂B₄O₇ until pH 9.2.), filter (0.22 µm) and store at 4°C.
5. Permeabilization solution (T-PBS): PBS containing 0.5% Triton X-100.
6. 2% Paraformaldehyde containing 0.2% Triton X-100.
7. Dry ice in styrofoam container.

2.2. Immunofluorescence Staining

1. Phosphate-buffered saline (PBS) (*see Section 2.1*).
2. T-PBS (*see Section 2.1*).
3. Blocking solution: 4% Block Ace (Dainippon Pharmaceutical, Tokyo, Japan) in PBS. Dissolve 4 g of Block Ace powder in 100 mL of PBS, aliquot and keep at -20°C.
4. Detection buffer: PBS containing 0.2% BSA (bovine serum albumin).
5. Washing solution: PBS containing 0.1% Tween-20.
6. Primary antibody: Anti-human α-tubulin (Cedarlane, Ontario, Canada).
7. Secondary antibody: Alexa fluor 594 rabbit anti-mouse IgG antibody (Molecular Probes, Carlsbad, CA, 2 mg/mL) (*see Note 4*).
8. DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, Carlsbad, CA, fluoropure grade, 10 mg). Dissolve contents of the vial in 2 mL of water to make stock solution (5 mg/mL), aliquot and keep at -20°C.
9. DAPI-staining solution: 1 µg/mL of DAPI in Vectashield (Vector laboratories, Burlingame, CA). Dilute DAPI stock solution in Vectashield anti-fading solution at a concentration of 1 µg/mL and protect from light.
10. Humid chamber.

2.3. Fluorescence *in situ* Hybridization (FISH)

1. RNase solution: RNase A (Ambion, Austin, TX, RPA grade, 1 mg/mL). Dilute to 100 µg/mL in 2 × SSC as a working solution.
2. 20 × SSC stock: 3 M NaCl and 300 mM sodium citrate. Filter (0.22 µm) before storage at room temperature. Prepare working solution (2 ×) with water.

3. Ethanol series: 70%, 85%, and 95% absolute ethanol in water.
4. Formamide. Keep at -20°C.
5. 50% (v/v) Dextran sulfate stock solution: dissolve dextran sulfate in water with heating at 65°C and filter sterilize (0.22 µm).
6. 10 mg/mL Sonicated salmon sperm DNA.
7. Labeled probe DNA: Probe DNAs are labeled by nick-translation. The Bio-Nick labeling system (Invitrogen, Carlsbad, CA) is used for labeling with biotin-14-dATP, and the DIG-nick translation mix (Roche, Basel, Switzerland) is used for labeling with digoxigenin (DIG)-11-dUTP.
8. Hybridization mixture: 50% (v/v) formamide, 10% (v/v) dextran sulfate, 100 ng/µL salmon sperm DNA, and 0.1–0.2 µg labeled probe DNA in 2 × SSC. Prepare 15 µL of hybridization mixture per slide.
9. Blocking solution: 4% Block Ace (Dainippon Pharmaceutical, Tokyo, Japan) in 4 × SSC. Dissolve 4 g of Block Ace powder in 100 mL of 4 × SSC, aliquot and keep in freezer at -20°C.
10. Detection buffer: 1% Block Ace in 4 × SSC.
11. Anti-biotin and anti-DIG: For goat anti-biotin (Vector laboratories, Burlingame, CA), dissolve in 1 mL water to make stock solution (1 mg/mL), aliquot and keep at -20°C. For mouse anti-digoxigenin (DIG) (Roche, Basel, Switzerland), dissolve in 1 mL of water to make stock solution (0.1 mg/mL), aliquot and keep at -20°C.
12. Secondary antibody: Alexa fluor 488 rabbit anti-goat IgG antibody (Molecular Probes, Carlsbad, CA, 2 mg/mL). Alexa fluor 594 rabbit anti-mouse IgG antibody (Molecular Probes, Carlsbad, CA, 2 mg/mL).
13. Detection solution: For biotin-labeled DNA probes, use anti-biotin in detection buffer at a final concentration of 5 µg/mL. For DIG-labeled DNA probes, use anti-DIG in detection buffer at a final concentration of 0.8 µg/mL.
14. Secondary detection solution: For biotin-labeled DNA probes, use Alexa fluor 488 rabbit anti-goat antibody in detection buffer at a concentration of 20 µg/mL. For DIG-labeled DNA probes, use Alexa fluor 594 rabbit anti-mouse antibody in detection buffer at a concentration of 20 µg/mL (*see Note 4*).
15. DAPI: 4',6-diamidino-2phenylindole, dihydrochloride (DAPI) (10 mg). Dissolve contents of the vial in 2 mL of water to make stock solution (5 mg/mL), aliquot and keep at -20°C.

16. DAPI staining solution: 1 µg/mL of DAPI in Vectashield (Vector laboratories, Burlingame, CA). Dissolve DAPI stock solution in Vectashield anti-fading solution at a concentration of 1 µg/mL.
17. 1% Pepsin solution: Dissolve 10 mg of pepsin in 1 mL of 0.9% NaCl (pH 1.8). 0.9% NaCl is adjusted to pH 1.8 with hydrochloric acid.
18. Paper cement.
19. Humid chamber.

2.4. Cryosectioning

1. Paraformaldehyde fixative: Prepare as in **Section 2.1**, step 4.
2. Phosphate-buffered saline (PBS) (*see Section 2.1*).
3. 16%, 18%, 20% Sucrose in PBS.
4. Embedding medium: Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan).
5. Positively charged microscope slide (25 × 75 × 1.0 mm).
6. PBS containing 0.1% Tween-20.

3. Methods

In this section, methods for preparing meiotic chromosomes in *Daphnia* are described, and the procedures for immunofluorescence staining, FISH, and cryosectioning are also detailed. Because good-quality chromosome preparations are essential for reliable results in cytogenetic analysis, different methods are used depending upon the type of meiocyte being studied. Oocyte chromosomes (**Fig. 14.1A**) are observed in individual oocytes after releasing accumulated yolk granules from the ovary using gentle manual pressure on a coverslip and capillary action. Oocytes prepared by this method are free from the opaque surrounding yolk granules, which cause low visibility and severe background problems in FISH and immunofluorescence staining. Spermatocyte chromosomes are prepared using a conventional squash method (**Fig. 14.1B,C**). Cryosectioning is an easy and fast way to prepare sections compared with paraffin wax sections and resin sections. To test the suitability of these chromosome preparations for cytogenetics, immunofluorescence staining was performed with anti- α tubulin antibody (**Fig. 14.2**), and FISH was performed with either IGS (**Fig. 14.3**) or single-copy cosmid clones (**Fig. 14.4**). These procedures could be easily modified for use with various types of antibody and DNA probes.

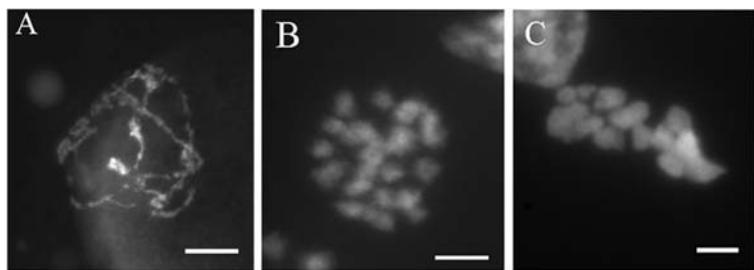


Fig. 14.1. DAPI-stained meiotic chromosomes prepared from *D. pulex*. (A) Prophase I (Pachytene stage) chromosomes prepared from an oocyte. Homolog pairing can be seen. Scale bar is 10 μ m. (B) Late prophase I chromosomes prepared from a spermatocyte. Chromosomes are thick and exist as 12 paired bivalents. Scale bar is 5 μ m. (C) Metaphase I chromosomes from a spermatocyte. Twelve paired chromosomes are observed. Scale bar represents 2 μ m.

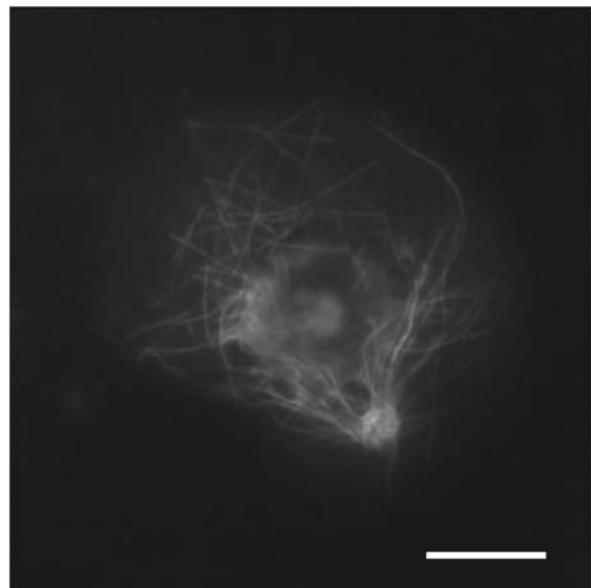


Fig. 14.2. Immunofluorescence staining of α -tubulin in the cell of an oocyte cluster in early vitellogenesis. DNA was counter-stained with DAPI. The radiation of α -tubulin from the centrosome region is clearly seen. The cell is either a nurse cell or an oocyte, but this can not be determined at this timepoint. In the early vitellogenesis, it is not possible to differentiate oocytes from nurse cells by just staining DNA. Scale bar represents 10 μ m. A color version of this figure is available on the companion CD for this volume.

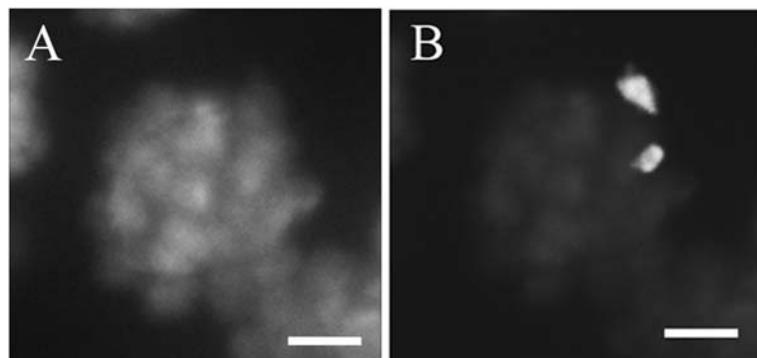


Fig. 14.3. FISH using IGS as a probe to chromosomes prepared from a spermatocyte. (A) DAPI-stained chromosomes are shown at early prophase I. (B) IGS signals (white) on counter-stained chromosomes (gray). Two separate signals were detected. Scale bar represents 5 μm .

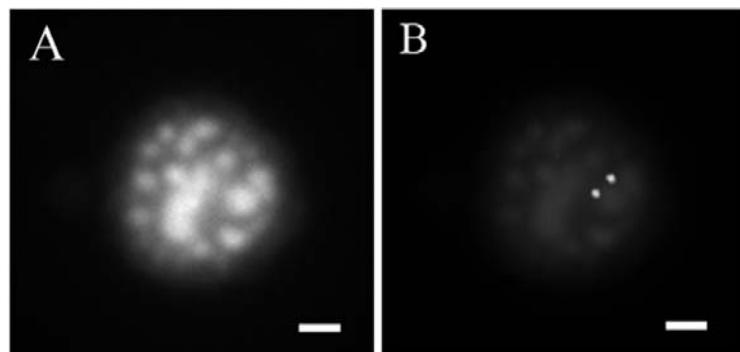


Fig. 14.4. FISH using a cosmid clone as probe. Chromosomes were prepared from testes by cryosectioning. (A) DAPI-stained chromosomes of a spermatogonium. (B) Cosmid clone signals (white) on counter-stained chromosomes (depicted in gray). Scale bar represents 2 μm .

3.1. Preparation of Oocyte Chromosomes

Females with vitellogenic ovaries (full of dense, dark green yolk) may be undergoing either meiosis or parthenogenesis. Discerning the difference between meiotic or parthenogenetic ovaries may take some practice, and is based upon differences in the relative color and appearance of yolk. During sexual reproduction, the ovaries are both darker and more homogeneous (Fig. 14.5A,C) than during parthenogenesis. By comparison, parthenogenetic ovaries (Fig. 14.5B,D) are a much lighter shade of green, and are distinguished by the presence of numerous, variably sized lipid droplets which are usually yellow or orange in color (see Note 5). These lipid droplets are associated with oocyte clusters of three nurse cells and an oocyte; each parthenogenetic ovary typically contains from two to several dozen of these clusters, in contrast to meiotic ovaries, in which there is always a single oocyte cluster maturing.

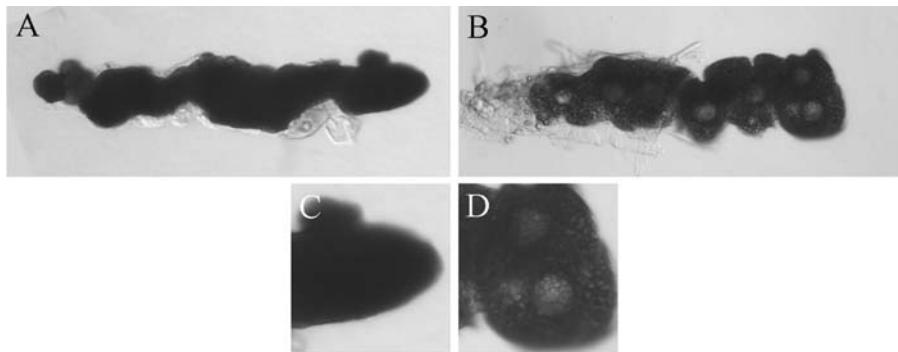


Fig. 14.5 Dissected vitellogenic ovaries of *D. pulex*. (A) Meiotic ovary. (B) Parthenogenetic ovary. (C) Magnified view of meiotic ovary. (D) Magnified view of parthenogenetic ovary. Parthenogenetic ovaries are a much lighter shade of green, and are distinguished by the presence of numerous, variably sized lipid droplets.

1. Fix the animals in 4% paraformaldehyde for 20 min and wash in PBS.
2. Dissect the animal in PBS on a glass slide with super-fine tip forceps under a dissection microscope, and pick out an ovary. The paired ovaries are lateral to the gut, and are connected to the epipodites, or swimming appendages, on the ventral surface of the animal (Fig. 14.6). These appendages (also called phyllopods) and associated debris must be carefully removed from the slide (*see Note 6*).

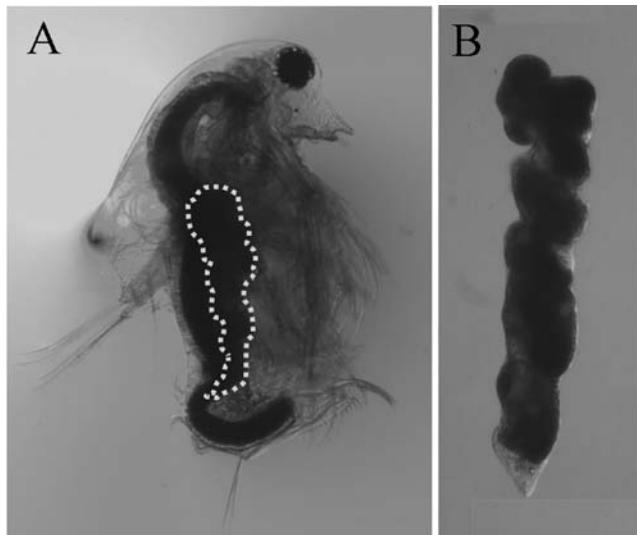


Fig. 14.6. (A) Dissected female of *D. pulex* lacking carapace. The dashed white line indicates the location of an ovary. The paired ovaries are lateral to the gut, and are connected to the epipodites, or swimming appendages, on the ventral surface of the animal. (B) Dissected vitellogenic ovary.

3. Tear the tip of the ovary with forceps, and lower a coverslip gently onto the ovary (see Note 7).
4. Remove excess PBS by placing a piece of filter paper at one end of the coverslip, and gently adding water under the other end of the cover slip. Accumulated yolk granules and the mature oocyte cluster are slowly flushed from the ovary by the pressure of the cover slip and capillary action of the filter paper (Fig. 14.7). Repeat the cycle of adding and absorbing water until the mature oocyte cluster cells are free from accumulated yolk granules (see Note 8). Mature oocyte cluster cells can be seen easily under a dissection microscope (Fig. 14.7).

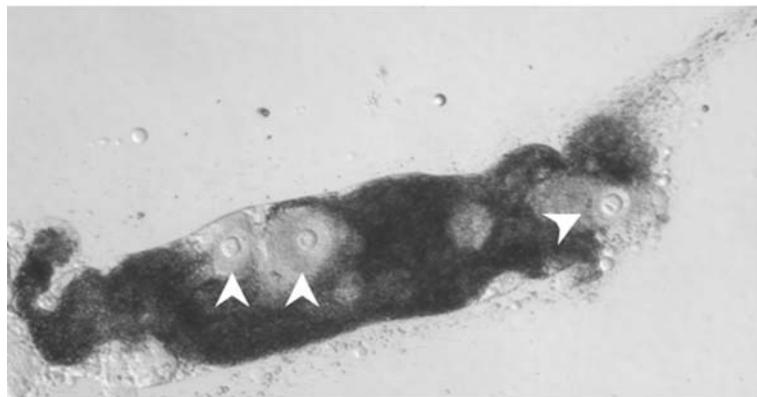


Fig. 14.7. Vitellogenic ovary during removal of accumulated yolk granules. Yolk granules are flushing out from the tip of the ovary (top right). Three oocyte cluster cells (arrowheads) are clearly visible in the ovary. One oocyte cluster cell (right cell) is about to release from the ovary.

5. After isolating the oocyte cluster cells under a cover slip, replace water with 2% paraformaldehyde containing 0.2% TritonX-100, and leave the slide for 10 min (see Note 9).
6. Replace the 2% paraformaldehyde containing TritonX-100 with 4% paraformaldehyde by placing a piece of filter paper at one end of the coverslip and adding 4% paraformaldehyde.
7. Put the slides on dry ice with the coverslip side up and leave for at least 30 minutes. Remove the coverslip with a razor-blade and air dry. Slides can be stored for at least one month at room temperature.

3.2. Preparation of Spermatocyte Chromosomes

1. Fix the males in 4% paraformaldehyde for 20 min and wash in PBS.

2. Dissect the animal in PBS on a glass slide with super-fine tip forceps, and pick out testis. The testes are lateral to the gut and are quite elongated, but have a much smaller diameter than the gut (**Fig. 14.8**). It may be helpful to stain some animals with DAPI in order to clearly see the testes, which will be filled with spermatocytes, and thus provide a clear morphological marker. Remove debris from the slide.

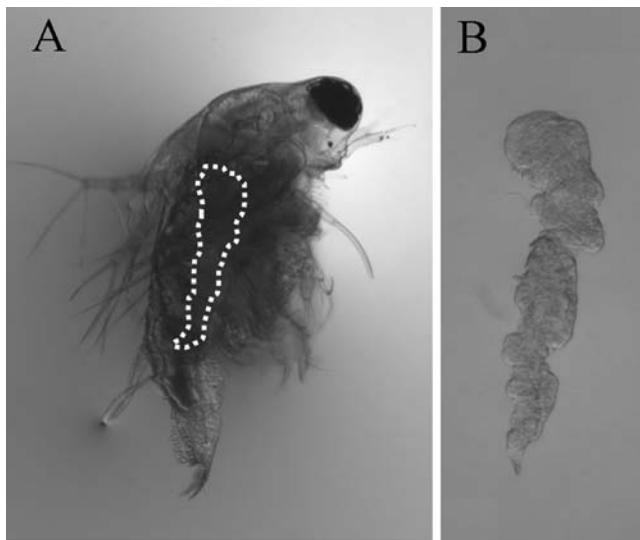


Fig. 14.8. (A) Dissected male of *D. pulex* lacking carapace. The dashed white line indicates the location of a testis. The paired testes are lateral to the gut and are elongated. A testis is much smaller in diameter than a vitellogenic ovary. **(B)** Dissected testis.

3. Replace PBS with PBS containing 0.5% Triton-X and incubate at room temperature for 10 min.
4. Replace the fluid with water and incubate for 10 min.
5. Replace the fluid with 4% paraformaldehyde and gently tear testis with forceps.
6. Put the coverslip on testis and gently apply pressure with thumb.
7. Put the slides on dry ice with the coverslip side up and leave at least 30 min. Remove coverslip with razor blade and air dry.
1. Wash the slides in PBS twice for 5 min in a Coplin jar and remove excess fluid by tilting the slides and wiping with a kimwipe.
2. Apply 100 µL of T-PBS to the target area, cover with parafilm and incubate for 10 min at room temperature in a humid chamber.

3.3. Immunofluorescence Staining

3. Wash the slides twice in PBS for 5 min.
4. Apply 100 µL of 4% Block Ace in PBS, cover with parafilm, and incubate in a humid chamber for 1 h at room temperature.
5. Remove parafilm and remove excess fluid by tilting the slide. Apply 100 µL of primary antibody in PBS containing 0.2% BSA, cover with parafilm and incubate in a humid chamber for 1 h at room temperature (*see Note 10*).
6. Wash in PBS for 10 min, PBS containing 0.1% Tween-20 for 10 min, and PBS for 10 min.
7. Remove excess fluid by tilting the slide, apply 100 µL of fluorescent dye-conjugated secondary antibody in PBS containing 0.2% BSA, cover with parafilm and incubate in a humid chamber for 1 h at room temperature protected from light.
8. Wash twice in PBS for 20 min protected from light.
9. Wipe excess fluid, apply 15 µL of DAPI in Vectashield (1 µg/mL), cover with coverslip and leave it for at least 10 min protected from light (*see Note 11*).
10. Observe using a suitable microscope.

3.4. Fluorescence *In Situ* Hybridization (FISH)

3.4.1. Pretreatment of Chromosome Preparations

1. Add 100 µL of RNase to the target area, cover with parafilm and incubate for 1 h at 37°C in a humid chamber.
2. Remove parafilm and wash in 2 × SSC for 5 min.
3. Wipe excess fluid, add 100 µL of 1% pepsin solution, cover with parafilm, incubate for 5 min at room temperature in a humid chamber, immerse in water for 1 min, and wash in 2 × SSC for 5 min (*see Note 12*).
4. Dehydrate in 70%, 85%, and 95% ethanol series for 5 min each and air dry.

3.4.2. Denaturation and Hybridization

1. Apply 15 µL of hybridization mixture directly to the prepared area on the slide, cover with a coverslip and seal with paper cement.
2. After paper cement is completely dry, place the slide on a heat block at 80°C and incubate for 6 min.
3. Incubate the slide at 37°C in a humid chamber for 15–72 h, depending on the concentration of probe and size of target site. For highly repetitive sequence, overnight incubation is enough. For single copy sequence, longer incubation will be required.

3.4.3. Washing and Detection

1. Remove paper cement carefully with forceps and immerse in 2 × SSC in a Coplin jar to float off the coverslip. After the coverslip is removed, transfer the slide into fresh 2 × SSC and incubate for 5 min.

2. Transfer slides into prewarmed 50% formamide, 2 × SSC and leave for 15 min at 37°C.
3. Wash slides in 2 × SSC twice for 10 min, and in 4 × SSC for 5 min.
4. After wiping excess fluid, apply 80 µL of blocking solution, cover with parafilm and incubate for 15 min at 37°C in a humid chamber.
5. Remove parafilm and wipe excess blocking solution carefully by tilting slides. Apply 80 µL of primary antibody in 1% Block Ace, 4 × SSC, cover with parafilm and incubate for 1 h at 37°C in a humid chamber.
6. Wash in 4 × SSC for 10 min, 4 × SSC containing 0.1% Triton X-100 for 10 min, and 4 × SSC for 10 min with gently shaking on an orbital shaker.
7. Wipe excess fluid, apply 80 µL of fluorescent dye-conjugated secondary antibody in 1% Block Ace, 4 × SSC, cover with parafilm and incubate for 1 h at 37°C in a humid chamber protected from light.
8. Wash in 4 × SSC for 10 min, 4 × SSC containing 0.1% Triton X-100 for 10 min, and 4 × SSC for 10 min in a Coplin jar protected from light with gently shaking on an orbital shaker.
9. Leave the slides in 2 × SSC for 5 min, wipe excess fluid carefully, apply 15 µL of DAPI in Vectashield (1 µg/mL) (Vector laboratories), cover with a coverslip and incubate for at least 10 min protected from light (*see Note 11*).
10. Observe using a suitable microscope.

3.5. Cryosectioning

3.5.1. Preparation of Cryostat Sections of Whole Animals

1. Fix the animals in 4% paraformaldehyde in a microtube overnight and wash three times in PBS in a microtube for 5 min with rocking on a rocker.
2. Immerse the animals in 16% sucrose and leave for 20 min with rocking, transfer to 18% sucrose and leave for 20 min with rocking, and then transfer to 20% sucrose for 20 min with rocking.
3. Put in 50% embedding medium (OCT:40% sucrose=1:1) and incubate for 20 min with rocking.
4. Prepare a mold (1 cm × 1 cm × 1 cm) with two layers of aluminum foil, and fill the mold with 100% embedding medium. Immerse the animals in 100% embedding medium in the mold and leave for 20 min.
5. Transfer four to ten animals to another mold filled with 100% embedding medium and arrange the direction of animals with the tips of forceps. Freeze at -80°C.

3.5.2. Sectioning

1. Remove aluminum foil from the frozen OCT block in the cryochamber at -20°C. Apply embedding medium to the cryotome platform and affix the OCT block on it before the embedding medium has time to freeze. Equilibrate the block for 10 min.
2. Clamp the platform containing the specimen onto the microtome of the cryostat, and cut the block until the surface of the animal is exposed.
3. Cut 20 µm sections, and gently lay the sections onto positively charged microscope slides and air-dry at room temperature. Store at -20°C until use. Slides can be stored at -20°C for at least one month.
4. Before using prepared sections, wash the slides in PBS three times for 5 min to remove embedding medium.

4. Notes



1. Positively charged slides ensure the adhesion of chromosomes, cells, and sections to slides during all procedures although they do tend to cause more background in immunofluorescence staining and FISH compared with uncharged slides. Therefore, positively charged slides are recommended for all types of preparation.
2. The size of the coverslip is not critical, but the amount of solution applied to the slide should be carefully matched to the volume underneath the coverslip. Volumes described here are for a coverslip of dimensions 18 × 32 × 0.15 mm. If a different size of coverslip is used, the volume of solution used must be adjusted accordingly.
3. This type of forceps (Dumont #5SF) may not be essential, but this super-fine tip is very helpful for reducing dissection time and obtaining clean preparations.
4. Numerous fluorescent dye-conjugated antibodies are available. Choose as required for a particular application.
5. Usually, the reproductive cycle of *Daphnia* is synchronized within a beaker. That is, most animals are typically undergoing either parthenogenetic or sexual reproduction at a given time. Note also that there are critically important differences between different strains and species of *Daphnia*. For example, there are strains of *D. pulex* in which females make their resting eggs (ephippia) parthenogenetically, rather than meiotically. This phenomenon is not seen in the widely studied congener *D. magna*.

6. Remove as much debris from the slide as possible with forceps. Too much debris may cause severe background in FISH and immunofluorescence staining, or obstruct viewing of target cells or chromosomes.
7. After placing a coverslip on the ovary, the ovary should be in the center of the coverslip. If the ovary is near the end of the coverslip, the target oocyte can possibly be lost during the subsequent flushing steps. Add an appropriate amount (up to 30 µL) of PBS to the ovary before tearing, such that the whole region of the ovary is totally soaked in PBS. However, too much PBS may cause the ovary to drift to the end of the coverslip.
8. Dispersed yolk granules may obstruct observation of cells or chromosomes, and detection of signals of target protein and probe DNA. Remove all yolk granules surrounding the oocyte. Tapping the coverslip gently with the tip of a pencil is an effective way to get yolk out of the oocyte.
9. This treatment gives good permeabilization for penetration of antibody and DNA probes in immunofluorescence staining and FISH.
10. Dilution of antibody should be optimized, and will be antibody dependent.
11. DAPI is a known mutagen and should be handled with care.
12. Pepsin treatment is not essential, but it helps to clean the cytoplasm covering the target. However, longer treatment times may cause morphological artifacts.

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Chapter 15

Immunofluorescent Microscopic Study of Meiosis in Zebrafish

Nazafarin Kochakpour

Abstract

In recent years, zebrafish (*Danio rerio*) has been used as a model vertebrate organism for studies of human disease, development, and genetics. This chapter describes detailed methods for the preparation of whole-mount meiotic oocytes and spermatocytes as well as cryostat sectioning of ovaries and testes for immunofluorescence microscopy studies of zebrafish.

Key words: Zebrafish, immunofluorescence microscopy, oocyte, spermatocyte, synaptonemal complex, cryostat, whole-mount spread preparation, antibody.

1. Introduction

Zebrafish (*Danio rerio*) is a model organism particularly suited for toxicological, developmental, and genetic studies because their embryos are transparent and they develop externally. Thus, the effects of different treatments can be observed directly. Moreover, they are small, cost-effective organisms with a short generation time (1).

Meiotic chromosomes have been studied in zebrafish males by Traut and Winking (2001) using comparative genomic hybridization (CGH) on mitotic and meiotic chromosomes (2). Later, Wallace and Wallace (2003) studied synaptonemal complexes (SC) in both sexes using light and electron microscopy (3). They reported that despite their different phenotypes, males and females have the same meiotic karyotype – 25 chromosomes with no heteromorphic sex chromosome. Females, however, do display longer synaptonemal complexes than males (3).

Recently, one of the fundamental aspects of sexual reproduction – meiosis – has become accessible through the immunocytology of chromosome synapsis, recombination, and segregation. The regulation of genetic exchanges can be analyzed in spermatocytes and oocytes with antibodies to synaptonemal elements, to centromeres and to recombination-associated proteins (4). Application of this method to male (5) and female zebrafish (6) has demonstrated a skewed distribution of recombination sites along the chromosomes and the lack of recombination sites in mutant MLH1 (Mut L homologue 1) males (7). Here, methods for zebrafish immunocytology of meiosis are presented.

2. Materials

2.1. Spermatocyte and Oocyte Spreads

1. 3×1 inch, 12-well microscope slides (Thermo Electron Corporation, Pittsburgh, PA) or 3×1 inch pre-cleaned coated microscope slides.
2. 2% BSA (bovine serum albumen): Dissolve 2 g BSA in 100 mL of distilled water with agitation at room temperature. Store in aliquots at -20°C . Once defrosted in the refrigerator, samples can be kept at 4°C for a couple of weeks.
3. Hypotonic solution: 1:2 phosphate-buffered saline (PBS, Roche): nanopure water. PBS can also be made as a 10 \times stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4 , and 18 mM KH_2PO_4 . Adjust to pH 7.4 with HCl if necessary and autoclave before storing at room temperature. Prepare working solution by diluting one part stock solution with nine parts distilled water.
4. Borate buffer: Stock: 0.05 M sodium borate in distilled water. Dilute 1:4 to use. Adjust to pH 9.2 with 0.5 N NaOH.
5. Paraformaldehyde: Freshly prepare 2% (w/v) paraformaldehyde in nanopure water for each experiment by heating to just below 60°C with agitation in the fume hood. Filter and adjust pH to 8 with borate buffer and cool the solution to room temperature. Add sodium dodecyl sulfate (SDS) to the final concentration of 0.03% (500 μL of 60 mg/mL SDS in 100 mL of paraformaldehyde) before use. If ovaries are dissected from young adult females, use 4% paraformaldehyde prepared in hypotonic solution with 0.06% SDS. No SDS is required when post-fixing cryostat sections in 2% paraformaldehyde.

6. Photo-Flo 200 (Kodak, Rochester, N.Y.): Prepare 0.4% (v/v) solution in water and adjust pH to 8 with borate buffer.
7. 1.3% (w/v) Dulbecco's Modified Eagle's Medium (DMEM, Sigma): 1.35 g DMEM, 0.07 g NaHCO₃, 112 µL sodium lactate in 100 mL nanopure water. Use a stir bar to dissolve components, then filter and adjust pH to 7.5 with borate buffer.
8. 2 mg/mL Collagenase in 1.3% DMEM.
9. Trypsin
10. Trypsin inhibitor
11. BSA
12. 1 mg/mL DNase I
13. A piece of 100 µm plastic mesh.
14. A 21-gauge, a 23-gauge needle and a 3 mL syringe are required if young adult females are used.

2.2. Cryosections

1. Embedding medium for frozen tissue specimens to ensure optimal cutting temperature OCT (Tissue-Tek, USA).
2. Isopentane
3. Liquid Nitrogen
4. Dry ice
5. Acetone
6. Cork and block holder
7. Cryotome apparatus

2.3. Immunostaining

1. Antibody dilution buffer (ADB): 10% goat serum, 3% BSA, and 0.05% Triton X-100 in PBS. Store in 50 mL aliquots at -20°C. Keep aliquots in the fridge once thawed.
2. Wash solution I: 0.4% Photo-Flo 200 in PBS.
3. Wash solution II: 0.03% Triton X-100 in PBS.
4. Blocking solution: 10% ADB in PBS (10% goat serum, 3% BSA, and 0.05% Triton X-100 in PBS).
5. Primary antibody.
6. Sodium azide. Store a 10% stock solution at 4°C. Work with caution: azide is highly toxic.
7. Secondary antibody.
8. ProLong Antifade mounting agent (Molecular Probes).
9. DAPI (4, 6-diamidino-2-phenylindole). Keep a 10 mg/mL stock of DAPI in water at -20°C. Add to the mounting agent to a final concentration of 4 µg/mL.

3. Methods

Only a small proportion of oocytes in an ovary are primary oocytes that are undergoing meiosis. The remaining oocytes of adult ovaries contain large amounts of yolk protein in their cytoplasm. The albumin of the yolk binds nonspecifically to antibodies, producing a strong fluorescent background. The use of young adult females (90–150 days old) diminishes this problem as they have noticeably fewer mature oocytes. Alternatively, the primary oocytes of the adult can be separated and isolated without releasing the yolk of the other oocytes. Here, we describe methods to prepare spreads of meiotic oocytes using either young or adult females.

3.1. Whole-Mount Preparation

3.1.1. Whole-Mount Preparation of Oocytes Using Adult Females

1. Sacrifice ten adult female zebrafish by submersion in ice-cold water for 10 min. Remove the heads and open the animals on the dorsal side from head to dorsal fin. Once the air bladder and the digestive organs are removed, ovaries are visible on both sides of the body (*see Note 1*).
2. Incubate the dissected ovaries in 10 mL of 2 mg/mL collagenase in 1.3% DMEM with agitation at 32°C for 20 min. Allow ovaries to settle for 3 min. Discard the supernatant and wash the ovaries three times with fresh 1.3% DMEM. For the last wash, make the volume of DMEM up to 10 mL.
3. Add 5 mg trypsin (final concentration: 0.5 mg/mL) to the cells and incubate for 10 min at 32°C with shaking.
4. Inactivate trypsin by adding 1 mg/mL trypsin inhibitor, 0.5 mg/mL BSA, and an additional 5 mL of DMEM and incubate for a further 5 min.
5. Add 150 µL of 1 mg/mL DNaseI (final concentration of 10 µg/mL) and gently pipette the oocytes up and down 100 times using the blunt end of a 10 mL plastic pipette. Then pass the oocytes through a 100-µm mesh screen.
6. Centrifuge the filtrate at 353*g* for 3 min and resuspend in 200 µL hypotonic solution. Place suspension on a pre-coated microscope slide. Allow the cells to settle down for 20 min at room temperature.
7. Fix the cells by placing the slide in 2% paraformaldehyde for 3 min. Wash three times in 0.4% Photo-Flo 200 for 1 min each. Air dry at room temperature for about 30 min. (Paraformaldehyde should be used in the fume hood and should be discarded in a hazardous waste container.)

3.1.2. Whole-Mount Preparation of Oocytes Using Young Adult Females

1. Sacrifice five young female zebrafish, about 90–150 days old (19–22 mm from head to caudal fin) by immersion in ice-cold water for 10 min. Remove the heads and open up the animals with a pair of fine scissors on the dorsal side from head to dorsal fin. After removing the air bladder and the digestive organs, ovaries are visible on two sides of the body and are noticeably smaller than the ovaries of older females. Keep the dissected ovaries wet with PBS during the dissection.
2. Place the ovaries in a microtube and add 300 µL PBS. To separate the oocytes from each other, gently pass and expel them 15-times each, first through a 21-gauge and then a 23-gauge needle using a 3 mL syringe. The use of a syringe and needle has the advantage over a pipette in that air pressure in the syringe prevents the cells from sticking and thus minimizes cell loss.
3. Spin down the cells at 353g for 3 min, resuspend them in 200 µL hypotonic solution and place on a pre-coated microscope slide. Allow the cells to settle for 20 min at room temperature.
4. Add 200 µL of 4% paraformaldehyde with 0.06% SDS to the slide. Reduce the volume of the liquid by allowing partial evaporation at room temperature in a fume hood for about 5 h, or under vacuum for about 2 h. Duration of evaporation may vary depending on the humidity or the vacuum power. The point is that the slide should not over-dry as this will result in a strong fluorescent background.
5. Wash the slides three times for 1 min each in 0.4% Photo-Flo 200. These slides can be used for immunostaining right away or can be air-dried and stored at –20°C for later use.

3.1.3. Whole-Mount Preparation of Spermatocytes

1. Wash a multi-well slide with soap, rinse thoroughly with water, and wipe it to dry. Coat the wells with 2% BSA and air-dry at room temperature in a clean, dust free area.
2. Sacrifice one young adult male zebrafish by submerging in ice-cold water for 10 min. Remove the head and open the animal on the dorsal side from head to dorsal fin using a fine pair of scissors. After removing the air bladder and the digestive organs, testes can be found as two long, narrow, whitish organs on each side of the body.
3. Cut the dissected testes with a razor blade several times and macerate in 100 µL PBS using a surgical spatula or flat head tweezers.
4. Cover each well of the 12-well slides with 30 µL of hypotonic solution. Add one µL of the cell suspension to each well and mix with hypotonic solution using a pipette tip. Cell membranes burst due to hypotonicity and the nuclei settle on the slide after 20 min.

5. Fix the cells by putting the slide in 2% paraformaldehyde for 3 min then washing three times in 0.4% Photo-Flo 200 for 1 min each. Air-dry the slides at room temperature for about 30 min. At this stage the slides can be stored at -20°C for later use. (Paraformaldehyde should be used in the fume hood and should be discarded in a hazardous waste container.)

3.2. Cryostat Sectioning

3.2.1. Freezing Method for Cryostat Sectioning

Freezing tissue directly in liquid nitrogen causes air bubbles to form around the tissue preventing instant freezing. Therefore, the use of cold isopentane is a better method of freezing the tissues.

1. Place a 3 mm thick slice of cork on an aluminum block holder with a drop of embedding medium for frozen tissue specimens (OCT). Add a drop of OCT to the cork.
2. Pour 25 mL of isopentane into a 50 mL glass beaker and hang it over the wide mouth of a thermos containing liquid nitrogen. Lower the beaker into the liquid nitrogen and wait till the isopentane starts to freeze. The bottom of the beaker will turn white.
3. Dissect the tissue (ovary or testis) and mount it on the OCT (*see Note 2*).
4. Immerse the tissue and the block holder into the isopentane for 30 s and store it at -80°C . To avoid any subsequent thawing and freezing, keep the frozen tissues on dry ice while transferring them to -80°C to prevent ice crystal damage.

3.2.2. Cryostat Sectioning

1. Turn on the cryostat at least 1 h before use and set the chamber temperature to -20°C . Place a covered container of acetone in the chamber as well as a brush and a clean knife blade.
2. Prepare 2% paraformaldehyde and 0.4% Photo-Flo 200 as described above but without SDS.
3. Place the block holder into the chuck, position it properly, and tighten it. Put the knife in the knife holder and tighten it. In different microtomes either the knife or the block are mobile. Arrange the apparatus such that the tissue is at the knife edge. Set the cutting thickness to 6–8 μm . Lower the anti-roll bar and start cutting. The plastic anti-roll bar should be set such that the edges of the bar and the knife are level.
4. It is easier to pick up sections one at a time. Transfer the section from the knife to the slide by touching the room-temperature slide to the section. The section melts onto the slide, transferring the tissue. Keep the slide at room temperature while the next section is cut. A brush can be used to remove tissue left-overs from the knife. Keep the brush inside the chamber.
5. After collecting the required number of sections, place the slide into the cold acetone for 30 s to remove the OCT from the sections (*see Note 3*).

6. Wash the slide in PBS for 5 min at room temperature and post-fix with paraformaldehyde for 3 min. Wash three times for 1 min each in 0.4% Photo-Flo 200.
7. Air-dried slides can be used for immunostaining or they can be stored at -20°C for later use.

3.3. Immunofluorescent Staining

1. Wash the slide in wash solution II and I for 10 min each at room temperature. Use a stir bar (*see Note 4*).
2. Incubate the slide in blocking solution for 10 min at room temperature with a stir bar.
3. Prepare an appropriate concentration of primary antibody in ADB (*see Note 5*).
4. Incubate the slide in primary antibody for 3 h or overnight at room temperature in a humid chamber in the dark (*see Notes 6–8*).
5. Repeat the washing and blocking as in steps 1–2. Prepare appropriate secondary antibodies and apply to the slide and incubate at 37°C for 1 h (*see Note 9*).
6. Wash the slide for 10 min each in wash solution I, II, and I respectively followed by two 1-min washes in 0.04% Photo-Flo 200 in nanopure water (pH 7.5).
7. Air-dry the slide for about 1 h in a dust-free area and mount it with antifade mounting agent and a coverslip. **Figure 15.1** shows a fluorescent micrograph of one spermatocyte (Fig. 15.1a) and one oocyte from zebrafish (Fig. 15.1b).

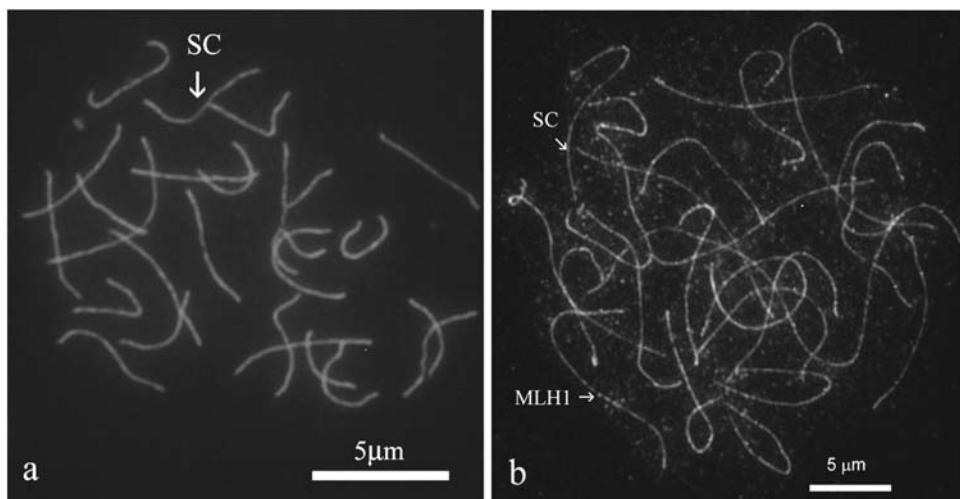


Fig. 15.1. Immunofluorescent micrograph of one spermatocyte and one oocyte spread nucleus. (a) A spermatocyte nucleus displaying 25 SCs labeled with mouse anti-SYCP1 antibody (central elements of Synaptonemal complex) detected with secondary antibody conjugated to rhodamine. (b) An oocyte nucleus stained with antibodies to SCs and to MLH1. A color version of this figure is provided on the companion CD for this volume.

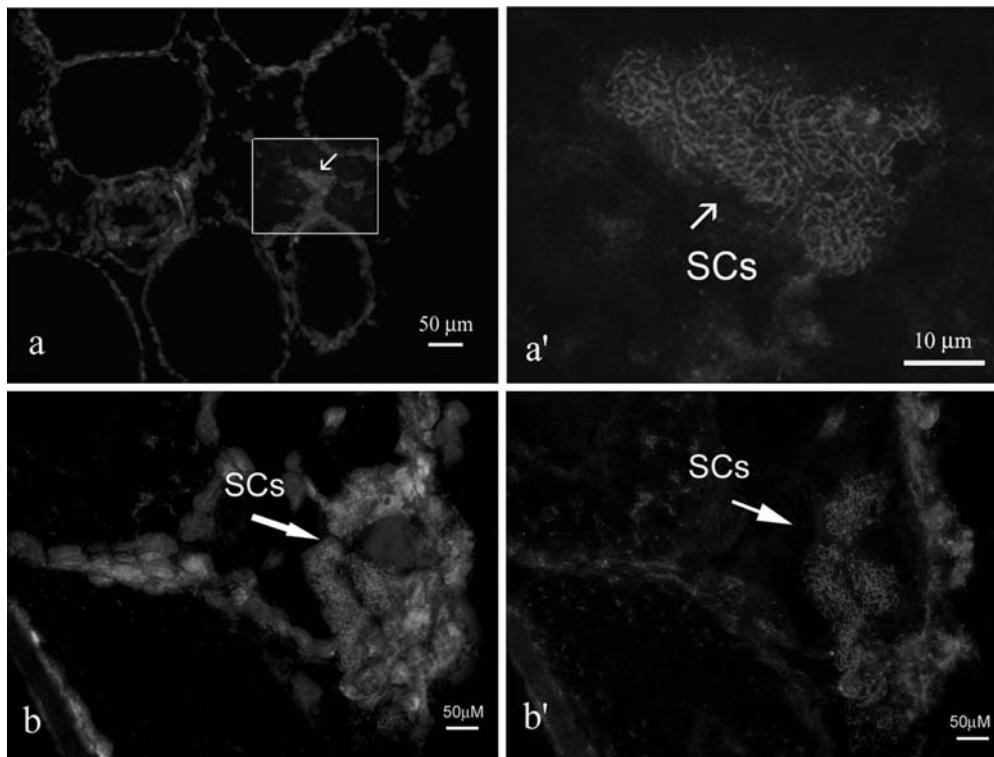


Fig. 15.2. Immunofluorescent micrograph of cryostat cross sections of an adult zebrafish ovary with the ventral side oriented to the right. (a) DAPI highlights the nuclei of follicular cells surrounding oocytes. (a') A higher magnification of the insert from the image in (a). SCs are visualized using rabbit anti-SYCP1 antibody conjugated to secondary antibody with rhodamine. (b) The superimposed image of DAPI and rabbit anti-SYCP1 antibody (b'). Primary oocytes are found in groups of two to six among oocytes in other stages. Usually only one or two nests of oocytes are seen in each cross section of ovary. *A color version of this figure is provided on the companion CD for this volume.*

All nuclei display 25 SCs with longer SCs in oocytes. Figure 15.2 represents cryostat cross-sections of zebrafish ovaries. SCs are visualized using rabbit anti-SYCP1 antibody (central elements of synaptonemal complex) identifying the primary (meiotic) oocytes. Nuclei of follicular cells surrounding oocytes are stained with DAPI. Primary oocytes are found in groups of 2–6 among oocytes in other stages. Usually only one or two batches of oocytes are seen in each transverse section of ovary.

4. Notes



1. Avoid drying the tissues by continually wetting them with PBS during the dissection.
2. If the orientation of the tissue is important, mark the block holder and orient the tissue as desired (horizontally or

vertically). Be careful not to lose the orientation of the tissue during the dissection. To obtain cross sections, tissues should be oriented vertically. Since both ovaries and testes are very soft tissues in zebrafish, to orient them vertically, hold the tissue above the cork with forceps and pour a drop of OCT onto it.

3. It is important not to disturb the previously transferred sections while collecting a newly cut section from the knife. Keep the slide perpendicular to the knife and start transferring the sections from the top of the slide, progressing down the slide for each new section.
4. If using a frozen slide, it must be brought to room temperature as quickly as possible to avoid condensation. To do so, place the back of the slide on the inside of your wrist till it reaches room temperature.
5. If using antibodies against mouse proteins on zebrafish, increase the concentration used for mouse by 5–10-fold. We use a 1:150 dilution of polyclonal antibodies against central (SYCP1) and lateral elements (SYCP3) of mouse synaptonemal complexes to visualize SCs. Commercial monoclonal mouse MLH1 antibody is used at 1:50 (BD Biosciences).
6. Add sodium azide to a final concentration of 0.02% if incubating in primary antibodies overnight (1 µL of 10% sodium azide in 500 µL).
7. The advantage of using a multi-welled slide is that small quantities of different antibody cocktails can be used in various wells. To prevent mixing of antibodies in adjacent wells, the spaces between them should be wiped dry using the edge of a slide wrapped in a piece of Kimwipe tissue.
8. To minimize the amount of required antibody when using a regular microscope slide, place the slide face side down on a piece of parafilm during incubation. Keep the nuclei from resting directly on the parafilm by placing two strips of parafilm under the edges of the slide.
9. If there are different antibody cocktails on each slide, rinse the slide with 0.04% Photo-Flo 200 in PBS from a squeeze bottle before dipping it into the wash bath.

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Chapter 16

Staging of Mouse Seminiferous Tubule Cross-Sections

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Abstract

Spermatogenesis is a cyclic process during which, within each epithelial area, various generations of germ cells undergo a series of developmental steps according to a fixed time schedule. The cycle of the seminiferous epithelium can be subdivided into stages. In the mouse, 12 such stages have been described that can be distinguished from one another by steps in spermatid development. The best way to recognize the stages in seminiferous tubule cross-sections is to use Bouin's-fixed testes of normal mice and sections stained with the Periodic acid Schiff (PAS) technique and hematoxylin. Unfortunately, this is not always possible. Sometimes PAS staining cannot be used, such as when immunohistochemistry is carried out. Moreover, not all germ cell types may be present in some instances, as in young or mutant mice. We summarize here all stage-identifying criteria that can be used in the ideal situation as well as in hematoxylin-only stained sections and/or when germ cell types are missing.

Key words: Cycle of the seminiferous epithelium, testis, mouse, spermatogenesis, staging.

1. Introduction

In mammals, the epithelium of the seminiferous tubules undergoes a repetitious series of changes known as the cycle of the seminiferous epithelium. This cycle has been defined as “a complete series of the successive cellular associations appearing in any one given area of the tubule” (1). Cross-sectioned seminiferous tubules contain multiple germ cell types that proliferate, undergo meiosis, and differentiate into spermatozoa. The organization of these germ cells within the seminiferous tubules has been studied extensively in many mammalian species, especially rodents (1–5). In 1956, Oakberg proposed the subdivision of the cycle of the mouse seminiferous epithelium into 12 stages,

based on the first 12 steps of the development of spermatids, a process called spermiogenesis (3). Oakberg also determined the duration of the cycle of the seminiferous epithelium to be 8.6 days and that of its composing stages which appeared to take a variable length of time. Russell et al. (5) published a practical guide for the histological evaluation of the testis of the rat, mouse, and dog.

Male germ cells can be subdivided into three main types. First are spermatogonia that include stem cells and that go through a series of about ten mitotic divisions (6–8) (**Table 16.1**). Most generations of spermatogonia do not show heterochromatin in their nuclei and are called “A” spermatogonia, among which reside the spermatogonial stem cells called “A-single” spermatogonia (A_s). The last couple of spermatogonial generations do show nuclear heterochromatin and are called “intermediate” (In) or “B” spermatogonia, depending on the amount of heterochromatin. The final spermatogonial division yields the second type of germ cells, spermatocytes. The spermatocytes traverse the G1 and S phase of the cell cycle and then enter the prophase of the first

Table 16.1.

Associations of germ cell types present in the various stages of the cycle of the seminiferous epithelium in mouse. Abbreviations: preL – spermatocytes before the start of the meiotic prophase; L – spermatocytes in leptotene phase of the meiotic prophase; Z – zygotene; P – pachytene; D – diplotene; numbers 1 through 16 – steps in the development of round spermatids to spermatozoa that are released into the tubule lumen during epithelial stage VIII. When two types of spermatogonia are mentioned in the same stage, this means that in that stage one generation of spermatogonia divides into the subsequent one. The A_{al} spermatogonia differentiate into A_1 spermatogonia without a concomitant division (3, 5–7).

stages / cell types	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
<i>spermatids</i>	13	13	14	14	15	15	16	16				
<i>spermatids</i>	1	2	3	4	5	6	7	8	9	10	11	12
<i>spermatocytes</i>	P	P	P	P	P	P	P	P	P	P/D	D	meiotic division I and II
<i>spermatogonia</i>	A_3 / A_4	A_4 / In	In	In / B	B	B / preL	preL	preL / L	L	L / Z	Z	Z / P
<i>spermatogonia</i>							A_1	A_1	A_1 / A_2	A_2	A_2 / A_3	A_3
<i>spermatogonia</i>	$A_s \xrightarrow{\text{ }} A_{pr} \rightarrow A_{al}$						A_{al}					

meiotic division. The meiotic prophase can be subdivided into leptotene, zygotene, pachytene, and diplotene stages. Before their entry into leptotene, the spermatocytes are called pre-leptotene. The spermatocytes carry out the two meiotic divisions and give rise to haploid round spermatids that through a lengthy process, called spermiogenesis, transform into elongated spermatids. Spermiogenesis can be subdivided into 16 subsequent steps, the first 12 of which were used by Oakberg to subdivide the mouse epithelial cycle into 12 stages (3) (**Table 16.1**).

A proper identification of the epithelial stages is of key importance, for example when one wants to follow the expression of proteins/mRNAs during germ cell development or when one wants to analyze the spermatogenic arrest in mutant mice. Most studies describing the epithelial stages are based on Periodic Acid Schiff (PAS) and hematoxylin-stained sections and are based primarily upon the changes of the acrosome and nuclear morphology of the younger generation of spermatids. Unfortunately, in a number of occasions PAS-hematoxylin stained sections are not available, such as with immunohistochemistry, or characteristic germ cell types are missing, as in young mice and some mutant mice, making stage identification much more difficult. Therefore, we now have summarized all stage-identifying characteristics, including ones not depending on PAS staining, which can be used to determine the stage of the epithelial cycle in a tubule cross-section.

2. Materials

1. 70%, 80%, 95%, and 100% Ethanol.
2. 4% Formaldehyde, phosphate-buffered (Klinipath, 4078-9001, The Netherlands).
3. Bouin's fluid: mix 150 mL of saturated picric acid, 50 mL of 37% formaldehyde, and 10 mL of glacial acetic acid.
4. Mayer's hematoxylin: Mix 1 g hematoxylin and 50 g potassium alum in 1,000 mL of distilled water overnight at room temperature. Add 1 g citric acid, 50 g active charcoal, and 0.2 g sodium iodide, then boil the solution, cool, and filter.
5. Paraffin.
6. 1% Periodic acid.
7. Schiff's reagent: 0.5 g pararosaniline in 15 mL of 1 N HCl and 0.5 g potassium bisulfate in 85 mL of distilled water.
8. Xylene.

3. Methods

3.1. Sample Preparation and Staining

1. Fix mouse testes in either Bouin's fluid or 4% phosphate-buffered formaldehyde (pH 7–7.4) for 24 h at room temperature and then put in 70% ethanol.
2. Embed testes in paraffin.
3. For PAS-hematoxylin staining, cut 5-μm-thick testis sections and mount on normal glass slides.
4. Deparaffinize in xylene and hydrate in an alcohol series (100%, 95%, 80%, and 70%).
5. Incubate slides in 1% periodic acid for 30 min at room temperature and then wash in running water for 10 min.
6. Incubate for 40 min in Schiff's reagent, and then wash in running water for 10 min.
7. Rinse slides in distilled water in between steps.
8. Counterstain slides with Mayer's hematoxylin for 3 min.
9. Wash slides in running water for 15 min, then dehydrate in alcohol and clear in xylene.
10. For hematoxylin-stained sections, omit the incubations with periodic acid and Schiff's reagents.

3.2. Distinguishing Stages in PAS-Hematoxylin Stained Sections

This section describes staging for mouse testes fixed in Bouin's fluid, embedded in paraffin, sectioned, and stained with PAS and hematoxylin. Our descriptions follow Oakberg's 12-stage classification scheme [(3) and **Table 16.1**].

1. Stage I: One generation of spermatocytes is present and two generations of spermatids, the oldest of which are elongating while the youngest generation of spermatids are still round and are newly formed by the division of the secondary spermatocytes in the preceding stage XII. Stage I round spermatids, which are by definition in step 1 of their development, can be discerned from secondary spermatocytes by their smaller size. Step 1 spermatids show the initial appearance of a proacrosomal vesicle but no PAS-positive acrosomic granules are present yet (**Fig. 16.1i**). In addition, early pachytene spermatocytes and a few A spermatogonia are present (**Tables 16.1** and **16.2**).
2. Stage II: This stage is characterized by the appearance in the younger generation of spermatids of two to three small PAS-positive acrosomic granules in the proacrosomic vesicle (**Fig. 16.1ii**). In the pachytene spermatocytes, which are still near the basal membrane, the sex body is formed. The A4 spermatogonia divide into intermediate spermatogonia

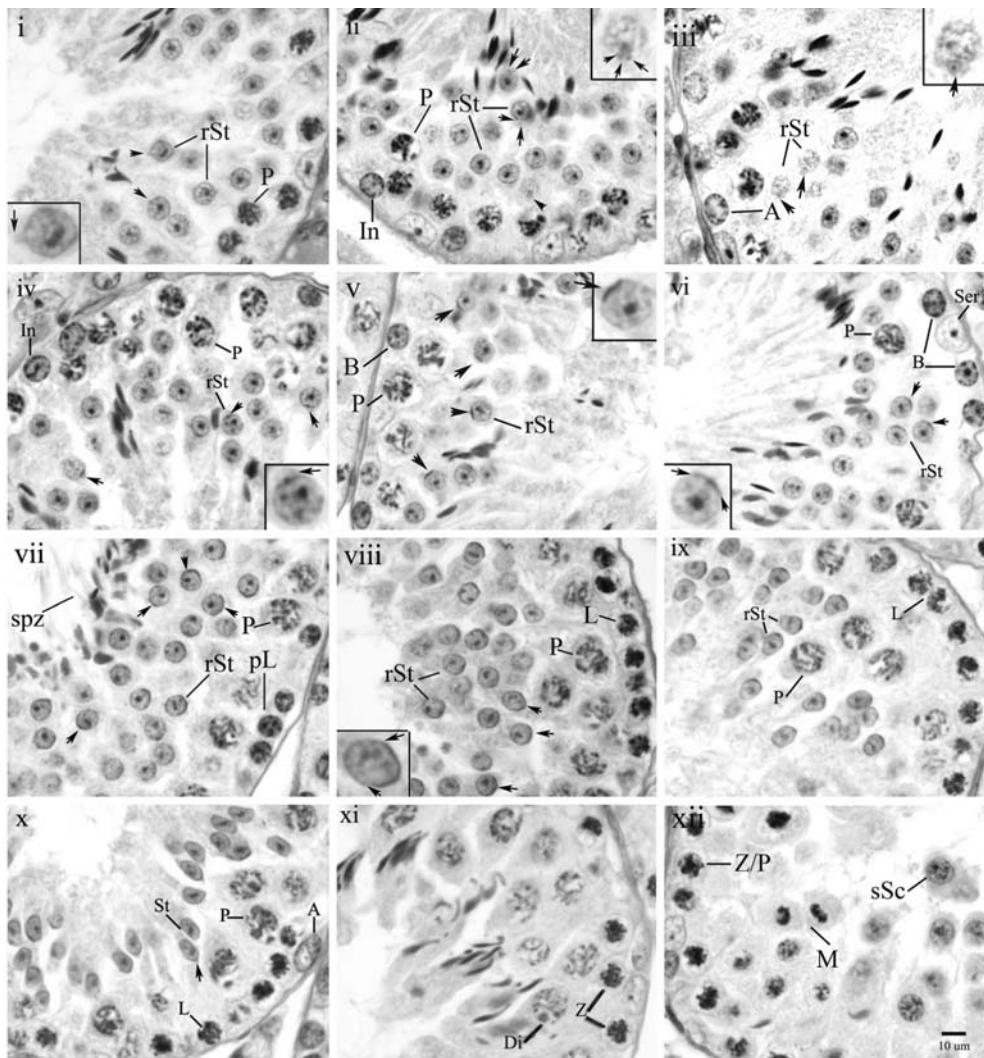


Fig. 16.1. PAS-hematoxylin-stained mouse testis showing different stages of spermatogenesis. (i) Late stage I shows the initial appearance of proacrosomal vesicle (arrows). (ii) Stage II, in which two or three proacrosomal granules are present (arrows). (iii) Stage III, step 3 spermatids in which the PAS-positive granules have coalesced into one granule that does not yet touch the nuclear membrane (arrows). (iv) Stage IV, the PAS-positive granule now touches the nuclear membrane and flattens at that side. (v) Stage V, the PAS-positive granule has now started to form a rim along its side that does not yet bend to follow the curvature of the nucleus. (vi) Stage VI, the acrosome now forms a cap that covers up to one-third of the nuclear circumference. The elongated spermatids have now moved towards the tubule lumen. (vii) Stage VII, the acrosome continues to spread out over the nucleus. Most of the round spermatids have not yet oriented themselves with their acrosome pointing to the basal membrane of the tubule. (viii) Late stage VIII. The acrosome of most of the round spermatids now faces the basal membrane of the tubule. In this tubule spermiation has already taken place and the elongated spermatids have left the epithelium. (ix) Stage IX, the nuclei of the spermatids have started to elongate. (x) Early stage X shows the continuous elongation and bilateral flattening of the spermatid heads. (xi) Stage XI shows darkly stained spermatid heads that already have compacted considerably and are very thin. (xii) Stage XII showing meiotic divisions and some secondary spermatocytes. A – type A spermatogonia; In – intermediate spermatogonia; B – type B spermatogonia; pL – preleptotene spermatocytes; L – leptotene spermatocytes; Z – zygotene spermatocytes; P – pachytene spermatocytes; Di – diplotene spermatocytes; M – meiotic divisions; sSc – secondary spermatocytes; rSt – round spermatids; Ser – Sertoli cells. A color version of this figure is available on the companion CD for this volume.

Table 16.2.
Determination of the stages of the cycle of the seminiferous epithelium in tubule cross sections under different circumstances with respect to histological staining and germ cell types present. Spg - spermatogonia; prel - spermatocytes before the start of the meiotic prophase; spc - spermatocytes; spt - round spermatids; espf - elongating spermatids.

Stages	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
<i>Normal epithelium PAS-hematoxylin stained</i>	No PAS positive granules in spt	2-3 PAS positive granules near the nucleus in spt	PAS-positive granules coalesce to one round granule	Acrosomal cap starts to form. Edges of cap still straight	Acrosomal cap covers up to 1/3 of spt nuclear surface. Esp line up at the tube lumen.	Acrosomal cap covers more than 1/3 of spt nuclear surface. Random orientation of cap towards basal membrane	More than half of the acrosomal caps orientated towards basal membrane	Rspf have started the elongation process and are no longer completely round	Spt are now somewhat elongated but did not yet get flattened	Espt now also flattened	Sp in meiotic divisions. Some tubules show secondary spc	
<i>Normal epithelium hematoxylin-stained</i>	Rspf and espf present Few and only A type spg present	In spg present	Late or mitotic B spg present, espf still embedded in epithelium	B spg and espf at tube lumen	preL present and espf not yet spermated	Post spermatiation, espf still round	As in PAS-stained material	As in PAS-stained material	As in PAS-stained material	As in PAS-stained material		
<i>No spermatids present</i>	One generation of spc present Few and only A type spg present	In spg present	Late or mitotic B spg present	Late or mitotic B spg	Leptotenes present Two generations of spermatocytes	Zygotene spc present Two generations of spermatocytes	Zygotene spc present Two generations of spermatocytes					
<i>Spc arrest after reaching pachytene stage</i>	Early pachytene	In spg present	Late or mitotic B spg present	Late or mitotic B spg	Leptotenes present	Zygotenes/early pachytene present						

(In), which have ovoid nuclei and a prominent rim of heterochromatin along the inner nuclear membrane. Hence, with respect to spermatogonia, one can see big A spermatogonia in late G2 phase, spermatogonial mitoses and early In spermatogonia.

3. Stage III: When the PAS-positive acrosomic granules that are still seen in stage II spermatids coalesce into one round granule, the epithelium is in stage III (**Fig. 16.1iii**).
4. Stage IV: The round acrosomal granule present in stage III moves towards the nuclear membrane of the spermatids and flattens where it touches the nuclear membrane (**Fig. 16.1iv**). With respect to the spermatogonia, one can recognize big In spermatogonia that are close to their division into B spermatogonia, mitoses of In spermatogonia and/or (small) early B spermatogonia. B spermatogonia show more heterochromatin than the In spermatogonia (**Fig. 16.1iv**). Mid-pachytene spermatocytes have obvious sex bodies and from this stage onwards start to move away from the basal membrane to localize more towards the lumen of the tubules.
5. Stage V: In stage V, the acrosomal granule of the round spermatids forms a flat rim around its perimeter that during this stage remains straight (**Fig. 16.1v**).
6. Stage VI: The acrosomal rim spreads over the nucleus of the round spermatids to cover maximally one-third of the nuclear surface. As the rim bends to follow the nuclear surface, a cap is formed (**Fig. 16.1vi**). The B spermatogonia divide into preleptotene spermatocytes. Hence, large premitotic B spermatogonia, mitotic B spermatogonia and small, newly formed preleptotenes can be found in stage VI. Furthermore, whereas in stage V the elongated spermatids are still embedded deep in the seminiferous epithelium, in stage VI they move towards the tubule lumen but are not yet released.
7. Stage VII: In stage VII, more than a third of the spermatid nuclear surface is covered by the acrosomal cap. The majority of the round spermatids have not yet orientated themselves in such a way that their acrosomal caps point to the basal membrane (**Fig. 16.1vii**).
8. Stage VIII: Stage VIII can be distinguished from stage VII by the fact that in this stage most of the round spermatids are pointing with their acrosomal cap towards the tubule basal membrane. Furthermore, during stage VIII, spermiation occurs and the elongated spermatids are released into the lumen as spermatozoa (**Fig. 16.1viii**). In stage VIII, the preleptotene spermatocytes go through S phase and in late stage VIII, these cells enter the leptotene phase of the meiotic prophase (**Fig. 16.1viii**). The latter transition is

characterized by the appearance of thin threads of chromatin (see the example of a leptotene cell in **Fig. 16.3** below). From this point up to stage XII, two generations of prophasic spermatocytes are present.

9. Stage IX: Stage IX starts when the round spermatids of stage VIII start to elongate and are no longer round. The nucleus becomes more flattened (**Fig. 16.1ix**).
10. Stage X: The spermatids continue elongation and become bilaterally flattened. However, the spermatid nuclei do not yet start to compact. The chromatin threads in the spermatocytes become thicker and heavily stained as the spermatocytes go from leptotene to zygotene phase (**Fig. 16.1x**).
11. Stage XI: The nuclei of elongated spermatids begin to compact and become more darkly stained. Elongation becomes more extreme (**Fig. 16.1xi**).
12. Stage XII: Stage XII is characterized by the presence of spermatocytes in either the first or second meiotic division and/or secondary spermatocytes (**Fig. 16.1xii**).

3.3. Distinguishing Stages in Hematoxylin-Stained Sections

In various circumstances it is not possible to apply PAS staining, for example after immunohistochemistry. In that case it becomes more difficult to determine the stage of the epithelial cycle and the result will be less accurate. If a good fixation method can be used, like Bouin's or diluted Bouin's (9) one can rely on determination of the generation of differentiating spermatogonia present (**Table 16.2**).

1. Stages I and II: If a tubule contains two generations of spermatids (round and elongating ones) and the elongated spermatids have not yet aligned themselves at the tubule lumen, it can be concluded that the tubule is at a point between the start of stage I and stage VI, in which the elongated spermatids move to the lumen. Furthermore, if the pachytene spermatocytes are still small and close to the basal membrane one can assume the tubule is before stage V.

To further define the epithelial stage of such a tubule section that is not stained with PAS, one has to determine the type of spermatogonia present. Therefore one has to be able to distinguish A, In, and B spermatogonia from one another. In stages I and II, low numbers of A spermatogonia can be found that may be in mitosis as A3 and A4 spermatogonia divide in stages I and II, respectively. The division of the A4 spermatogonia renders In spermatogonia that can be identified by the presence of heterochromatin. However, in the newly formed In spermatogonia the latter may be difficult to see (**Fig. 16.2i and ii**).

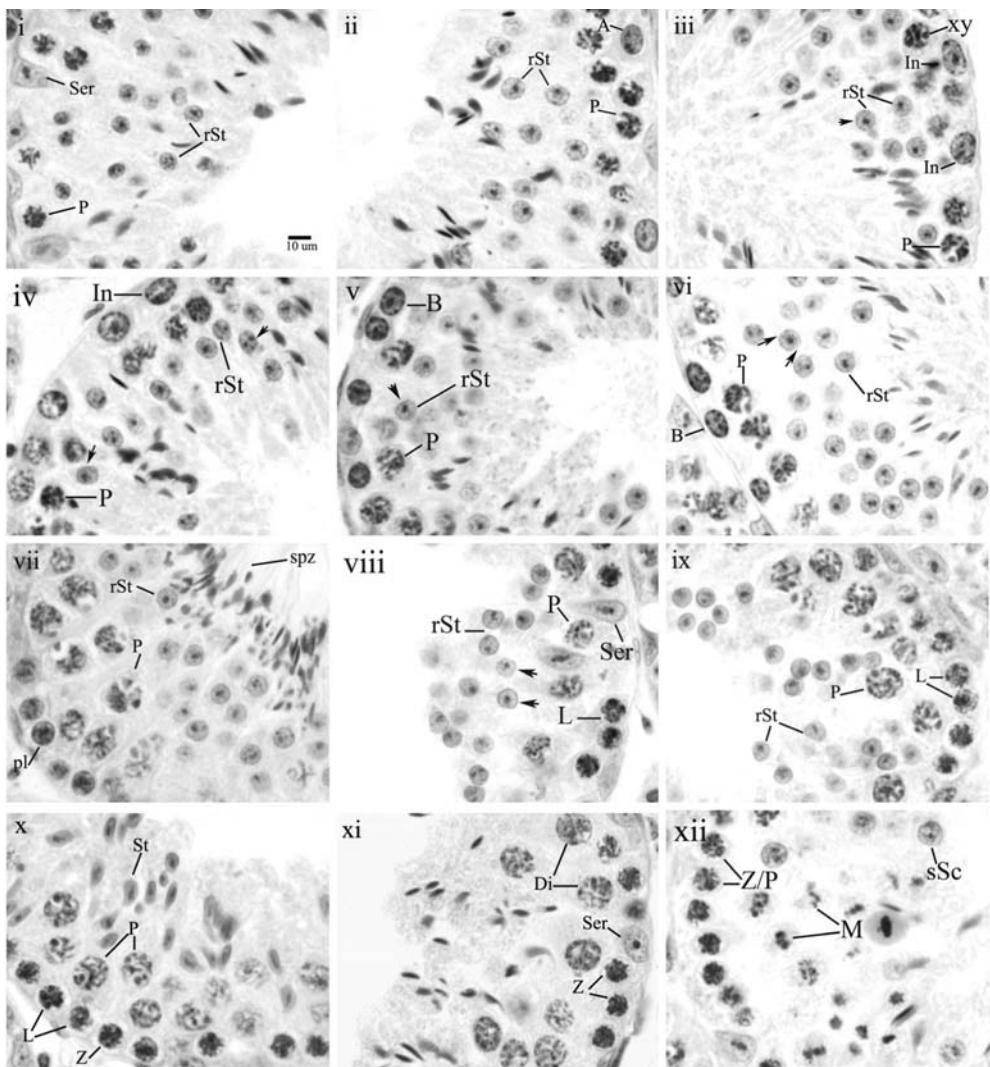


Fig. 16.2. Hematoxylin-stained mouse testis section showing different stages of spermatogenesis. (i) Early stage I. The very first part of stage I can be distinguished by the newly formed spermatids which are smaller in size compared to later in stage I. (ii) Second half of stage I through stage II. Early pachytene spermatocytes are present along with A type spermatogonia (arrows). (iii) Late Stage III is characterized by mid-pachytene nuclei with an obvious sex body (xy). Intermediate spermatogonia can be identified by their heterochromatin. (iv) Stage IV. An inward curved area in the nuclear surface of round spermatids made by the acrosomal vesicle can be seen in stages III and IV. Furthermore, mitotic or nearly mitotic In spermatogonia can be seen as well as newly formed small B spermatogonia that still have a telophasic appearance. (v) Last part of stage IV and stage V. B spermatogonia are present that display more heterochromatin at the nuclear rim. (vi) Stage VI. The B spermatogonia are close to or in mitosis into preleptotene spermatocytes. The elongated spermatids move towards the lumen. (vii) Last part of stage VI through the middle of stage VIII. This part of the cycle is characterized by the presence of preleptotene spermatocytes as well as elongated spermatids about to be released into the tubule lumen. (viii) Late stage VIII. The elongated spermatids have left, whereas the younger generation of spermatids are still round. At the end of stage VIII, the preleptotene spermatocytes start to enter meiotic prophase (Leptotene). (ix) Stage IX, the nuclei of the spermatids have started to elongate. (x) Early stage X shows the continuous elongation and bilateral flattening of the spermatid heads. (xi) Stage XI shows darkly stained spermatid heads that already have compacted considerably and are thin. (xii) Stage XII showing meiotic divisions and some secondary spermatocytes. A – type A spermatogonia; In – intermediate spermatogonia; B – type B spermatogonia; pL – preleptotene spermatocytes; L – leptotene spermatocytes; Z – zygotene spermatocytes; P – pachytene spermatocytes; Di – diplotene spermatocytes; M – meiotic divisions; sSc – secondary spermatocytes; rSt – round spermatids; Ser – Sertoli cells. A color version of this figure is available on the companion CD for this volume.

2. Stages III/IV: Stage III can be distinguished from stage II by the presence of In spermatogonia (**Fig. 16.2iii**). In spermatogonia have ovoid nuclei and a prominent chromatin envelope due to a rim of heterochromatin along the inner nuclear membrane. Some heterochromatin may extend inside the nucleus (see the example of In spermatogonium in **Fig. 16.3**). With optimal fixation, an inward curved area in the nuclear surface of round spermatids made by the acrosomal vesicle can be seen in stages III and IV (**Fig. 16.2iii and iv**). In the first half of stage IV the In spermatogonia are in late S or G2 phase of the cell cycle and show large nuclei with clearly visible heterochromatin. These cells also enter mitosis in stage IV. Unfortunately, the increase in size of the In spermatogonia is gradual and does not provide the observer with a very accurate way to distinguish the border between stages III and IV. At the mitosis of the In spermatogonia, B spermatogonia are formed that are at first telophasic and then become small early B spermatogonia that have an oval-shaped nucleus with more heterochromatin than the In spermatogonia around the nuclear rim (compare examples of In and B spermatogonia in **Fig. 16.3**). Early pachytene spermatocytes in stages I and II show nuclei that generally have a round shape with large chromatin cords that leave interchromosomal spaces (see example of early pachytene in **Fig. 16.3** “eP”). In contrast, in stages III–V the early spermatocytes gradually develop into mid-pachytene spermatocytes that show crowded chromatin patches and obvious sex bodies (see example of mid pachytene in **Fig. 16.3** “mP”). However, because the transition from early to midpachytene is very gradual, pachytene spermatocytes cannot be used to stage the epithelial cycle in any detailed way.

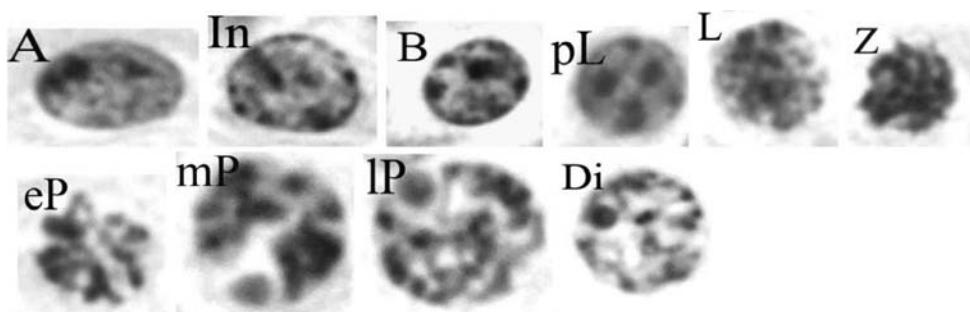


Fig. 16.3. Morphology of the nuclei of different germ cell types. A – type A spermatogonia; In – type intermediate spermatogonia; B – type B spermatogonia; pL – preleptotene spermatocytes; L – leptotene spermatocytes; Z – zygotene spermatocytes; eP – early pachytene spermatocytes; mP – midpachytene spermatocytes; lP – late pachytene spermatocytes; Di – diplotene spermatocytes. A color version of this figure is available on the companion CD for this volume.

3. From the second half of stage IV until the middle of stage VI:
In the second half of stage IV and in stage V, B spermatogonia are present that show large chromatin patches along the nuclear envelope. In early stage VI, the B spermatogonia are enlarged as they approach the mitotic division that will convert them into preleptotene spermatocytes (preL). In addition, stage VI can be recognized by the behavior of the elongated spermatids. These cells approach the end of their development into spermatozoa and in stage VI move towards the tubule lumen where they line up at the lumen side of the epithelium. In stage VI, B spermatogonia divide into preleptotene cells (**Fig. 16.2iv–vi**).
4. From the middle of stage VI until the middle of stage VIII:
The preleptotene spermatocytes formed in the second half of stage VI are smaller than B spermatogonia and show a round nucleus with some chromatin clumps (**Figs. 16.2vii,viii** and **16.3 “pL”**). In the latter half of stage VIII, chromosome threads start to appear indicating that these cells have entered the leptotene stage of meiotic prophase. At about the same time as the entrance into meiotic prophase, the elongated spermatids are released into the tubule lumen as spermatozoa, a process called spermiation.
5. Stages IX through XII: Fortunately, the changes in the shape of the spermatids that start the elongation process can be appreciated just as well with or without PAS staining as is also the case for the meiotic divisions. Therefore for this part of the epithelial cycle, stage recognition in sections stained only with hematoxylin is essentially similar to that in sections stained with PAS-hematoxylin (see **Section 3.2**, steps 9–12) (**Table 16.2**) (**Fig. 16.2ix–xii**).

3.4. Distinguishing Stages when There are no Spermatids

In young mice and in some mutant mice with a spermatogenic arrest, no spermatids are present. This makes it impossible to use these later cells to determine the epithelial stage of tubule cross-sections. In that situation, an important first assessment is whether one or two generations of spermatocytes in meiotic prophase are present. If there is one such generation, the tubule is in a part of the epithelial cycle from stage I till the first half of stage VIII, whereas if there are two, it is in a stage from the second half of VIII till XI. Stage XII remains equally recognizable (**Tables 16.1** and **16.2**). Stage XII can still be distinguished by the presence of spermatocytes in meiotic divisions or secondary spermatocytes (**Table 16.2**).

Stages I–VIII can be distinguished in the same way as in normal adult mouse testis sections stained with only hematoxylin (see **Section 3.3**, steps 1–4), except that spermatid behavior in stage VI and spermiation in stage VIII can no longer be used as

additional helpful information. However, spermatogonial cell types and numbers and the presence of preleptotene spermatocytes remain available to establish a stage recognition similar to that in hematoxylin-stained sections (**Table 16.2**).

In the absence of spermatids, stage recognition from the second half of stage VIII to stage XI becomes much less precise. One can identify the end of stage VIII by the start of the leptotene phase of the spermatids but the transition to zygotene in stage X cannot be pinpointed very accurately as at the light microscopic level this occurs rather gradually and until stage XII there are no further stage-identifying criteria available.

3.5. Stage Recognition in Case of Spermatogenic Arrest at the Spermatocyte Level

Mutations in genes encoding proteins involved in DNA damage repair or proteins with a specific role in the meiotic process often cause an arrest of spermatogenesis at the level of spermatocytes in epithelial stage IV (10–17). In these sections it will be possible to discern stages III to X by criteria as described for the situation with no spermatids (**Table 16.2** and **Fig. 16.4ii–vi**) (*see Section 3.4*). However, for the period from the second half of stage X (where the leptotene spermatocytes enter zygotene stage) to stage II, where In spermatogonia are formed, it is no longer possible to reliably subdivide the cycle into stages (**Fig. 16.4i**). During this period,

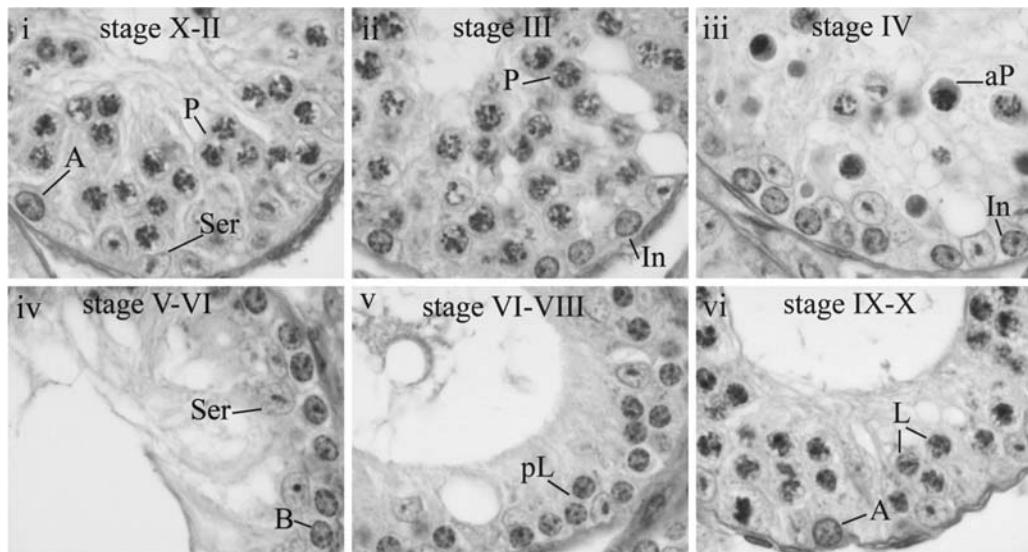


Fig. 16.4. Identification in *Mdc1*^{-/-} mouse testis with spermatogenesis arrested at stage IV. (i) Second half of stage X through stage II. It is not possible to be more specific for this part of the epithelial cycle. In all these stages, A spermatogonia and zygotene/early pachytene spermatocytes are present. It is hazardous to try to discern zygotenes as well as pachytene at the light microscopic level because spermatocyte development may be abnormal. (ii) Stage III can be identified by intermediate spermatogonia. (iii) Stage IV, mitotic or nearly mitotic In spermatogonia can be seen and in this case apoptotic pachytene as well. (iv) Stage V to the middle of stage VI, B spermatogonia can be distinguished. (v) Late stage VI through stage VIII is characterized by the presence of preleptotene spermatocytes. (vi) Stage IX–X, leptotene spermatocytes can be identified. *A color version of this figure is available on the companion CD for this volume.*

there will just be one generation of spermatocytes present and low numbers of A spermatogonia, providing no clue to distinguish epithelial stages.

3.6. Distinguishing Spermatogonial Cell Types in Sections

As will be clear from the above (*see Sections 3.3–3.5*), as soon as the developmental steps in spermatid development cannot be distinguished properly, much of the stage recognition relies on the presence of spermatogonial cell types. Among the spermatogonial cell types, A spermatogonia are of little use because they are present in relatively low numbers throughout the epithelial cycle. Special techniques are necessary to discern different A spermatogonial cell types from one another (18) and these techniques restrict the experimental possibilities with the material. In and B spermatogonia can be used to determine the epithelial stage, though less precisely than with round spermatid development.

Unfortunately, it takes some experience to immediately recognize In and B spermatogonia or even preleptotene spermatocytes in any individual tubule section. The best way to learn to do this is by starting to learn to recognize leptotene spermatocytes, because they are the easiest to distinguish. Then, proceed backwards to distinguish preleptotene spermatocytes. An important point is to study both the morphology and density of these cells (numbers per Sertoli cells). When one feels confident in recognizing (pre)leptotene spermatocytes, one can proceed to find B spermatogonia that are close to or already in mitosis (i.e., becoming preleptotene spermatocytes), marking stage VI. Again, it is important to study both the morphology and density of the B spermatogonia, the number of which will be about half of that of the (pre)leptotene spermatocytes. Then one proceeds further back to study younger B spermatogonia which are smaller the earlier they are in their cell cycle. Finally, one will be able to see In spermatogonia dividing into B spermatogonia, marking stage IV. The number of In spermatogonia per Sertoli cell will again be half that of the B spermatogonia. Subsequently, one can get acquainted with the morphology of younger In spermatogonia, marking stage III and the latter half of II.

The ease with which one will be able to recognize the morphology of the In and B spermatogonia and (pre)leptotene spermatocytes depends heavily on the quality of the fixation. With Bouin's fluid it will be easiest. It will be more difficult using formaldehyde, especially when combined with antigen-retrieval procedures, and will likely be impossible in cryosections. Fortunately, the cell density criterion is often quite helpful.

3.7. Final Considerations

The possibilities to distinguish epithelial stages and germ cell types in cryosections have not been discussed here. Because of the very poor morphology, it is generally not possible to be accurate. In case it concerns a testis with full spermatogenesis one may see

elongated spermatids. When these are present, it will be an area somewhere in stages XI until the middle of VIII and when they are absent, it will be in stages ranging from the second half of stage VIII to stage X. Furthermore, when the elongated spermatids are at the tubule lumen it will be an area somewhere between the second half of stage VI and spermiation in the middle of stage VIII.

Clearly, the most precise identification of the epithelial stage and the developmental step of the various types of germ cells present in a particular area can be achieved in sections of a normal, optimally fixed and PAS-hematoxylin-stained testis (*see Section 3.1*). Certainly, for people who want to learn to distinguish germ cell types and stages of the epithelial cycle, it will be best to study this type of material first until this no longer gives problems. On the basis of this knowledge it will be possible to distinguish stages and cell types in more complicated situations.

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Chapter 17

Isolation and Short-Term Culture of Mouse Spermatocytes for Analysis of Meiosis

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Abstract

Understanding meiosis is facilitated by in vitro experimental approaches, but this has not been easily applicable to mammalian meiocytes. Available methods for in vitro analysis of mammalian oocytes are generally limited to experimental analysis of the late prophase period. Short-term cultures of male germ cells have been useful for analysis of earlier meiotic prophase pathways, as well as onset of the meiotic division phase, but no studies have achieved reliable spermatogenesis in vitro. Here we describe a method for preparing highly enriched pachytene spermatocytes from mouse testicular cell suspensions using cell-size fractionation by sedimentation through a bovine serum albumin gradient at unit gravity. We also provide a procedure for short-term culture of spermatocytes and the pharmacological induction of the prophase-to-division phase transition.

Key words: Mouse, spermatogenesis, meiosis, spermatocytes, germ cell enrichment, in vitro culture, G2/MI transition, okadaic acid.

1. Introduction

1.1. Challenges for In Vitro Experimental Analysis of Meiosis in Mice

Experimental analysis of mammalian meiosis has been frustrated by lack of malleable in vitro systems, except those for analysis of late prophase mouse oocytes (1, 2), and by practical and anatomical obstacles to the study of mammalian meiocytes (for example, the early prophase stages of oogenesis occur during fetal development). The focus of the methods presented here will be on the analysis of the male meiotic cells, spermatocytes, in the mouse. Spermatocytes are accessible in testes of both pubertal and adult individuals, but are present in tight associations with surrounding somatic cells, the Sertoli cells.

The difficulties for manipulation of meiosis *in vitro* are best viewed in the broader context of the success – and, more specifically, lack thereof – in achieving mammalian spermatogenesis *in vitro* (3). Despite many imaginative and technologically savvy attempts, there has been no repeatable, robust success in producing fully differentiated haploid spermatids by culture of diploid precursor germ cells. Promising results with culture of rat germ cells have been reported (4–6), but these methods have not been generally applied, nor has there been a detailed analysis of the fidelity of the meiotic process in the cultured germ cells. Thus, the bottom line is that it has been extraordinarily difficult to recapitulate the whole process of mammalian meiosis *in vitro*. One biological issue underlying this problem lies in the morphological interactions and bi-directional signaling pathways by which germ cells and Sertoli cells communicate. Intimate contact between germ cells and Sertoli cells is initiated when seminiferous cords are formed early in testicular morphogenesis. After birth, both Sertoli cells and germ cells proliferate, but on different schedules. In the mouse, Sertoli cells proliferate during fetal testis enlargement and in the perinatal period, but in the second week after birth, their proliferation ceases. Conversely, male germ cells become mitotically quiescent after their migration to the fetal gonad. Soon after birth they resume proliferation and migrate to the basal lamina of the seminiferous tubule, where spermatogonial stem cells reside in niches that enable their slow proliferation and periodic differentiation into spermatogonia. As spermatogonia proliferate, cytokinesis is incomplete, so that cohorts of spermatogonia (and spermatocytes and spermatids) are present as syncytia of synchronously differentiating cells conjoined by narrow intercellular bridges (in the mouse, for instance, primary spermatocytes undergo meiotic prophase in a syncytium of over 500 cells) (7). During pubertal development, Sertoli cells interact and, under androgen regulation, establish specialized cell-cell junctions that are the anatomical basis for the basal and adluminal compartments of the seminiferous epithelium (8). Indeed, male mice lacking components of the tight junctions are infertile (9, 10). A critical event of early meiotic prophase is that germ cells, during the earliest stage of meiotic prophase, pass through these junctions to exit the basal compartment and establish themselves in the adluminal compartment as primary spermatocytes (11, 12). It is in the context of the environment of the adluminal compartment that germ cells subsequently complete the complex events of meiotic prophase and undergo the two meiotic divisions that segregate chromosomes to form haploid germ cells, as well as carry out the subsequent cellular and nuclear remodeling processes of spermiogenesis.

During the first wave of spermatogenesis, when meiosis is first initiated in the developing testis, the germ cells and the surrounding Sertoli cells organize into the intimate structural and temporal associations that ensure successful meiosis and continuous sperm

production (13). Sertoli cell products, such as glial cell line-derived neurotrophic factor, GDNF, control germ cell numbers and survival during early developmental stages (14). During development, Sertoli cells are responsive to hormones (FSH, testosterone and perhaps even thyroid hormone regulate their maturation) (15), supply a number of important proteins and glycoproteins for differentiating germ cells, and may engage in metabolic cooperativity with the germ cells (16–19). All of these processes are likely mediated and/or facilitated by junctions between Sertoli cells and germ cells (12). Numerous analyses suggest that survival of germ cells is regulated by their interactions with Sertoli cells (20), and death of pachytene spermatocytes in the first round of spermatogenesis may be a mechanism to adjust relative numbers of germ cells to Sertoli cells (21, 22). Thus, in the testis, germ cell–somatic cell interactions are of prime importance, and Sertoli cells likely regulate and coordinate metabolic, environmental and signaling pathways crucial to the progress of meiosis. Given the importance of this physiological context, it is not surprising that it has been so difficult to recapitulate mammalian meiosis *in vitro*. Nonetheless, there are some promising approaches using isolated male germ cells that, with understanding of their limitations, have provided avenues for experimental analysis.

1.2. Enrichment of Mouse Spermatogenic Cells

The approaches for short-term culture of mouse spermatocytes described in this chapter depend on enrichment of germ cells. The stage-specific enrichment of mouse testicular germ cells entails release of germ cells from the somatic cells of the seminiferous epithelium and size fractionation by sedimentation at unit gravity through a bovine serum albumin (BSA) gradient. This method was first described by Bellv  more than thirty years ago (23, 24) and was pivotal for profiling developmental gene expression of male germ cells. We, as well as all workers in this field, are indebted to Bellv  and his colleagues for the development of these procedures. Our methods are adapted with little change from this pioneering work and readers are referred to a comprehensive methodology review by Bellv  (25) for further background and references. Other methods for enriching selected populations of male germ cells are available (e.g., fluorescence-activated cell sorting or seminiferous tubule microdissection), but this method is most widely used.

The sedimentation protocol we describe here provides fractions of germ cells enriched (but not purified) for particular developmental stages; these stages are separated in the sedimentation gradient on the basis of germ cell size. Of relevance to meiosis, stages that can be successfully enriched include leptotene/zygotene spermatocytes, juvenile (early to mid-) pachytene spermatocytes, and adult pachytene spermatocytes (all sub-stages), but it is also possible to enrich spermatogonia and round spermatids. Because

the preparative protocol includes a protease digestion step and deliberate dispersion of cells, the intercellular bridges established during spermatogonial proliferation are disrupted and germ cells are recovered as single cells; in fact, the formation of symplasts is deliberately minimized in the procedure. The effect of disruption of cytoplasmic bridges on the coordination of the meiotic events, which ordinarily occur in a syncytium of more than 500 cells, is not known. Another consequence of the relatively harsh treatment during the isolation process is digestion of surface proteins and removal of cellular appendages such as the developing spermatid tail. Furthermore, the effect of the isolation procedures on metabolism of germ cells is largely unexplored. Together, possible metabolic and membrane alterations, disruption of the intercellular bridges, and removal of the intimately surrounding Sertoli cells, are factors contributing to the limited viability during culture of spermatocytes enriched by these methods. In our experience, germ cells isolated in this manner retain high viability for 24 h in appropriate culture media, but lose viability rapidly thereafter (see below).

1.3. Short-Term Culture of Mouse Spermatocytes

The short-term culture methods described here were developed for testing function of meiotic cells by in vitro methods (26) and are based on culture media developed by others for analysis of male germ cells (27). The major issues that must be considered in assessing the function of spermatocytes under these conditions is that they lack their surrounding Sertoli cells and are no longer interconnected with other spermatocytes in large syncytia (see above). There have been few attempts to systematically enhance culture media for spermatocytes. However, despite viability limited to 24–36 h, the methods described here have proven useful to examine and manipulate key steps in late meiotic prophase. Unlike oocytes, release of spermatocytes from their surrounding somatic cells does not prompt the onset of the meiotic division phase. Morphologically normal chromosome synapsis is maintained under these culture conditions (26) and patterns of expression of recombination and cell cycle-related proteins in cultured spermatocytes are similar to those of spermatocytes freshly isolated from testes (28, 29). The discovery that the phosphatase inhibitor okadaic acid (OA) prompted premature onset of the meiotic division phase in cultured spermatocytes (30) suggested that onset of the transition out of meiotic prophase into the division phase (the G2/MI transition) is normally controlled by cell autonomous factors and that the phosphatase inhibitor abrogates mechanisms that prolong meiotic prophase. In vitro experimental analyses determined that spermatocytes acquire competence to undergo the G2/MI transition by condensing bivalent chromosomes at the mid-pachytene stage, roughly coincident (but not causally related) to the accumulation of the spermatocyte-specific histone H1t (also

known as HIST1H1T) (31). Subsequent analyses based on this culture system have defined requirements and regulators of the G2/MI transition, such as topoisomerase II and metaphase promoting factor, MPF (28, 30–33). Data on expression of these proteins by spermatocytes cultured with OA compare well with data from analysis of stage-selected segments of seminiferous tubules (34–36). However, an important issue to bear in mind is that a normal meiotic spindle is not formed in the presence of OA; thus the outcome of the pharmacologically induced and precocious meiotic division, and fidelity of chromosome segregation, cannot be determined. Indeed, mid-pachytene spermatocytes, when induced to undergo the meiotic division phase by micro-injection into oocytes, exhibit abnormalities of chromosome segregation (37); this suggests that OA treatment abrogates some normal controls and events in meiotic maturation. In spite of this limitation, short-term culture of spermatocytes and experimental induction of the G2/MI transition are useful strategies for analysis of meiotic chromosome dynamics and phenotype analysis of spermatocytes deficient for putative meiotic genes (33, 38, 39).

2. Materials

2.1. Isolation of Male Germ Cells from Adult Mice

1. Tabletop clinical centrifuge with adjustable speed and temperature.
2. Gyrotatory water bath shaker.
3. 1 L filter system, 0.45 µm cellulose acetate.
4. Two 50-mL, one 500-mL, and two 1,000-mL Erlenmeyer flasks.
5. 8-cm magnetic stir bar.
6. Magnetic stirrer.
7. Tubing, 1.2 mm × 1.8 mm × 2,000 mm.
8. Nalgene 180 clear PVC tubing, 1.6 mm-I.D. × 0.8 mm-W.T. × 3.2 mm-O.D.
9. Nalgene 180 clear PVC tubing, 6.4 mm-I.D. × 1.6 mm-W.T. × 9.5 mm-O.D.
10. Hemacytometer.
11. Fraction collector suitable for collection of 10-mL fractions (e.g. Frac-920, GE Healthcare).
12. Solid rubber stoppers that fit 50-mL Erlenmeyer flasks.
13. 16 × 100 mm disposable glass culture tubes.
14. Microscope slides.

15. 22 × 22 mm #1 cover glasses.
16. 35 mm Petri dish.
17. Parafilm.
18. High vacuum grease.
19. 15-mL and 50-mL polystyrene conical tubes.
20. Disposable 3-mL plastic pipettes with compressible bulbs.
21. 6-in glass Pasteur pipettes.
22. Collagenase from *Clostridium histolyticum*, type IV (Sigma Aldrich, C5138).
23. Trypsin from bovine pancreas (Sigma Aldrich, T9201).
24. DNase I from bovine pancreas, lyophilized (USB, 14365).
25. Albumin, bovine, fraction V – electrophoresis grade (Sigma Aldrich, A7906).
26. Dextrose.
27. 80-μm Nitex nylon mesh (Sefar America, 3-80-37).
28. MEM essential amino acids solution, without L-glutamine, 50 × .
29. MEM nonessential amino acids solution, 100 × .
30. Penicillin-streptomycin-glutamine, 100 × .
31. Trypan blue solution.
32. 5% CO₂ in air, gas cylinder.

Specialized glassware:

33. Two 1,200-mL fleakers, one fleaker (fleaker 1) with one glass outlet at the base, the other (fleaker 2) with two glass outlets at the base.
34. 3-way micrometering stopcock with socket joint (ProScience Inc., GlassShop, Canada, 56700-800).
35. 3-way double oblique stopcock with solid glass plug (Kimble Kontes, 833000-0002).
36. 50-mL glass syringe barrel used as cell-loading chamber.
37. 180-mm sedimentation chamber (STA-PUT chamber), with stainless steel baffle (ProScience Inc., GlassShop, Canada).

*Stock solutions for the Krebs-Ringer bicarbonate (KRB) media
(can be prepared in advance and stored at 4°C for several weeks):*

38. 9% NaCl.
39. 1.15% KCl
40. 6.5% NaHCO₃
41. 2.1% KH₂PO₄

42. 3.8% MgSO₄·7H₂O

43. 1.2% CaCl₂

Solutions prepared the day before the procedure and stored at 4°C:

44. Enriched Krebs-Ringer Bicarbonate medium for germ cell isolation. Prepare from stock solutions as described in **Table 17.1**.

Table 17.1.
Enriched Krebs-Ringer Bicarbonate medium

Stock solution	2,000 mL	Final concentration
9% NaCl	156.0 mL	120 mM
1.15% KCl	62.4 mL	4.8 mM
6.5% NaHCO ₃	65.2 mL	25.2 mM
2.1% KH ₂ PO ₄	15.6 mL	1.2 mM
3.8% MgSO ₄ ·7H ₂ O	15.6 mL	1.2 mM
1.2% CaCl ₂	23.4 mL	1.3 mM
100 × Pen/Strep/Glu	20.0 mL	1 ×
50 × essential amino acid	40.0 mL	1 ×
100 × nonessential amino acid	20.0 mL	1 ×
Dextrose	4.0 g	11.1 mM
Double-distilled H ₂ O to	2,000 mL	—

45. 0.5% (w/v) BSA: 1.5 g BSA into 300 mL KRB

46. 2.0% (w/v) BSA: 11.0 g BSA into 550 mL KRB

47. 4.0% (w/v) BSA: 22.0 g BSA into 550 mL KRB

48. 2 mg/mL DNase I: dissolve DNase I with double-distilled water and store in 50-μL aliquots at -20°C.

2.2. Culture of Spermatocytes

Use reagents suitable for cell culture.

1. Standard laboratory cell culture incubator.
2. Hemacytometer.
3. Tabletop clinical centrifuge with adjustable speed and temperature.
4. Billups-Rothenberg modular incubator chamber (Billups-Rothenberg Inc., MIC-101).
5. Four-well Nunclon culture dishes (NUNC, 176740).

6. 5% CO₂ in air.
7. Minimum Essential Medium alpha (MEM α) (powder), with L-glutamine, without ribonucleosides and deoxyribonucleosides.

Solutions required:

8. MEM α : Prepare the medium following the procedure of the manufacturer, but add 50 mg streptomycin sulfate and 75 mg penicillin G per liter of medium. The medium can be stored at 4°C for two months.
9. Spermatocyte culture medium: Place 145 μ L DL-Lactic acid sodium salt, 2.5 mL fetal bovine serum, and 0.295 g HEPES into a 50 mL conical tube and fill with MEM α to 50 mL. Adjust pH with 1 N NaOH to 7.0–7.2. This medium can be stored at 4°C for a couple of weeks.
10. 300 μ M OA stock solution: Add 100 μ L absolute ethanol to one vial of OA (ammonium salt, 25 μ g) and store at –20°C for up to 2 months. This can be dispersed into suitably sized aliquots to be thawed when needed; in our experience, thawing the stock solution once or twice is not detrimental within the storage period of 2 months at –20°C.

3. Methods

3.1. Isolation of Male Germ Cells from Adult Mice

3.1.1. Preparation the Day Before

These procedures are based on those previously developed and described by Bellvé (25).

1. *Glassware and plasticware preparation:* Thoroughly wash all glassware and plasticware coming into contact with germ cells, including Erlenmeyer flasks, 1,200-mL fleakers, three-way glass stopcocks, 50-mL syringe barrel and sedimentation chamber, with soap and water, rinse and dry (*see Note 1*). Seal glassware, magnetic stir bar, stainless steel baffle and PVC tubing in autoclave bags, autoclave and store until required. Immerse the three-way plastic micrometering flow valve and tubing of the gradient outlet in 70% ethanol overnight. The following day, rinse the pieces with distilled water and air-dry.
2. Prepare the Krebs-Ringer bicarbonate (KRB) medium (*see Section 2.1*, step 44). Aerate the medium by bubbling 5% CO₂ in air through a sterile cotton-tipped 10 mL pipette for 20 min at room temperature; this adjusts the pH between 7.2 and 7.3. Sterilize the medium by vacuum-filtration using the 0.45- μ m filtration unit.

3. Prepare the BSA solutions (*see Section 2.1*, steps 45, 46, and 47) and store at 4°C.
4. Weigh 20.0 mg of collagenase and 20.0 mg of trypsin; place in individual 15-mL conical tubes and store at -20°C in a dessicator until the next day.
5. *STA-PUT sedimentation system assembly:* Assemble the system in a 4°C cold room as diagrammed in **Fig. 17.1**.

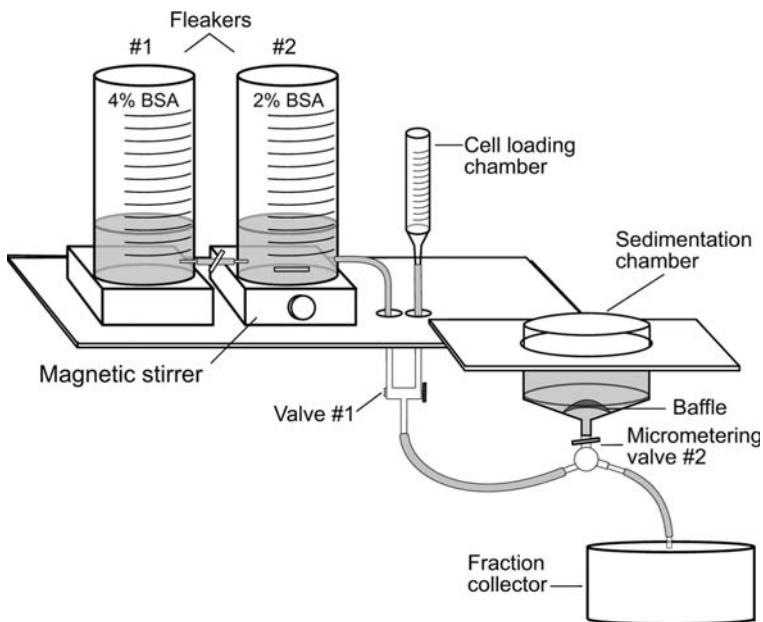


Fig. 17.1. Schematic representation of the STA-PUT sedimentation apparatus used to separate different populations of male germ cells at unit gravity. The system is set up as described in **Section 3.1.1.** (step 5), and a detailed description of the loading procedure is given in step 16 of **Section 3.1.2.**

- a. Connect the two 1,200-mL fleakers (fleaker 1 and fleaker 2) with 10 cm of Nalgene 180 clear PVC tubing (6.4 mm-I.D.). Place clamp 1 on the tubing between the two fleakers to prevent premature mixing of the 2% and 4% BSA solutions. Place the 8-cm magnetic stir bar in fleaker 2 and place the magnetic stirrer under fleaker 2. Ensure that fleakers 1 and 2 are leveled by adjusting the height of fleaker 1 accordingly.
- b. Connect the second outlet of fleaker 2 via 25 cm of Nalgene 180 clear PVC tubing (6.4 mm-I.D.) to the inlet of the three-way double oblique stopcock (valve 1 – assemble the stopcock after spreading a bit of vacuum grease on its surface).
- c. Connect the 50-mL barrel of the glass syringe to the other outlet of the three-way glass stopcock (valve 1) via 15 cm of Nalgene 180 clear PVC tubing (6.4 mm-I.D.).

- d. Assemble the three-way glass/plastic micrometering flow valve (valve 2 – spread a bit of vacuum grease on its surface). Attach valve 2 to the base of the sedimentation chamber using parafilm and a clip.
- e. Connect valve 1 to valve 2 using 50 cm of Nalgene 180 clear PVC tubing (6.4 mm-I.D.).
- f. Place a 1,000- μ L pipette tip on the other outlet of valve 2. Connect one end of the collection tubing to the tip and the other end to the fraction collector. The collection tubing is made up of 70 cm of Nalgene 180 clear PVC tubing (1.6 mm-I.D. – end connected to the tip) connected to 5 cm of tubing (1.2 mm \times 1.8 mm – end connected to the fraction collector).
- g. Carefully place the stainless steel baffle over the opening at the base of the sedimentation chamber. Ensure that the baffle is perfectly leveled or it will disrupt the formation of the gradient.

3.1.2. Germ Cell Isolation Procedure

This entire procedure takes about 8 h.

1. Before starting, set temperature of shaking water bath to 32°C and add ~20 mL of KRB medium in each of the two 50-mL Erlenmeyer flasks – leave on ice until step 3.
2. *Retrieval of testes:* Kill 6–12 male mice, aged about 8 wk, by cervical dislocation. Lay each animal on its back; sterilize the ventral surface with 70% ethanol. Remove testes from the abdomen with a pair of surgical scissors. Holding the fat pad with fine forceps, rinse each testis in KRB medium in a 35 mm Petri dish on ice.
3. *Preparation of testes:* place testes on a sheet of Parafilm and detunicate by gently grasping the tunica albuginea with two pairs of fine-tipped tweezers. Avoid using excessive force when detunicating testes to minimize the formation of symplasts, which severely affect the purity of resultant germ cell populations. It will take roughly 10 min to kill the mice and prepare the testes for the next step.
4. *Collagenase digestion (0.5 mg/mL):* place half of the detunicated testes in one of the 50-mL flasks containing 20 mL of KRB medium, the other half in the other 50-mL flask. Dissolve the pre-weighed 20 mg of collagenase in 4 mL of KRB medium by vortexing (use 2 mL from each flask, being careful not to pick up any testicular content as the medium is withdrawn); redistribute 2 mL back to each flask. The collagenase must be added only after the testes are detunicated.

5. Briefly gas the flasks with 5% CO₂ in air and cover with rubber stoppers. Incubate for 20 min in the 32°C water bath with shaking at 225 rpm. At the end of the incubation, the testes should have dispersed into lengths of seminiferous tubules (like spaghetti).
6. Setting the flasks on an angle on ice, allow the tubules to settle to the bottom. Decant and discard the supernatant fluid. Wash the tubules by adding 20 mL KRB medium to each flask; swirl flasks around and allow tubules to settle by gravity again. Repeat this step twice for a total of three washes to eliminate a maximum number of interstitial cells, which are found in the discarded supernatant.
7. *Trypsin digestion (0.5 mg/mL)*: after the third wash, pour 20 mL of fresh KRB medium into each 50-mL flask. Dissolve the pre-weighed 20 mg of trypsin by the same procedure used in step 4 and distribute 2 mL back to each flask. Add 5 µL of 2 mg/mL DNase I to each flask and mix by swirling flasks.
8. Briefly gas the flasks with 5% CO₂ in air and cover with rubber stoppers. Incubate for 13 min in the 32°C water bath with shaking at 225 rpm. After the digestion, tubules will look similar to those in step 5, but will be more fragile.
9. Using a plastic transfer pipette cut off at the second mark to create an opening of ~3 mm, gently pipette tubules up and down for 3 min to free the germ cells (*see Note 2*). The presence of clumped “strings” of cells indicates cellular aggregation due to the release of DNA from damaged cells. Addition of a few µL of DNase I will help eliminate some of the clumps, as will the next steps (steps 10, 13, and 15).
10. Filter the cell suspension through an 80-µm Nitex mesh into a 50-mL conical tube. Gently pour in 0.5% BSA-KRB to complete the volume to 50 mL. Equally distribute the cell suspension into eight disposable 16 × 100 mm round bottom glass culture tubes.
11. Centrifuge the filtered cells for 7 min at 500g in a tabletop centrifuge set at 4°C.
12. During step 11, prepare the following solution and keep on ice: add 30 µL of 2 mg/mL DNase I to 150 mL of 0.5% BSA in KRB medium.
13. After the first spin, discard supernatant, resuspend cells by gently tapping on the side of the tube and then slowly add three Pasteur pipetfulls of 0.5% BSA supplemented with DNase I (prepared in step 12). Shake the tubes gently as the solution is added to avoid clumping. If clumping does occur, resuspend cells with a plastic transfer pipette cut off at the second mark as in step 9.

14. Centrifuge cells again for 7 min at 500*g* and wash them as in step 13. Repeat twice for a total of three washes. After the final spin, discard the supernatant and add ~5 mL of 0.5% BSA in KRB medium without DNase I to one tube. Resuspend cell pellet by flicking, transfer suspension to next tube and resuspend the second pellet by flicking; repeat this procedure to sequentially pool germ cells from the eight glass tubes. Transfer the cell suspension to a 50-mL conical tube and bring volume to 25 mL with 0.5% BSA in KRB medium without DNase I.
15. Filter cells with an 80- μm Nitex mesh into a 50-mL conical tube. Count cells with a hemacytometer using a 1:10–1:20 dilution and determine cell viability using Trypan blue dye exclusion (viability should be over 95%). Although a maximum number of 1×10^9 germ cells can be loaded onto a SP-180 STA-PUT chamber, often better separations are obtained with 5×10^8 or fewer cells (*see Note 3*). As a guide, $\sim 5\text{--}9 \times 10^7$ germ cells can be obtained per adult CD1 mouse. Once cell counts are determined, dilute the appropriate number of cells to 25 mL using 0.5% BSA in KRB medium. Cells are now ready to load onto the STA-PUT apparatus.
16. *Preparing the sedimentation apparatus for loading of the germ cells:*
 - a. Add 550 mL of 4% BSA in KRB to the rear fleaker (fleaker 1), and 550 mL of 2% BSA in KRB to the front fleaker (fleaker 2). The clip placed on the tube between the two fleakers prevents premature mixing of the two solutions.
 - b. Fill the tubing connecting fleaker 2 and the inlet of the three-way stopcock (valve 1) with the 2% BSA solution; remove all bubbles in the tubing by squeezing it.
 - c. Add 50 mL of KRB medium (or as much as needed) in the cell-loading syringe and fill all tubing connecting the syringe and the chamber inlet (valve 2) with this medium. Squeeze the tubing to remove air bubbles. The apparatus is ready for cell loading.
 - d. Load 50 mL of KRB medium through the syringe into the STA-PUT apparatus. Adjust the flow rate to 1 mL per 3 s using the micrometering screw of the three-way stopcock at the base of the sedimentation chamber. This KRB medium will form a layer on top of the cells, protecting them from direct contact with air.
 - e. As the volume of KRB media nears the bottom of the syringe, carefully add the 25 mL of cell suspension to the syringe before it empties to prevent air bubbles from entering the tubing. Load cells at the rate of 1 mL/3 s; the cells

should load within a couple of minutes. **From this point on carefully protect the apparatus from any vibrations (see Note 4).**

- f. As the last of the cell suspension is entering valve 1, immediately switch the flow through stopcock valve 1 so the BSA solution enters behind the cells, preventing air bubbles from entering the tube. Remove the clip between fleakers 1 and 2 to allow the two BSA solutions to mix and the gradient to form. Turn on magnetic stirrer placed under fleaker 2; ensure mixing is thorough but not too vigorous to avoid excessive incorporation of air. This is time zero; begin collecting fractions 2 h and 30 min after this time. The entire gradient should load within 45 min (± 15 min); adjust flow with micrometering screw (see Note 5).
17. After all fluids have entered the sedimentation chamber, close valve 1 and turn off the magnetic stirrer. Let cells sediment for ~ 1 h 45 min.
18. Load the fraction collector with sequentially numbered disposable 16 \times 100 mm round bottom glass culture tubes.
19. *Collection of fractions:* At time 2.5 h post-zero, begin collecting 10 mL fractions (~ 300 drops/tube using the Frac-920 fraction collector). Carefully control flow rate with the micrometering stopcock so that each fraction collects in about 43–45 s. The last fraction should be collected approximately 4 h after first loading the cell suspension.
20. After fraction collection, centrifuge fractions 25–95 (generally, fractions 1–24 do not contain cells) at 500*g* for 7 min at 4°C. Aspirate all but 0.5 mL of supernatant. Resuspend cell pellets by gently flicking tubes and keep on ice.
21. *Analysis of fractions:* Analyze fractions with differential interference contrast (DIC) light microscopy using a 40 \times objective. See Fig. 17.2 for examples. Fractions are examined in

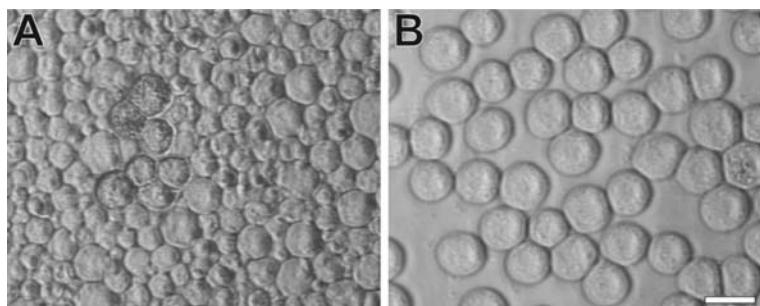


Fig. 17.2. Germ cells before and after enrichment by unit gravity sedimentation. Panel (A) illustrates a mixed germ cell preparation from adult testes prior to loading into the STA-PUT chamber; note the range of cell sizes. Panel (B) illustrates the fraction of pachytene spermatocytes enriched by cell-size fractionation. Bar = 15 μm .

increments of five to find those containing the cells of interest. For purposes of comparison, aliquots can be removed from two fractions at a time and placed side-by-side on a microscope slide. Cover each sample gently with a 22 × 22 mm coverslip (samples are not meant to mix on the slide). Cells are identified on the basis of distinctive morphological criteria and differences in cell size – the use of a calibrated reticule in an ocular lens of the microscope is strongly recommended to establish cell size (*see Note 6*).

22. Once fractions containing the large pachytene spermatocytes (**Fig. 17.2B**) have been identified, aliquots from surrounding fractions are also examined to select fractions for pooling. Evaluate the purity of the fractions by counting the numbers of pachytene and nonpachytene cells out of a total of 100 cells. Fractions of the upper and lower limits of the peak of pachytene spermatocytes can be included or excluded depending on the need for yield versus maximal purity (*see Note 7*).
23. Once the window of pachytene spermatocytes is identified, pool the appropriate fractions of germ cells into a disposable 16 × 100 mm round bottom glass culture tube. Centrifuge for 5 min at 220*g* at 4°C. Aspirate supernatant and resuspend cells in 5 mL of KRB medium. Centrifuge and wash two more times.
24. After the final wash, resuspend cells in 5 mL of KRB medium. Taking an aliquot of the cell suspension, prepare a 1:2–1:5 dilution with KRB medium. Count cells and determine the purity of the enriched pachytene spermatocyte preparation with a hemacytometer. Cells are ready to be used for short-term culture (*see Note 8*).

3.2. Short-Term Culture of Spermatocytes

1. Before starting, ensure that the incubator is stably adjusted to 32°C and prepare the MEMα, the spermatocyte culture medium and the OA stock solution as described (*see Section 2.2*, steps 8, 9, and 10).
2. *Preparation of cells for culture:* After determining the cell count (*see Section 3.1.2*, step 24), centrifuge the 5-mL spermatocyte suspension at 500*g* for 7 min at 4°C. Resuspend the cell pellet in MEMα culture medium at a concentration of 2.5×10^6 cells/mL.
3. Place desired volume of cell suspension into wells of the four-well dish, ensuring that the cells are well suspended before pipetting. An optimal volume is approximately 1 mL/well (2.5×10^6 cells/well) (*see Note 9*).
4. Place the covered four-well dishes in a Billups-Rothenberg modular incubator chamber. Perfuse this with humidified 5% CO₂ in air, and seal outlets.
5. Incubate at 32°C for the desired time period (*see Note 10*), after which cells can be experimentally treated.

6. Open the modular incubators. From each dish, resuspend the cells by gentle pipetting and remove a small aliquot of cells for viability assessment by Trypan blue dye exclusion (*see Note 11*), as well as any cells desired for cytological or biochemical assays.
7. *Treatment with OA for experimental induction of the G2/MI transition:* Add OA to the culture medium at a concentration of $4 \mu M$ (*see Note 12*). After addition of OA, mix the medium by pipetting gently several times. Return the culture dishes to the modular incubator chamber, perfuse with gas as previously described (step 4) and return to the incubator at $32^\circ C$ for desired time (*see Note 13*).
8. At the end of the culture period, resuspend the cells by gently pipetting, and collect cells for final viability assessment and desired cytological or biochemical analyses (*see Note 14*). *See Fig. 17.3* for examples of cultured cells before and after OA treatment immunolabeled with antibodies recognizing meiotically relevant proteins.

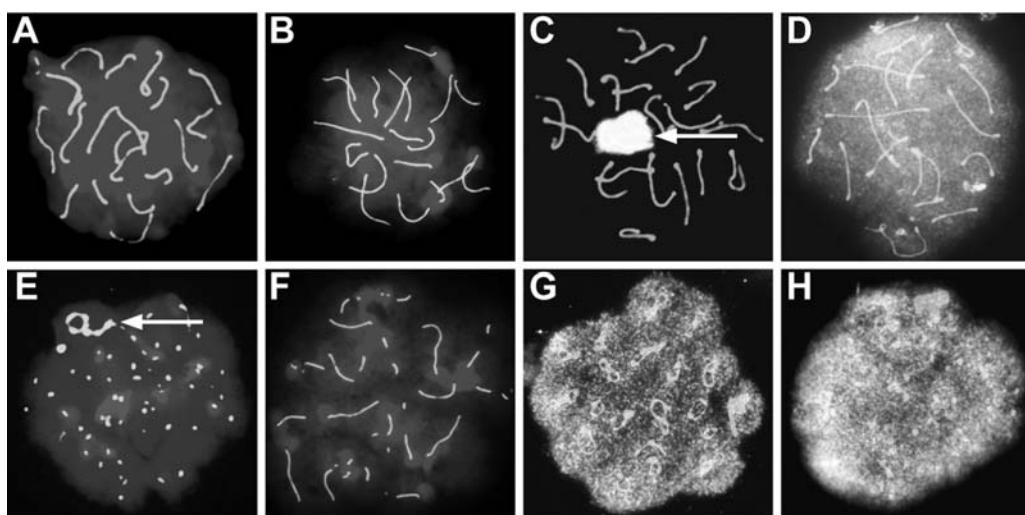


Fig. 17.3. Examples of immunolabeled surface-spread chromatin of spermatocytes after short-term culture, with and without OA treatment. Panels (A–D) are images of cultured pachytene spermatocytes before OA treatment; panels (E–H) are images of cultured spermatocytes treated with OA to induce the G2/MI transition; panels (A) and (E) depict labeling with anti-SYCP3 before and after OA treatment; and panels (B) and (F) depict labeling with anti-SYCP1 before and after OA treatment. (A) When labeled with antibody against SYCP3, a protein of the lateral element of the synaptonemal complex, cultured pachytene spermatocytes exhibit a typical pattern. (B) When labeled with antibody against SYCP1, a protein of the central element of the synaptonemal complex, the synaptonemal complexes of all autosomal chromosomes of cultured pachytene spermatocytes are labeled, revealing that synapsis is maintained in culture. (C) This image reveals the typical labeling of the XY body (arrow) by anti- γ H2AX in cultured spermatocytes. (D) Antibody recognizing histone H1t labels most cultured spermatocytes in the chromatin surrounding the synaptonemal complexes, which are labeled with anti-SYCP3. (E) After induction of the G2/MI transition with OA, the labeling with anti-SYCP3 is typical of metaphase I, where SYCP3 accumulates in centromeric regions; the condensed sex chromosome axes are still obvious (arrow). (F) Soon after induction of the G2/MI transition with OA, the labeling pattern with anti-SYCP1 reflects desynapsis during the diplotene stage. (G) Immediately after OA treatment, the labeling pattern of phosphorylated histone H3 is typical of that of diplotene spermatocytes, seen in the chromatin surrounding the axes of the synaptonemal complex, labeled with anti-SYCP3. (H) After OA treatment, when SYCP3 signals accumulate in the centromere regions, labeling with anti-phosphorylated histone H3 is intense throughout the chromatin of metaphase I spermatocytes.

4. Notes



1. If cells begin to adhere to the surface of glassware (usually the sedimentation chamber), acid-wash the glassware to remove cellular debris that has accumulated over time.
2. Glass Pasteur pipettes can also be used for this step, but the tips must be fired to round off any sharp edges.
3. If the concentration of germ cells loaded onto the gradient is too high, a “raining” effect (25) occurs: the cells cluster into small droplets that rapidly sediment down through the gradient, giving the appearance of rain showers; this markedly reduces the level of purity achieved. It is recommended to start with 2.5×10^8 cells until experience is gained in both preparing the germ cells and applying the cell suspension to the gradient.
4. Vibrations (such as those from carelessly shutting the cold room door) will disrupt the formation of the gradient and cause germ cells to separate inefficiently, markedly reducing the level of purity achieved. If using a shared cold room, it is wise to alert other users.
5. If the cells and the BSA are loaded too slowly, cells will settle on the base of the sedimentation chamber; if loaded too quickly, the turbulence created while passing the stainless steel baffle will disrupt those cells already layered beneath the KRB.
6. Pachytene spermatocytes are spherical and have a diameter of 12–18 μm . Their appearance is granular, with a thin ring of cytoplasm and a large nucleus with thick strands of bivalent chromosomes (**Fig. 17.2B**). Spermatids (developmental steps 1–8), also referred to as round spermatids, are spherical but are much smaller than pachytene spermatocytes, their diameter ranging between 8 and 10 μm . Round spermatids have clear cytoplasm, much smaller nuclei with homogeneous chromatin and, most distinctively, a prominent central nucleolus. Although the fraction number in which these different cell types will be found varies from one manipulator to the other, the order in which the cells should appear should always be as follows: in the earliest fractions, Sertoli cells (identified by the presence of lipid droplets) and larger multi-nucleated symplasts (made up of several round spermatids), followed by pachytene spermatocytes, then round spermatids and finally elongating/condensing spermatids mixed with residual bodies.
7. Usually, a peak of pachytene spermatocytes of 10–15 fractions of more than 80% purity can be obtained. The pooled fractions usually yield a total number of pachytene spermatocytes of 1 to 3×10^7 . The main contaminants are multi-nucleated symplasts and Sertoli cells.

8. The isolation procedure described in **Section 3.1.2.** is designed for the retrieval of pachytene spermatocytes from adult male mice. Leptotene/zygotene spermatocytes as well as juvenile (early) pachytene spermatocytes can also be isolated using the same apparatus and procedure with the following modifications. In step 2, use 60–90 17-day-old male mice and in step 3, gently tease apart the tubules with fine forceps after detunification. Additionally, filter the cell suspension through a 53- μm Nitex nylon mesh in steps 10 and 15 instead of an 80- μm mesh (Sefar America, 3-53-41). Leptotene/zygotene spermatocytes are spherical and have a granular appearance with visible strands of thickened chromatin. Their diameter varies between 8 and 12 μm . Pachytene spermatocytes are as described in **Note 6**. In our experience, the early prophase spermatocytes obtained from juvenile mice do not remain viable as long as do pachytene spermatocytes from adult mice.
9. Volume can be adjusted to facilitate addition of any treatment solution. For example, we use 986.7 μL per well for culture when planning an addition of 13.3 μL OA (*see Note 12*).
10. Generally, an overnight (up to 12 h) culture period before any treatment allows spermatocytes to recover from the cell-enrichment procedure.
11. For viability assessment, transfer 20 μL of cell suspension to 80 μL of PBS in a 1.5 mL Eppendorf tube; after gently mixing, put 20 μL of cell suspension into 20 μL of Trypan blue in another 1.5 mL tube; place 20 μL of this (which is a 10 \times dilution of the original cell concentration) in a hemacytometer for viability determination, blue cells being those that are dead.
12. To achieve a final concentration of 4 μM , 13.3 μL OA stock solution is added to 986.7 μL culture volume.
13. Generally, by 5 h of OA treatment at 32°C, 70–80% of the spermatocytes progress to metaphase I.
14. To investigate the dynamics of G2/MI transition, spermatocytes can be collected at different time intervals during the 5-h culture period. Collected samples can be either used for cytological preparations, or lysed for biochemical analyses.

Acknowledgments

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Chapter 18

Isolation and Analyses of Enriched Populations of Male Mouse Germ Cells by Sedimentation Velocity: The Centrifugal Elutriation

Marco Barchi, Raffaele Geremia, Roberto Magliozzi, and Enrica Bianchi

Abstract

The studies of molecular events that occur in single cell types within a tissue often require the disaggregation of the tissue into a single cell suspension, followed by isolation of distinct cell populations. The germinal epithelium of mammals is composed of several cell types, which divide mitotically, before entering meiosis. In this chapter, we describe the isolation of five mouse germ-cell fractions by centrifugal elutriation, and characterize them by their DNA content (flow cytometry), cell morphology (DAPI staining of nuclei, Giemsa staining of squashed cells) and deposition of stage-specific meiotic markers (SYCP3, H1t, SAM68) on chromosome spreads and whole cells. Within 2 h it is possible to obtain enriched populations of elongated spermatids (up to ~50% of the fraction), round spermatids (up to ~80%), primary spermatocytes (up to ~89%), and secondary spermatocytes (up to ~17%). Furthermore, most of the collected spermatocytes of the primary spermatocyte fraction are in early-mid pachytene stage as judged by chromosome spreads, enriched up to ~89%. Elutriation and techniques used for characterization of germ cell fractions are described.

Key words: Centrifugal elutriation, sedimentation velocity, germ cell purification, meiosis, mouse, germ cells, SYCP3, H1t, SAM68, testis, spermatogenesis.

1. Introduction

In mammals, the male germinal epithelium is composed of somatic Sertoli cells and germ cells that can be divided into at least three categories: mitotic (spermatogonia), meiotic (primary and secondary spermatocytes), and differentiating germ cells (spermatids and sperm). At the base of the seminiferous tubule, stem cells divide mitotically to replenish the stem-cell population and give rise to

differentiating spermatogonial cells. The latter divide and give origin to different sub-classes of spermatogonia, until they perform a last cycle of DNA duplication (pre-leptotene stage) followed by the formation of meiotic primary spermatocytes. The latter (leptotene, zygotene, pachytene, diplotene, and diakinesis-stage cells) are thus tetraploid (4C), and after a long maturation process enter the first meiotic division and divide to give rise to secondary spermatocytes (2C). Following a second meiotic division, secondary spermatocytes form haploid (1C) round spermatids which, after morphological changes (spermiogenesis), form morphologically mature sperm.

All the described cell types differ functionally, but also physically, by size and shape. During the last forty years, physical cell characteristics have been successfully used to obtain purified germ cell populations for biochemical studies. At least two methods have been used: sedimentation at unit gravity (Staput) (refer to Chapter 17 in this volume) and centrifugal elutriation. Both methods use cell size (thus sedimentation velocity) as criteria for cell fractionation. The former method, using a bovine serum albumin (BSA) gradient, allows an efficient cell separation by sedimentation velocity under unit gravity force. This method allows the isolation of enriched fractions of bone marrow cells (1) as well as rat and mouse germ cells (2–4). However, its principal limitations are both the relatively long time required (4–5 h) and the limited amount of cells that can be fractionated ($\sim 8 \times 10^7$ cells) [e.g., see (5)]. Centrifugal elutriation, in contrast, separates cells by velocity sedimentation at greater than unit gravity. Thus, for an equivalent cell separation, it requires less time (1–1.5 h). Furthermore, the amounts of cells that can be fractionated are of the order of $3\text{--}5 \times 10^8$, allowing the recovery of a higher amount of cells per fraction. The purpose of this chapter is to describe the centrifugal elutriation technique using the JE-6B and JE-5.0 elutriator systems (Beckman Coulter Inc) (see Note 1) and to characterize purified mouse germ cell fractions using flow cytometry (FACS), cell morphology (squash/Giemsa staining) and deposition of cell-type-specific markers (SYCP3/H1t/SAM68).

1.1. Principles of Cell Separation by Elutriation

The principles of cell separation are based on Stokes' law of slow sedimentation of a sphere through a viscous noncompressible fluid medium. Under this law, the sedimentation velocity of a particle (S_v) depends on the diameter (size) of the particle (d) and its density (ρ_p), the density (ρ_f) and viscosity (n) of the fluid, the radial position of the particle (r) and the angular velocity (Ψ) in radians/second:

$$S_v = d^2(\rho_p - \rho_f)/18n \times \Psi_r^2$$

Since the volume parameter is raised at the second power and because cell populations often do not differ much with respect to

density, cell separation by sedimentation velocity is mostly based on size (*d*) differences. Although Stoke's law accurately describes the behaviour of rigid spherical particles, it is somewhat less accurate in describing the *Sv* of cells which are not rigid and not always spherical (e.g., sperm). Thus, a more generalized form of Stoke's law that takes account of cell shape has also been described (6, 7).

Centrifugal elutriation in the JE6B and JE-5.0-elutriator systems combines two separation force technologies: centrifugation, the process of sedimentation under the influence of a *centrifugal force* field generated by the rotor, and *counterflow force*, generated by a pump-derived washing fluid (8, 9)(Fig. 18.1).

Separation takes place into a funnel-shaped elutriation chamber embedded in the rotor (Fig. 18.2). While the rotor is spinning (*centrifugal force*), a suspension of cells is pumped at a preset flow rate (generated by the pump) into the narrow end of the elutriation chamber. As fluid traverses through the chamber toward the axis of rotation, its velocity decreases as the chamber walls become wider (Fig. 18.2), thus creating a velocity gradient from the

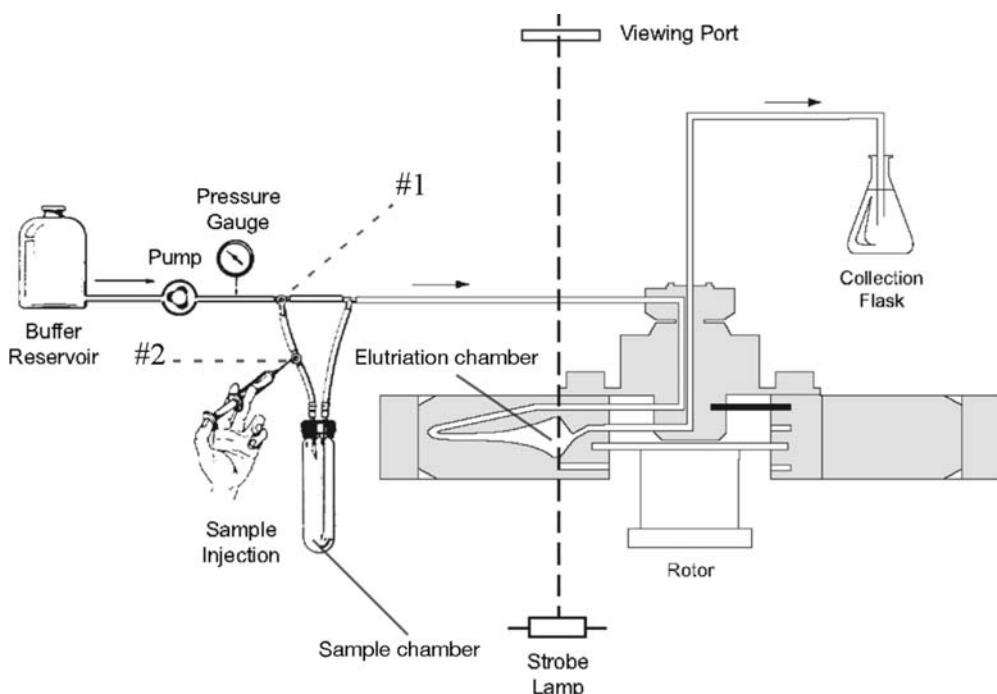


Fig. 18.1. Elutriation in the JE elutriator system (Beckman Coulter, Inc.). During the elutriation, the EKRB buffer contained in the buffer reservoir is pumped into the elutriator system, filling up the sample and elutriation chambers, and collected into a collection flask. When the system has been filled and the rotor is spinning, the sample is injected into the sample chamber through the injection valve (#2). At this time, the bypass valve (#1) is closed and the EKRB buffer does not flow into the sample chamber. Once the sample has been fully injected, valve #2 is closed, while valve #1 is switched to be open. In this manner, the EKRB buffer flows into the sample chamber and the cell suspension enters into the elutriation chamber. Cell gradient formation in the elutriation chamber can be monitored by watching through a viewing port on the centrifuge door, while cells are lit by a strobe lamp. (Figure reproduced with permission of Beckman Coulter, Inc.).

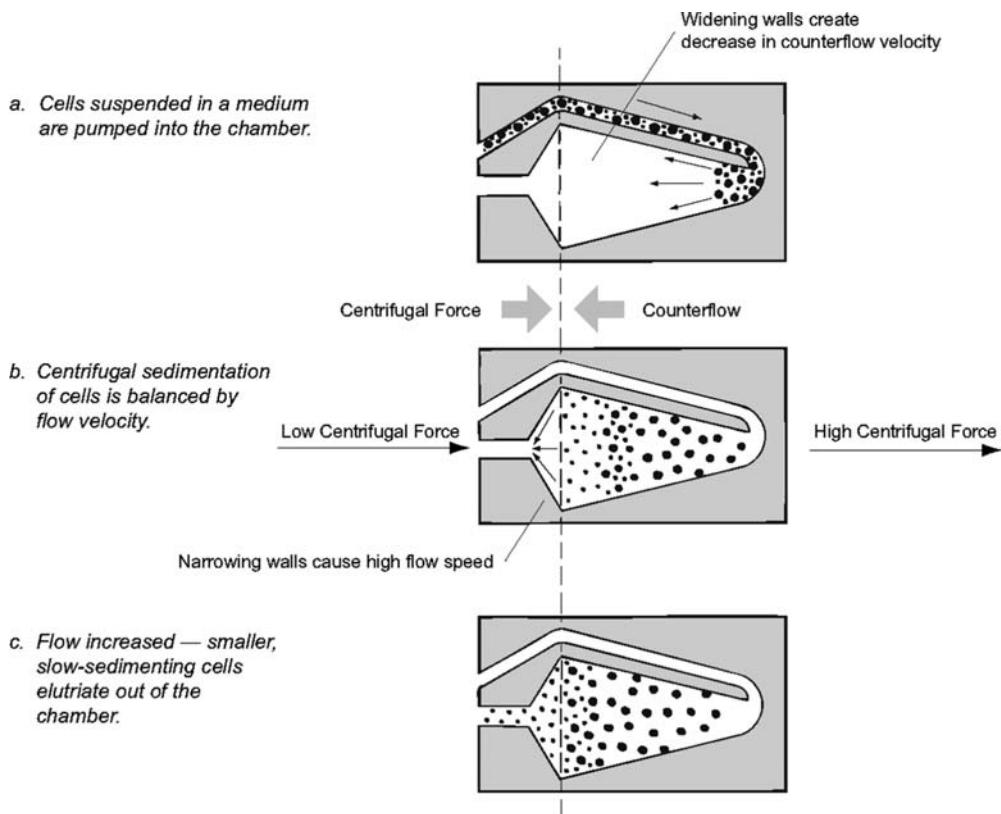


Fig. 18.2. Distribution of cells with different sedimentation velocities within the elutriation chamber. (a) Cells are pumped into the chamber through the peristaltic pump-generated force which generates a counterflow that is opposite to the centrifugal force of the rotor. Because of the widening of the elutriation chamber, the counterflow force decreases at the elutriation boundary (dotted line). (b) The centrifugal force, which influences the sedimentation rate of the cells, also varies within the chamber. It is low close to the axis of rotation and high at the opposite end of the elutriation chamber. (c) According to Stokes' law, the sedimentation velocity of the cells is mostly based on size differences, so that smaller cells flow first to the elutriation boundary and can be elutriated out of the chamber. (Figure reproduced with permission of Beckman Coulter, Inc.).

narrow end of the chamber to the widest part of the chamber (9) which is opposite to the centrifugal force. The centrifugal force also creates a velocity gradient. This is low in proximity to the centrifugation axes and increases in proximity to the narrowed end of the elutriation chamber (Fig. 18.2). The widest part of the chamber is the elutriation boundary. Following the introduction into the elutriation chamber of a suspension of cells with different size (d), cells migrate according to their sedimentation rate (S_v) to a position in the gradient where the effects of the *counterflow force* balance the sedimentation velocity. Smaller or less dense cells with a small sedimentation rate are quickly washed toward the axis of rotation, where they are caught in an increasing flow velocity caused by the rapidly narrowing chamber wall (9) (Fig. 18.2). Large or dense cells with high sedimentation rate, on the contrary,

remain near the inlet of the elutriation chamber where centrifugal force and sedimentation rate are high. Cells with an intermediate volume or density reach the equilibrium at the elutriation boundary (**Fig. 18.2**). By increasing the flow rate of the pump in gradual steps, successive fractions of large or dense cells can be washed out of the elutriation chamber (**9**) and collected in a collection flask or tubes (**Fig. 18.1**).

2. Materials

2.1. Animals

Testes should be obtained from three adult mice (~8 wks old) of the desired strain background. For illustration purposes, the description in this chapter uses Swiss CD1 mice. Typically, from three mice, a suspension of $\sim 3 \times 10^8$ monodispersed cells is obtained.

2.2. Elutriator and Accessory Material

1. The systems used for the germ cell separation described here are the JE-6B and JE-5.0 elutriation systems and rotors with a “standard chamber” (Beckman Coulter) (**Fig. 18.1**) (*see Note 1*).
2. Peristaltic pump, Masterflex (2–100 mL/min flow rate).
3. Pressure gauge, to monitor pressure variations in the system.
4. Silicone tubing (Masterflex), which is required to connect elutriator, buffer reservoir, peristaltic pump, and sample chamber (**Fig. 18.1**).

2.3. Testicular Germ Cell Suspension Preparation

1. Shaking water bath set at 30°C.
2. Refrigerated bench centrifuge.
3. Sterile blades (*see Note 2*).
4. 50 mL sterile polypropylene tubes (e.g., Falcon tubes).
5. Thoma’s chamber, for cell counting.

2.4. Elutriator Setting

1. Elutriator: Beckman Coulter JE-6B or JE-5.0.
2. Pipette aid.
3. 100 mL graduated cylinder.
4. Serological pipettes.
5. Timer.
6. 1 L of sterile water (*see Note 3*).

2.5. Elutriation

1. Sterile PBS: 17 mM NaCl, 2.68 mM KCl, 1.45 mM KH₂PO₄, 7.75 mM Na₂HPO₄, pH 7.4.
2. Minimal essential medium (MEM) (Life Technologies, Inc.).

3. DNase I: crude lyophilized powder (*see Note 4*).
4. Collagenase digestion medium: 25 mL of 0.25 mg/mL collagenase (type XI) supplemented with 0.05 mg/mL DNase I, in MEM (*see Note 4*).
5. Trypsin digestion medium: 25 mL of 1 mg/mL trypsin and 0.05 mg/mL DNase I in MEM (*see Note 4*).
6. Fetal calf serum (FCS).
7. Bovine serum albumin (BSA).
8. 1.5 L of sterile elutriation medium, EKRB: 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 11 mM glucose, essential amino acids (Life Technologies, Inc.), 1 × penicillin and streptomycin (Life Technologies, Inc.), 0.2% BSA. All components are diluted in water. Antibiotics and BSA are added to the solution the day of the experiment; the solution is then filtered through a 0.22 µm filter, and stored at 4°C.

2.6. Preparation of Cells for DNA-Content FACS Analyses

1. Cold PBS.
2. Cold 1% paraformaldehyde (PFA) in PBS. Make fresh from a 10% PFA stock in water.
3. 90% ethanol.
4. 2 mg/mL RNase A dissolved in water.
5. 1 mg/mL Propidium iodide (PI).
6. Water bath set at 37°C.
7. 4°C centrifuge.

2.7. Squash of Isolated Meiotic Cells and Giemsa Staining

1. Hypotonic solution: 75 mM KCl in water.
2. Fixing solution: methanol-acetic acid, 3:1.
3. 0.4% (w/v) Giemsa dye, dissolved in 0.15 mM NaH₂PO₄, 3% methanol.
4. Washing buffer: 0.15 mM NaH₂PO₄, 3% methanol.
5. SuperFrost microscope slides.
6. Water bath, or thermo block set at 37°C.
7. Refrigerated benchtop centrifuge.
8. Mounting media for histology

2.8. Chromosome Spreads Preparation

1. Five 50 mL glass Coplin jars.
2. Multi-Spot glass slides 3 in × 1 in with 12 wells of 6 mm dia. (Thermo Electron Corporation).
3. Plastic coverslips.

4. Phenol red indicator: 0.5% phenol red in water. Filter and store at room temperature indefinitely.
5. 50 mM borate buffer: dissolve sodium borate in water, adjust pH to 9.2 with 0.5 N NaOH.
6. 0.1 N NaOH in water.
7. pH indicator strips.
8. Warm stirring plate set at 50°C, stirring bar.
9. MEM (Life technologies, Inc).
10. 2% Paraformaldehyde (PFA) in water (*see Note 5*): Put 100 mL water into a small glass bottle and warm it to 50°C in a microwave (~1 min). Add 2 g of PFA and stir on a warm stirring plate set at 50°C. While shaking, add a few drops (2–4) of 0.5 N NaOH and stir for 10 more minutes. If PFA is not fully dissolved after 10 min, add one or two more drops of 0.5 N NaOH and stir for 10 more minutes. Repeat the procedure until PFA is completely dissolved and solution is crystal clear. Cool PFA solution to room temperature, on ice (or cold room) for about 30 min. Filter solution with 0.45 µm filter and add 100 µL of 0.5% phenol red indicator. Solution should appear a pink-violet colour. Using pH indicator strips, monitor the pH of the solution. It should be between 7.5 and 8.0. If pH is too low, adjust it with a few drops of 0.05 M borate buffer. Use 2% PFA as fixing solution-2 and to prepare fixing solution-1 (*see below*).
11. 60 mg/mL SDS: After dissolving SDS, add 30 µL of phenol red indicator per 100 mL of solution. Adjust pH to 8.2 with borate buffer.
12. Fixing solution-1: to 50 mL of 2% PFA, add 250 µL of 60 mg/mL SDS (final 0.03% SDS) (*see Note 6*). Place into a Coplin jar labelled *Fix-1*.
13. Fixing solution-2: 50 mL of 2% PFA solution. Put into a Coplin jar labelled *Fix-2*.
14. 0.05% Hypotonic NaCl solution: to 500 mL of water, add 2.5 g of NaCl and stir on stirring plate until dissolved. Adjust pH to 7.5–8.0 using borate buffer (*see Note 5*).
15. Photo-Flo 200 (Kodak, Rochester, NY).
16. 0.4% Photo-Flo washing solution: to 200 mL of water, add 800 µL of Photo-Flo solution and 200 µL of 0.5% phenol red indicator. Stir and, using pH strips, adjust pH to 7.5–8.0 with borate buffer (about three drops). Aliquot the 200 mL solution in three Coplin jars called *wash-1*, *wash-2*, and *wash-3* (*see Note 5*).
17. ProLong Gold® antifade reagent with DAPI (Molecular Probes).

2.9. Staining**Chromosome Spreads
with SYCP3 and H1t
Antibodies**

1. IgG-free BSA (Jackson ImmunoResearch).
2. Goat serum (has to be from the species in which secondary antibody is produced).
3. 10% Triton X-100 in PBS.
4. Antibody dilution buffer (ADB): 10% goat serum, 3% BSA, 0.05% Triton X-100, in PBS.
5. Humidified chamber. This can be made using a slide box with wet paper towels at the bottom.
6. Primary antibodies (rat anti-SYCP3, rabbit anti-H1t) diluted in ADB (*see Note 7*).
7. Secondary antibodies appropriate to detect the primary antibodies.

**2.10. Immuno-
fluorescence of
Isolated Germ Cell
Fractions**

1. PBS.
2. 4% PFA. Prepare fresh.
3. 0.1% Triton X-100 in PBS.
4. ADB.
5. Primary antibodies (rat anti-SYCP3 and rabbit anti-SAM68) diluted in ADB (*see Note 7*).
6. Secondary antibodies.
7. ProLong Gold® antifade reagent with DAPI (Molecular Probes).

3. Methods**3.1. Elutriator Setting**

The centrifugal force generated by the rotor and counterflow force generated by the pump must be set according to the cell-type that has to be separated. Different settings of rotor speed and pump flow rate have been used for mast cells (10), solid tumour cells (11), or germ cells from mice, hamster (12) and rats (7). Furthermore, different laboratories also used different settings for the same cell type [e.g. *see* (13, 14, 7)]. In our laboratory, in order to isolate mouse meiotic germ cells, we routinely collect five fractions using the elutriator settings described in **Table 18.1**. These settings have been established according to Stokes' law (*see Section 1.1*) and germ cell size (15).

**3.2. Pump Velocity
Setting**

Every time elutriation is performed, before mice are sacrificed, check the pump velocity setting parameters to be sure that under the current environmental conditions, the flow rates are as reported in **Table 18.1** (*see Note 8*).

Table 18.1
Elutriator settings

Fraction	Pump flow rate (mL/min)	Volume (mL)	Rotor speed (rpm)
1	13.5	200	3,000
2	17.9	150	3,000
3	31.3	150	3,000
4	23.2	150	2,000
5	40.0	150	2,000

1. Fill the elutriator with sterile water and start the pump, and then the rotor. Set the latter at 3,000 rpm.
2. Constantly monitor the pressure gauge assembly (**Fig. 18.1**) to be sure that there is not an increase of the system pressure over 5 psi. (*see Note 9*)
3. Once the pressure is stable, set the pump speed so that in one minute you collect 13.5 mL of water (fraction 1) as described in **Table 18.1**. Collect the water in a 100 mL graduated cylinder and measure the collected volume using a serological pipette. Adjust the pump speed to reach the desired flow rate. Repeat this operation for all pump flow-rates indicated in **Table 18.1**. At this point the elutriator is set, and the preparation of testicular cell suspension can be started.

3.3. Testicular Germ Cell Suspension Preparation

1. Sacrifice the animal according to the direction of the ethical commission of your country, and wash the abdomen of the animal with alcohol.
2. Open the abdominal cavity of the mouse, and grabbing the fat attached to the epididymis, pull out the testis from the pelvic cavity.
3. Strip off the albuginea membrane, and put decapsulated testis in a 50 mL falcon tube containing 20 mL of sterile 1X PBS. Use six testes from three adult mice (*see Note 10*).
4. Wash twice with 1X PBS.
5. Pour off PBS from the tube and fill with 24 mL of MEM.
6. Add 1 mL of 25X collagenase and digest for 10 min at 30°C in a water bath under constant shaking (~130 shakes/min). After this first step, the interstitial tissue between the seminiferous tubules is digested, and tubules appear as long filaments floating in the media. If tubules do not appear separated from each other, shake the tube gently by hand a few times. According to the degree of purity of the enzyme,

in some cases the digestion might result too aggressive and a fragmentation of the seminiferous tubules is observed. In this case, to avoid germ cell loss, perform the digestion in EKRB containing 0.3–0.5% BSA (*see* 2.5.8), so that the non-specific digestion activity can be partially neutralized by the BSA. Occasionally, DNA might be released from the cells. In this case add a few drops of DNase I and shake gently.

7. Wash tubules twice with MEM, letting them deposit at the bottom of the falcon tube (under unit gravity force) between each wash.
8. Transfer tubules to a Petri dish and chop them in pieces using a sterile blade (*see Note 2*).
9. Collect tubules using a serological pipette and pipette them up and down to further let germ cells exit out of the tubules.
10. Transfer tubule pieces into a falcon tube containing 23 mL of MEM and add 1 mL of 25X trypsin and 1 mL of 25X DNase I. Digest, under shaking (~130 shakes/min), for 30 min at 30°C (*see Note 11*). As in 3.3.6 this enzymatic digestion can be performed also in EKRB supplemented with 0.3–0.5% BSA.
11. Stop digestion by adding FCS to 10%.
12. Let tissue debris deposit at the bottom of the falcon tube for 5–10 min at room temperature.
13. Collect supernatant and transfer it to a new 50 mL falcon tube.
14. Centrifuge cells for 10 min at 1,200*g* at 4°C.
15. Pour off most of the media and gently tap the bottom of the tube to resuspend the cells.
16. Add 20 mL of EKRB and pipette cells up and down with a serological pipette (*see Note 12*).
17. Count the cells of the suspension using a Thoma's chamber. Be sure not to load into the sample chamber (**Fig. 18.1**) more than 5×10^8 cells, because an excessive amount of cells negatively affects the purity of the germ cell fractions. We typically load $3\text{--}5 \times 10^8$ cells.
18. Put cell suspension on ice.
19. Transfer the appropriate amount of cell suspension into a 30 mL sterile syringe for injection into the sample chamber (**Fig. 18.1**).

3.4. Elutriation

1. At the end of the elutriator setup procedure (**Section 3.2**), remove the sterile water from the elutriator by switching on the pump. Let all fluid come out of the elutriator system (including the sample chamber, **Fig. 18.1**). You can empty the sample chamber by putting it in the vertical position and switching the bypass valve (*see #1 in Fig. 18.1*) so that the liquid in the chamber is pumped out.

2. Fill the entire elutriator system with the EKRB buffer. To fill the sample chamber, put it upside down and switch the bypass valve to the position that allows EKRB flow into the chamber.
3. Start the rotor (3,000 rpm). Similarly to what was described previously, it is important to avoid increases of the pressure system over 5 psi (*see Note 9*).
4. Once the pressure of the system is stable, stop the rotor.
5. Then stop the pump.
6. Once cell suspension is in the 50 mL sterile syringe, restart the pump and then the rotor. Set the pump at the velocity that allows the EKRB to flow out of the system at the velocity of 13.5 mL/min.
7. Switch bypass valve so that the elutriation medium does not flow to the sample chamber and insert the syringe tip containing the cell suspension into the injection valve (*see #2 in Fig. 18.1*).
8. Open the injection valve and inject the cell suspension, watching the pressure gauge, to be sure that the pressure generated by the injection does not cause an excessive increase (over 5 psi) of the pressure in the system. To this end, the cell suspension has to be injected very slowly.
9. Once cell suspension is in the sample chamber, close the injection valve and pull out the syringe.
10. To allow cells to enter into the elutriation chamber, switch the bypass valve.
11. Once cell suspension has completely flowed into the elutriation chamber and the collection chamber is clear of cell suspension, start collecting fraction one. Collect four 50 mL tubes (200 mL total).
12. Collect consecutively fractions 2 (17.9 mL/min velocity flow) and 3 (31.3 mL/min velocity flow), changing the velocity of the pump before starting collection of the cells. For each fraction, collect 150 mL of cell suspension in three 50 mL tubes.
13. Before collecting fraction 4, slow down the pump to 23.2 mL/min and set the rotor at 2,000 rpm. Then, start collecting fraction 4 (three tubes of 50 mL each) (*see Note 13*).
14. Set the pump to 40 mL/min and collect fraction 5 (three tubes of 50 mL each).

At the end of the elutriation process you will have five total fractions (1–5), each of them composed of three or four sub-fractions of 50 mL each. These fractions can be further analyzed for their germ cell content by FACS analyses, Giemsa staining, staining of chromosome spreads and immunofluorescence.

3.5. Techniques for the Evaluation of the Purity of Germ Cell Fractions

The purity of cell fractions obtained by centrifugal elutriation can vary slightly from one experiment to the other. Purity depends on various factors: different efficiency of enzymatic digestion of the testis (*see Note 4*), different operator, different environmental conditions, and intrinsic variability of the machine. As a consequence, before proceeding to the experimental use of the isolated fractions, it is best to monitor the homogeneity of the collected cell fractions. As described in this section, several techniques can be used, each giving slightly different information. The choice depends upon the specific cell type and fraction to be characterized, and the time available for such characterization.

3.5.1. Analysis of DNA Content by FACS

One way to characterize enrichment of germ cell fractions is the analyses of the cells' DNA content. The purity of each 50-mL sub-fraction can be analyzed separately. Typically, most of the cells are eluted in the first two sub-fractions for each fraction, while the third sub-fraction does not usually contain a sufficient amount of cells for FACS analysis. As shown in **Table 18.2**, sub-fractions might be slightly different. In our hands, major differences are often observed among the sub-fractions of fraction 4. For instance, in typical experiments, 2C cells made up $23.8 \pm 14.6\%$ of sub-fraction 4.1 and $42.4 \pm 17.8\%$ of sub-fraction 4.2 (*see Table 18.2*). The FACS profile differences among sub-fractions of fractions 1, 2 and 5 are usually less pronounced. Sample FACS profiles for fraction 3, sub-fraction 4.1 and fraction 5 are shown in **Fig. 18.4A, D, G**, respectively. The limit of this technique is that it is not possible to distinguish between mitotic and meiotic cells, as well as between meiotic subtypes of cells contained in the fractions.

1. Centrifuge germ cell fractions at $1,200g$ for 15 min at 4°C .
2. Pour off the supernatants.
3. Tap the bottom of each tube to suspend the cells in the residual liquid.
4. Resuspend each fraction in 20 mL of sterile $1 \times$ PBS.
5. Count the cells using a Thoma's Chamber.
6. Collect an aliquot of 1×10^6 cells from each fraction, leaving the remainder of the cells on ice.
7. Centrifuge the aliquot of cells at $1,200g$ for 15 min.
8. Wash the pellet once with 1 mL of cold $1 \times$ PBS.
9. Resuspend the cells in 1 mL of 1% PFA in PBS and incubate for 30 min on ice.
10. Pellet fixed cells at $3,000g$ for 15 min.
11. Wash the pellet with 1 mL of cold $1 \times$ PBS.
12. Pellet the cells and resuspend in 300 μL of cold PBS

Table 18.2.
Yields and purity of germ cell fractions from elutriation of testis samples from three 2-month-old CD1 males

Fraction	No. of cells in fraction(millions)	% Haploid cells (1C) ^a	% Diploid cells (2C) ^a	% Tetraploid cells (4C) ^a	Cell types (%)	Cell subtype ^b (%)	Technique ^c
1 + 2	29.4±11.8 (n=3)	97.5	2.4	0.1	33.9±21 elongated spermatids ^d 0.6±0.8 sperm 65.2±22 residual bodies 0.4±0.1 others		3.5.2
3	45.1±8.4 (n=4)	93.6±1.3	4±0.5	2.1±1.2	7.6±5.9 primary spermatocytes 3.8±0.9 secondary spermatocytes 71.6±3.7 round spermatids ^e 9.3±6.9 elongated spermatids 4.2±1.5 others		3.5.3
4.1	15.4±8.5 (n=3)	62.1±23	23.8±14.6	13.8±8.7	22±3.7 primary spermatocytes 12.9±5.8 secondary spermatocytes 51.3±6 round spermatids 6.1±5.2 elongated spermatids 7.6±1.2 others	L: 9.2 Z: 36.2 P: 53.8 Di: 0.8	3.5.3 3.5.4 3.5.5

(continued)

Table 18.2.(continued)

Fraction	No. of cells in fraction(millions) (n=3)	% Haploid cells (1C) ^a	% Diploid cells (2C) ^a	% Tetraploid cells (4C) ^a	Cell types (%)	Cell subtype ^b (%)	Technique ^c
4.2	3.7±2.3	29.8±18	42.4±17.8	27.8±0.3	29.3±2.4 primary spermatocytes 13.5±1.6 secondary spermatocytes 44.9±11.5 round spermatids 0.7±0.9 elongated spermatids 11.6±4.1 others	L: 2.5 Z: 6.7 P: 89.1 Di: 1.7 7.9±6.9 others	3.5.3 3.5.4 3.5.5
5	1.3±3.6 (n=5)	0.9±0.4	3.7±2	95.3±2.3	79.3±14 primary spermatocytes ^f 1.9±1.3 secondary spermatocytes 8.0±6.2 round spermatids 1.8±1.7 elongated spermatids 7.9±6.9 others	L/Z: 4.8 P: 72.9 Di: 20.3 Diak: 0.8 MI: 0.8	3.5.3 3.5.4

^aDNA content as measured by FACS.

^bSubtypes of the primary spermatocyte population; L = leptronema, Z = zygonema, P = pachynema, Di = diplonema, Diak = diakinesis, MI = metaphase I.

^cThis column indicates the subheading(s) that describes techniques used for identification of cell types and subtypes.

^dAbout 99% of DAPI-positive cells of fractions 1 and 2 are elongated spermatids.

^eA higher enrichment (~80%) of round spermatids can be obtained by excluding sub-fraction 3.1.

^fThe primary spermatocyte content can be enriched up to 89.5% in sub-fraction 5.2.

13. Transfer the suspension into a 1.5 mL tube containing 700 μ L of 90% ethanol.
14. Store cells overnight at 4°C (*see Note 14*).
15. On the next day, pellet the cells at 3,000*g* for 15 min.
16. Resuspend (wash) the pellet in 1 mL of cold PBS.
17. Pellet the cells and resuspend in 200 μ L of PBS.
18. Add 200 μ L of RNase solution and incubate at 37°C for 20 min.
19. Add 1 μ L of PI solution and incubate at 37°C for 15 min in the dark.
20. Spin down cells for 10 min at 1,800–2,000*g*.
21. Resuspend in 100 μ L of cold PBS.
22. Immediately analyze by FACS.

3.5.2. DAPI Staining of Nuclei

A more accurate way to characterize germ cell fractions is to stain with DAPI. This is a very simple and fast technique (it takes about 10 min) that allows one to examine the morphology of the DAPI-stained nuclei, providing a rapid evaluation of the purity of germ cell fractions. **Figure 18.3** show the example of fraction 2 stained by DAPI, and observed by fluorescence merged with a phase contrast. Elongated spermatids at different stages of maturation are visible. These cells vary with respect to shape of the nuclei and stage of cell maturation, including the extent of sperm tail formation and elimination of residual cytoplasm (16). Furthermore, as observed by phase contrast, residual bodies are a consistent component of cells in this fraction (16). However, no spermatocytes or round spermatids are visible, indicating that, most of the haploid calls identified by FACS analyses of fractions 1 and 2, are elongated spermatides. **Figure 18.4C, F** show examples of fractions 3 and 5, respectively. Fraction 3 is strongly enriched for round spermatids, which appear as cells with small nuclei and one or two chromocentres (16). In contrast to spermatids, DAPI-stained primary spermatocytes in fraction 5 appear as cells with bigger nuclei and less condensed chromatin (*see Fig. 18.4F* in the color version of this figure, provided on the companion CD for this volume). Sometimes the sex chromatin is also visible, appearing as a dense body at the edge of the nucleus (not shown). As also revealed by FACS analyses (*see Section 3.5.1*) the evaluation of fraction 4 by DAPI staining reveals that it is not a homogeneous fraction, containing both round spermatids and spermatocytes (not shown). Using these morphological criteria, it is possible to quantify the enrichment of the isolated fractions for each cell type described.

1. At the end of the elutriation process, collect 2 mL of each fraction to be analyzed and spin it down for 10 min at 1,200*g*.
2. Resuspend (wash) the cells with cold 1 × PBS.

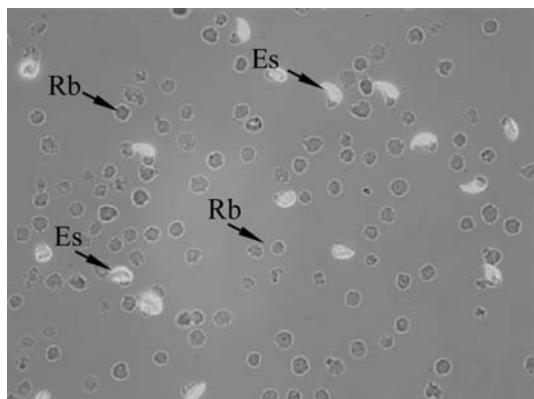


Fig. 18.3. Fraction 2 is enriched for residual bodies and elongated spermatids. Cells collected from fraction 2 were stained with DAPI (light grey), and analyzed by fluorescence/phase-contrast microscopy. Elongated spermatids (Es) appear as DAPI-positive cells with a sperm-like head without a sperm tail. This cell type represents >90% of the DAPI-positive cells in fraction 2 with no contamination from sperm. Sperm contamination (2.2%) is instead present in fraction 1. Residual bodies (Rb), are DAPI-negative bodies that represent residual cytoplasm released from the elongating spermatids during the spermiogenesis process. Rb are the main component of fraction 2 ($81\% \pm 9.7$), whereas they represent $49.5\% \pm 7.3$ of fraction 1. *A colour version of this figure is provided on the companion CD for this volume.*

3. Pellet the cells at $1,200g$ for 10 min.
4. Repeat step 2 and 3 once more.
5. Resuspend the cells in 100 μ L of $1 \times$ PBS
6. Spot cells on superfrost slides coated with poly-L-lysine. Let cells settle down on the slide for 10 min at room temperature.
7. Fix with 4% PFA for 8–10 min.
8. Wash twice for 5 min with $1 \times$ PBS.
9. Put a drop of ProLong Gold® antifade reagent with DAPI on top of the cells and apply a glass coverslip.
10. Let the slides dry overnight at room temperature.
11. Examine in a fluorescence microscope equipped with phase contrast.

3.5.3. Squash of Isolated Meiotic Cells and Giemsa Staining

The analyses of cell morphology by squash and Giemsa staining has the advantage, compared to DAPI staining of the nuclei, of permitting a more precise identification of the cell types in the fractions. For example, as indicated in **Fig. 18.4E**, this method easily allows the identification of secondary spermatocytes that are less identifiable by DAPI staining. Nevertheless it needs great time to be completed (about 2 h).

1. Collect $0.5\text{--}1 \times 10^6$ cells and centrifuge at $1,200g$ for 15 min at 4°C .
2. Resuspend cells in 1 mL of hypotonic solution.

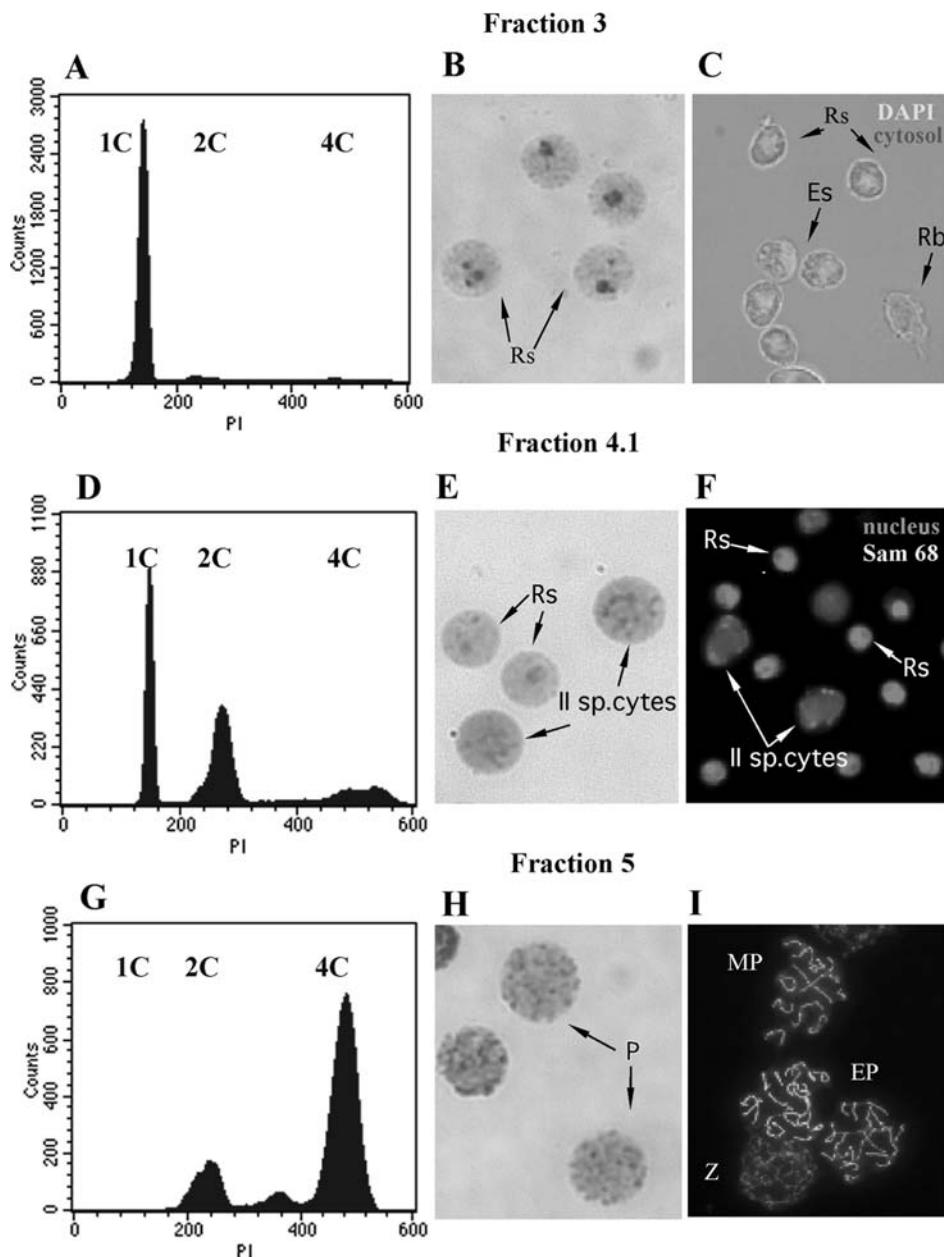


Fig. 18.4 (A–C) Analysis of fraction 3. As determined by FACS (panel A), fraction 3 is mainly composed of haploid (1C) cells. Cells from fraction 3 were squashed and stained by Giemsa (panel B) or with DAPI (white in panel C) and analyzed either by light microscopy or fluorescence/phase contrast microscopy. As shown, most of the cells in this fraction are round spermatids (Rs). Rb = residual body, Es = elongated spermatides. (D–F) Analysis of sub-fraction 4.1. Sub-fraction 4.1 contains a mixed population of 1C, 2C and 4C cells by FACS (panel D). As determined by Giemsa staining (panel E), this sub-fraction contains mostly 1C round spermatids (Rs) and 2C cells, including secondary spermatocytes (II sp.cytess). Staining (panel F) with anti-Sam68 (white), and DAPI (grey) revealed that most of the 1C cells are round spermatids, which contain Sam68 in the nucleus, while about 50% of 2C cells are secondary spermatocytes, which contain Sam68 in the cytoplasm. Some primary spermatocytes are also present. (G–I) Analysis of fraction 5. By FACS (panel G), the majority of cells in this fraction are 4C cells. Judging by the size of the cells and the appearance of the chromatin-following squash preparation and Giemsa staining (panel H), most of the cells are at pachytene/diplotene stages. Analysis

3. Incubate for 15–20 min at 37°C.
4. Resuspend the cells and fix them by adding 500 µL of methanol:acetic acid solution (3:1).
5. Resuspend by vortexing and incubate at 4°C for 1 h.
6. Centrifuge cells at 4°C for 10 min at 5,000g.
7. Wash pellets four times with 2 mL of methanol:acetic acid solution (3:1). Resuspend the pellet with each wash.
8. After the last wash, resuspend pellets in 200–500 µL of methanol:acetic acid solution (3:1).
9. Drop the cells from a height of 10–15 cm onto glass slides to allow spreading of the nuclei.
10. Stain slides with Giemsa solution for about 5 min.
11. Wash slides in washing buffer.
12. Dry the slides at room temperature.
13. Mount with glass coverslip using a histology mounting medium.
14. Observe nuclei by light microscopy.

3.5.4. Preparation of Chromosome Spreads and Staining with Anti-SYCP3 and Anti-Histone H1t

The morphological analyses of mouse meiotic germ cells by Giemsa staining is a valid method to distinguish between primary and secondary spermatocytes and round spermatids. However, it does not allow one to distinguish among pachytene spermatocytes at different sub-stages of prophase I. As indicated in **Table 18.2**, fraction 5 is enriched for primary spermatocytes. A detailed sub-stage classification of these cells can be done by staining chromosome spreads with antibodies against the synaptonemal complex (SC) protein 3 (SYCP3) and the testis-specific histone H1t. The SYCP3 protein is a component of the axial/lateral element of the SC. This protein first appears in leptotene as short stretches of SYCP3, while at pachynema it forms the lateral elements of the SC (17, 18). The histone H1t is a well-established marker that appears in prophase I from mid-pachytene onward, up to the round-spermatid stage (17, 19). The SYCP3 staining allows one to distinguish between leptotene, zygotene, pachytene, and diplotene cells, while the H1t marker allows one to identify the mid-late pachytene sub-population. To better characterize the spermatocyte cell

Fig. 18.4 (continued) of chromosome spreads stained with anti-Histone H1t, anti-SYCP3, and DAPI (panel I) confirmed that most of the 4C cells are primary spermatocytes at early pachynema (EP, with fully synapsed chromosomes but no H1t staining), mid pachynema (MP, with fully synapsed chromosomes plus H1t staining) or diplonema (not shown, with de-synapsing chromosomes and strong H1t staining), with a small contamination with primary spermatocytes at zygonema (Z, with partially synapsed chromosomes and no H1t staining). A colour version of this figure showing the SYCP3, DAPI, H1t and Sam68 staining of panels F and I, is provided on the companion CD of this volume.

composition of fraction 5, we collect the first two sub fractions (50 mL each) and analyze them separately. Sub-fractions 5.1 and 5.2 contains about 40% and 30% of cells at early-pachynema and mid-pachynema respectively. However, while sub-fraction 5.2 contains about 20% of cells at diplonema, these cells are rare in sub-fraction 5.1 (about 6%).

Chromosome spreads are performed as in (20) with some modifications (21):

1. Before spreading the testicular cells, clean the 6-mm-well glass slides with window cleaner (e.g. Windex or Vetril) using a tissue paper and rinse three times in water.
2. Wipe dry the slides with tissue paper.
3. Collect $0.5\text{--}1 \times 10^6$ cells from each fraction.
4. Wash cells twice with 500 μL of 1X PBS. Resuspend the pellet for each wash.
5. Resuspend cells in 200 μL of hypotonic solution.
6. Leave on ice for 5–10 min.
7. Place a 25 μL drop of hypotonic-treated cells on each 6 mm well of the glass slide.
8. Let nuclei deposit at the bottom of the well for 10 min at room temperature.
9. Immerse slides in the *Fix-1* Coplin jar and let cells fix for 3 min.
10. Transfer slides to the *Fix-2* Coplin jar and leave cells to fix for an additional 3 min.
11. Move slides to the *wash-1* jar and let slides wash for 1 min.
12. Repeat the same procedure with the *wash-2* and *wash-3* jars.
13. At the end of the last incubation, remove the excess of liquid by touching slides onto a paper towel, and let slides dry at room temperature for 10 min.
14. Use the slides immediately for immunostaining or store dry at -80°C for up to two weeks in a slide box.
15. Spot 25 μL of diluted primary antibodies (anti-SYCP3 and anti-H1t) on each well of the 6-mm glass slides containing the chromosome spreads.
16. Incubate slides at 37°C overnight in a humidified chamber.
17. The following day, wash slides in a 50 mL Coplin jar containing slide washing buffer-1 for 5 min (*see Note 15*).
18. Wash slides for an additional 5 min in slide washing buffer-2 (*see Note 15*).
19. Following this last wash, incubate slides with secondary antibodies diluted in ADB buffer solution, for 1 h at 37°C in the dark.

20. At the end of the incubation, wash slides for 5 min in slide washing buffer-1, and for an additional 5 min in slide washing buffer-2 (*see Note 15*) in the dark (*see Note 17*).
21. Dry the slides in the dark and mount them with a coverslip, using one to two drops of mounting medium.
22. Let mounted slides dry out overnight, at room temperature, in the dark in a slide holder.
23. Next day, analyze slides with a fluorescence microscope.

**3.5.5. Immunofluorescence
Staining with Anti-SYCP3
and Anti-SAM68**

SAM68 is an RNA binding protein expressed in the testis, which appears in the nucleus of pachytene spermatocytes, re-localizes to the cytoplasm of secondary spermatocytes, and shuttles back into the nucleus in round spermatids (22). Using the immunofluorescence technique described above (Section 3.5.4), we successfully used this marker to easily identify secondary spermatocytes contained in fraction four (*see Fig. 18.4F* on the color version provided on the companion CD).

1. At the end of the elutriation process, collect 2 mL of each fraction to be analyzed and spin down for 10 min at 1,200*g*.
2. Wash cells twice with cold 1 × PBS. Resuspend and pellet the cells for each wash.
3. Spot cells on superfrost slides coated with poly-L-lysine.
4. Let cells settle down for ten min at room temperature.
5. Fix with 4% PFA for 8–10 min.
6. Wash twice for 5 min with 1 × PBS.
7. Incubate cells for 1 h at room temperature with ADB buffer.
8. Incubate with primary antibodies overnight at 4°C.
9. Wash twice in 1 × PBS, followed by three 10-min washes in 1 × PBS.
10. Incubate with secondary antibodies for 1 h at room temperature.
11. Wash twice in 1 × PBS, followed by three 10-min washes in 1 × PBS.
12. Mount slides with mounting medium.

4. Notes



1. Currently the JE-6B elutriation system is no longer available from Beckman Coulter, and has been substituted by the JE-5.0 system with standard chamber. On the latter, the distance of the standard elutriation chamber inlet, and elutriation boundary, from the axis of rotation are identical to the

standard chamber previously used in the JE-6B model. However, the standard chamber capacity of the JE-5.0 model is slightly less than the standard chamber mounted on the JE-6B model (4 mL vs. 4.2 mL). Using the experimental settings described in this chapter, we found no significant differences between the two elutriator systems.

2. This step is optional, but it increases the amount of germ cells in the suspension.
3. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ·cm. This standard is referred to as “water” in the text.
4. Prepare a 25 × stock(s) of the enzyme(s) in MEM, and store them in 1 mL aliquots at –20°C. Alternatively, enzymes can be made fresh each time, which guarantees high-quality tissue digestion. To prepare the working solutions, add 1 mL of each enzyme to a 25 mL final volume of MEM and store on ice before use.
5. Solution is made fresh each time.
6. Depending on the degree of the chromatin dispersion desired, 0–0.06% SDS in PFA is used. The more SDS used, the greater the spreading. For rat and mouse, 0.03% SDS in the first fixing solution is generally used. For hamster, use 0.015%. For humans, use 0.06% (20).
7. Rat anti-SYCP3 (1:500) was a gift from Dr. Mary Ann Handel (Jackson laboratories). Rabbit anti-H1t (1:300) was a gift from Dr. Peter Moens (York University). Rabbit anti-Sam68 (1:400) was from Santa Cruz Biotechnology. All primary and secondary antibodies should be diluted in ADB buffer.
8. Performing an elutriator setting every time you start an elutriation is good practice to get reproducible results. The external temperature, in fact, can influence the variation of the diameter of the Masterflex tubing, causing slight variations of the pump velocity settings from one experiment to the other.
9. If a pressure increase occurs, stop the centrifuge (not the pump) until the rotor stops, and then re-start it. To prevent a system pressure increase, it is important that no trapped bubbles are present in the system. Therefore, it is also good practice to open and close the bypass valve (#1 in Fig. 18.1) when you re-start the rotor, to allow trapped bubbles to flow out of the system. If a bubble goes into the collection chamber, it creates resistance to free liquid flow, causing an increase of the system pressure. Under this condition, stop and re-start the rotor. When no bubbles are present in the system, the pressure gauge should point to 0.
10. If you are planning to put isolated cells in culture, the testis collection and enzymatic digestion of the tissue has to be performed under sterile conditions.

11. While trypsin digestion is ongoing, you can fill the elutriator with the EKRB elutriation buffer, as described in **Section 3.4**, steps 1–5.
12. If there is undigested, floating material still present in the suspension, add a few drops of DNase I solution and gently agitate the tube. Often, DNA that comes out of broken cells causes formation of cell aggregates that disappear after this additional DNA digestion.
13. Stop the pump only after the rotor has stopped.
14. If you are not processing the cells on the next day, you can store them at –20°C up to one month before processing.
15. To get the best result, put a stirring bar into the Coplin jar and stir at moderate velocity.
16. The secondary antibodies used in this work were from Jackson ImmunoResearch.
17. To cover the slides while they are washing, you can use a plastic beaker covered with aluminium foil, and put it upside down over the Coplin jar.
18. This technique has also the advantage that it requires a very small amount of cells, and it is thus particularly indicated in cases in which the amount of cells is a limiting factor.

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Chapter 19

Electron Microscopy of Mammalian Chromosomes

Peter Moens

Abstract

This chapter describes methods of preparation of meiotic nuclei for their analysis with electron microscopy. Procedures include surface spreading as well as fixation, staining, and sectioning of tissue and subsequent 3-D reconstruction analysis. Immunostaining protocols for electron microscopy are provided. The rationale for using electron microscopy in place of light or fluorescent microscopy for specific cytological analyses is presented.

Key words: Electron microscopy, immunoelectron microscopy, shadow casting, serial sectioning, 3-D reconstruction.

1. Introduction

In 1965, John and Lewis reviewed aspects of meiotic chromosome behavior in exhaustive detail from a light microscope perspective (1). With the commercial development of the electron microscope (EM), new perspectives emerged from observations by Fawcett (2) and Moses (3, 4). It was discovered that nuclei in prophase of meiosis of a large number of organisms had an unusual chromosome structure. Moses proposed that the axial complex between paired homologous chromosomes consists of two parallel-aligned lateral elements that are the cores of the paired chromosomes and a central element that lies between the lateral elements. He named the axial complex the synaptonemal complex (later renamed as the synaptonemal complex, SC). In keeping with this nomenclature, the term “core” is used here to describe the structural component of the unpaired chromosome and “SC” refers to the axial complex

Peter Moens is deceased

of the parallel-aligned cores, usually found between synapsed homologous chromosomes. The term “axial elements” referring to the cores of unpaired chromosomes is of uncertain origin and somewhat ambiguous since it may refer to the cores or the SCs.

From the time of their earliest discovery, the SCs have been implicated in the processes of synapsis and crossing over. Meyer (5) gave the initial evidence in support of recombinant functions in his 1964 report of *Drosophila melanogaster* spermatocytes and mutant *c(3)G* oocytes that fail to form SCs and are coordinately incapable of crossing over. Much later, it was found that C3G protein is a central element component of the SC (6–8). The relationship between SCs and crossing over was further sustained by the discovery that EM-defined, SC-associated dense nodules are distributed in accordance with crossover distribution (9). The coincident positions of SC-associated recombination nodules, RNs, with localized crossing over in *Allium* species (10), and in grasshopper species, provide evidence for roles of RNs and SCs in chiasma formation. Generally, in many organisms, SCs play significant roles in chromosome synapsis, recombination, and segregation. von Wettstein et al. (11) summarized in 1984 the explosion of new information on SCs in the 1960’s and 1970’s. Presently, ultrastructure as such plays a less prominent role but it has become a significant method for the detection and localization of functional elements during prophase of meiosis.

2. Materials

2.1. Preparation of Plastic-Coated Slides/ Grids

1. Microscope slides
2. Glass cleaner (e.g., Windex or Sparkleen)
3. Kimwipes or other lint-free tissue
4. 0.55% Petri dish plastic dissolved in chloroform. Store in a clean dry glass bottle with a glass stopper.
5. Masking tape
6. Evaporating dish
7. Parafilm
8. Tweezers
9. Grids

2.2. Fixation and Sectioning

1. *s*-Collidine buffer: Make stock solution with 2.67 mL pure *s*-collidine and distilled water to make 50 mL. Make 2 × strength (0.2 M) *s*-collidine buffer with 50 mL *s*-collidine stock solution, approximately 9 mL 1.0 N HCl for pH 7.4, and distilled water to make 100 mL. The 0.2 M stock solution is stored at 4°C and used at 0.1 M working concentration.

2. Glutaraldehyde: 2–5% in *s*-collidine buffer
3. 1% osmium tetroxide in *s*-collidine buffer. Mix approximately 400 μ L of 4% osmium tetroxide plus approximately 800 μ L of 0.2 M *s*-collidine buffer and 400 μ L distilled water. Make fresh each time.
4. Graded alcohol series: 30%, 50%, 80%, and 100% ethanol
5. Propylene oxide
6. Freshly made Epon or other plastic embedding medium
7. Ultramicrotome
8. Glass or diamond knife
9. 100–200 mesh copper or nickel grids
10. Single-hole EM grids
11. Plastic-coated grids
12. Parafilm
13. Electric glow carbon rods for evaporator (optional)

2.3. Surface Spreading

1. Plastic-coated glass slides
2. 1% (w/v) phenol red in water. Filter and store in a glass bottle
3. MEM (with Hanks salts, without L-glutamine), no more than 100 mL
4. 0.05 M Sodium borate buffer in distilled water (stock). Adjust to pH 9.2 with 0.5 N NaOH. Dilute 1:4 to use.
5. Hypotonic 100 mM NaCl, pH 7.5–8.0 with borate buffer. No more than 100 mL in a squeeze bottle is needed.
6. Phosphate-Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄. Adjust to pH 7.4 with HCl. Approximately 2 L are needed.
7. 500 mL PBS with 2 mL Kodak Photo-Flo 100 in a squeeze bottle
8. Paraformaldehyde: 100 mL 2% (w/v) paraformaldehyde in distilled water. Add one drop (about 40 μ L) 1 N NaOH and 100 μ L filtered 1% phenol red. Heat to 60°C until dissolved. Cool and filter. Adjust pH to 7.5–8.0 with borate buffer. Use with or without 0.03% (w/v) SDS in water.
9. 0.4% (v/v) Kodak Photo-Flo 200 in water
10. Filter paper
11. Lens paper
12. Dental wax
13. Flat-nosed tweezers
14. Coplin jars: 5 × 50 mL
15. Transfer pipettes and Eppendorf tubes
16. Nail polish

2.4. Silver Nitrate Visualization

1. 45% (w/v) silver nitrate in water
2. 70 mesh nylon membrane
3. Silver enhancement if required (e.g.: IntenSE®M silver enhancement kit, GE Healthcare).

2.5. Immunogold Electron Microscopy

1. 20–50 μ L of primary antibody
2. Plastic-coated slides
3. Cover slips (22 \times 60 mm or 22 \times 22 mm) or Parafilm
4. Gold, nickel, or copper grids
5. 10% (w/v) sodium azide in water
6. 0.05 M sodium borate buffer
7. 0.4% (v/v) Kodak Photo-Flo 200 in water with about 50 μ L 1% phenol red, pH 7.5–8.0 with borate buffer
8. PBS with 0.4% Kodak Photo-Flo 200, about 300 mL
9. ADB (antibody dilution buffer): 10% (v/v) goat serum, 3% (w/v) BSA, 0.1% (v/v) Triton X-100 in PBS. Make in batches and freeze in aliquots of 50 mL.
10. Blocking buffer: 10% ADB in PBS
11. Secondary antibody conjugated to 5, 10, or 15 nm gold
12. Distilled water with 0.4% Kodak Photo-Flo 200, pH 7.4 (adjust pH with borate buffer)
13. 1 mg/mL DNase I in MEM or PBS buffer. Freeze in 50 μ L aliquots. For working strength, use 50 μ L in 50 mL MEM.
14. 0.4% Osmium tetroxide. *Extreme caution: osmium vapor can fix the cornea of the eyes. Work in a fume hood.*

2.6. Shadow Casting

1. Palladium-gold wire I
2. Evacuator

3. Methods

3.1. Preparing Plastic-Coated Grids/Slides

1. Preparing the slides. To make plastic-coated slides, wash glass microscope slides with a commercial glass cleaner such as Windex or in a soap solution such as Sparkleen or Liquinox. Rinse very thoroughly in distilled water and wipe dry with a lint-free tissue such as a Kimwipe (*see Note 1*).
2. Adding the plastic coat. Working in a fume hood, tape a piece of masking tape to the dry, frosted end of the cleaned slide and dip the slide in a glass-stoppered glass jar containing 0.55%

Petri dish plastic dissolved in chloroform (*see Note 2*). Make sure the tape does not touch the liquid or it will detach from the slide. Withdraw the slide from the liquid and tape the slide to the jar such that the slide is above the liquid line but is still contained within the jar. Replace the cap of the jar and leave the slide to drain in the chloroform fumes for about 15–20 s. Place the slide on a slide rack to air dry. The plastic film should have a silvery interference color (*see Note 3*). To avoid the plastic film coming loose during staining and washing, put a strip of nail polish along the edges and across the slide to form compartments.

3. Removing the film from the slide. After the nuclei have been attached to the plastic film and have been processed (*see Section 3.3*), the film can be removed by scoring around the edges of the slide with a clean, pointed object. Fill a clean container such as an evaporating dish to the very top with distilled water. Wipe the surface with lens paper and touch the edge of the slide to the side of the water-filled container with the tip of the slide touching the water surface. Keep the slide in this position for a few seconds until the water surface tension starts to lift the film off the slide. Slowly push the slide further into the water such that the film floats off onto the water surface.
4. Placing the EM grids. EM grids can be placed directly onto the floating plastic film in the area of interest. Alternatively, the slide can be scanned with the light microscope before detaching the film, and the grids can be placed on the desired nuclei. The film is then floated off as in step 3, taking care not to disturb the grids. In either case, carefully lower a piece of Parafilm the size of a glass slide onto the floating grid-coated film, avoiding air bubbles. Leave for a minute or two before lifting first the edge of the Parafilm and subsequently the entire strip. Air dry and remove the grids with tweezers and store in a grid box.
5. Making plastic-coated grids. If desired, plastic coating without cells can be applied to grids in the above manner that are then stored in a grid box for future use. Nuclei or other material can be adhered to the plastic at a future date.
6. Stabilizing the film. For extra stable films, coat the grids by electric glow discharge carbon rods in a vacuum.

3.2. Sectioning

3.2.1. Fixation

1. For most purposes, small pieces of tissue (1–2 mm cubes or pellets) can be fixed in 2–5% gluteraldehyde in phosphate buffer for 1 h at room temperature.
2. For mammalian testes or ovaries, incubation in 0.1 M *s*-collidine buffer, pH 7.4, for 30–45 min with 5% gluteraldehyde gives good fixation.

3. If cellular organization within the seminiferous tubule is to be undisturbed, it is best to infuse the intact testis with the fixative for 30–45 min.
4. Release the tubules and fix for an additional 30 min.
5. Wash 3 × 15 min in 0.1 M *s*-collidine buffer.
6. Post-fix in freshly made 1% osmium tetroxide in 0.1 M *s*-collidine buffer for 1 h.
7. After rinsing in water, dehydrate in a graded alcohol series (15 min each of 30, 50, 80, and 100% ethanol). Leave in 100% ethanol for 1 h or overnight.
8. In a fume hood, incubate for 1 h in 1:1 100% ethanol and propylene oxide followed by 1 h in pure propylene oxide in a microtube.
9. Place the samples in 1:1 propylene oxide and freshly made Epon for 1 h. Poke a few holes in the microtube cap to allow gradual evaporation of the propylene oxide.
10. Remove the tissue from the Epon mix and blot it on a paper towel. Embed the tissue in freshly made Epon in a silicone mold or BEEM capsule. Polymerize for 48 h at 60°C.
11. Clean any spilled Epon by wiping with acetone. Allow surplus Epon to polymerize before discarding.

3.2.2. Sectioning and Collection on Grids

1. Cut thin sections on an ultramicrotome and float on the water surface behind the glass or diamond knife (*see Note 4*).
2. Collect sections on single-hole grids or 100–200 mesh copper grids.
3. Collect consecutive serial sections from the water surface by placing a single-hole, uncoated EM grid over the ribbon of sections. Remove the grid from the water bath.
4. Transfer the sections by placing a plastic-coated grid below the single-hole grid carrying the sections and draw the water from between the two grids with a triangle of filter paper (**Fig. 19.1A,B**).

3.2.3. Imaging and Analysis

1. Images can be obtained from single sections or from a series of sections.
2. The SC contents of a nucleus can be reconstructed from serial electron micrographs in a two-dimensional image.
3. Alternatively, the consecutive micrograph images can be entered as the Z planes in a computerized reconstruction program such as the free software NIH Image to produce a three-dimensional image that can be rotated in any direction to observe the organization of the SCs within the nucleus. A stereoscopic image of the nucleus can also be produced (12).

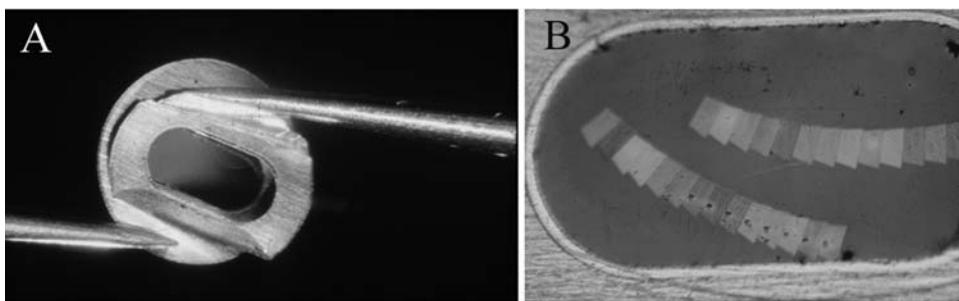


Fig. 19.1. (A) The transfer of the sectioned material from the single-holed grid to the plastic-coated grid. (B) Two ribbons of serial sections on a water bath.

3.3. Surface Spreading

1. After carefully removing all fat from the testis, prepare a testicular cell suspension by isolating the testes of the animal in MEM. Remove the tunica and rinse off the tubules with MEM (*see Note 5*). Cut the tubules with a new, grease-free razor blade on a piece of clean dental wax and squeeze with flat-tipped tweezers to expel the cells. Collect the suspension in an Eppendorf with 1.5 mL MEM and gently draw up and down 10–20 times through a wide-mouthing plastic transfer pipette. Allow the tubules to settle and collect the supernatant. Spin briefly at a low speed ($100g$ 3 min). Remove the clear supernatant and suspend the cells in the remaining liquid.
2. Fill a Petri dish to the brim with hypotonic 100 mM NaCl such that a convex surface is formed. Touch a 3–4 μL drop of testicular cell suspension to the convex water surface. The contents of the drop will be drawn across the water surface dispersing most of the cytoplasm. Gently lower a plastic-coated glass slide onto the water surface, leave it for about 10 s, then slowly roll the slide off the water bath.
3. Fix the nuclei that are now attached to the slide by immersing the slide for 3 min in 50 mL of 2% paraformaldehyde, pH 7.5–8.0, with or without 0.03% SDS, depending on the species. Move the slide to a fresh Coplin jar with 2% paraformaldehyde, pH 7.5–8.0, for an additional 3 min.
4. Wash three times for 1 min each in 0.4% Kodak Photo-Flo 200, then air-dry the slides. This causes the nuclei to break open and the contents to spread out.
5. For some applications such as immunogold electron microscopy, it can be useful to reduce the amount of chromatin by 10-min digestion with 1 $\mu\text{g}/\text{mL}$ DNase I in MEM or PBS buffer with magnesium ions.
6. To prevent accidental loss of the plastic film in subsequent treatments, fine tracks of nail polish can be applied to the slide along the edges and across the slide (Fig. 19.2).

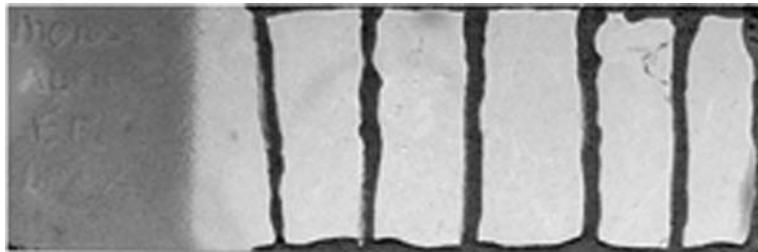


Fig. 19.2. A plastic-coated slide with nail polish strips to prevent accidental loss of the plastic film.

3.4. Immunogold Electron Microscopy

3.4.1. Preparation

1. Wash the slides 10 min in PBS with 0.4% PhotoFlo, 10 min in 0.02% Triton X100 and 10 min in blocking buffer. Place a stir bar in each Coplin jar and stir at slow speed during the washes.
2. Add 50 μ L or more of primary antibody (usually 10-fold more concentrated than would be used for fluorescence microscopy) to the entire plastic-coated slide carrying surface-spread nuclei. Cover with a cover slip or Parafilm. Alternatively, drops of antibody can be placed on individual gold or nickel grids that have nuclei on plastic film. For electron microscopy, different antigens can be visualized simultaneously. For example, the primary antibodies human anti-centromere, rabbit anti-MLH3, and mouse anti-MLH1, when applied together can later be detected with secondary goat anti-human antibody conjugated to 15 nm gold particles, goat anti-rabbit MLH3 antibody conjugated to 10 nm gold particles and goat anti-mouse MLH1 antibody conjugated with 5 nm gold particles (*see Fig. 19.3*).

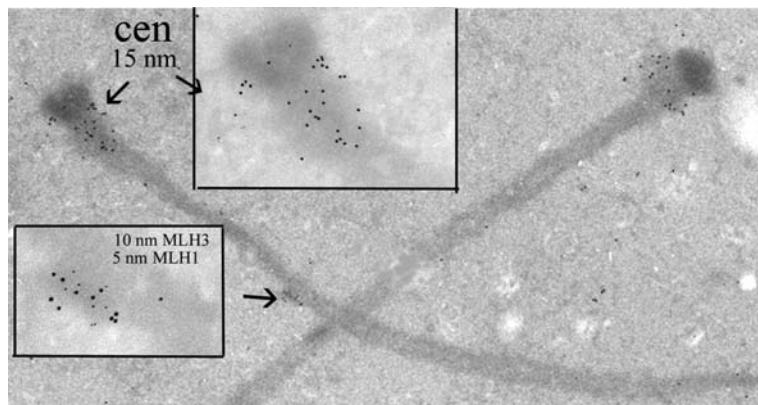


Fig. 19.3. Different sized gold particles conjugated to secondary antibodies identify specific antigens. The accuracy of EM localization is far greater than that of immunofluorescence, which can give a false impression of co-localization of proteins on a structure.

3. Incubate approximately 3 h at room temperature. Exposure times for slides or grids need not be long because the spread nuclei are on the surface. If longer incubations are performed (e.g., overnight), add 2 μ L of 10% sodium azide per mL of diluted antibody to prevent bacterial growth.
4. Repeat wash steps as described in step 1.
5. Add secondary antibody to the grids or slides and incubate for 1 h at room temperature or at 37°C.
6. Wash for 10 min each in PBS with 0.4% PhotoFlo, PBS with Triton X100, PBS with 0.4% PhotoFlo and then wash for 1 min in water with 0.4% PhotoFlo at pH 7.6. Air-dry the slides. Again, the use of stir bars during washes gives cleaner preparations.
7. Next, float the plastic film off on a water surface and place copper grids on the film as described above (see **Section 3.1**). Lift the film off the water surface with a sheet of Parafilm, air dry, and remove the grids to a grid box. The grids remain usable for years.
8. SCs are visible without staining, but EM visibility can be improved by exposing the grid to osmium tetroxide vapor for 10–30 min in a closed container. Alternatively, spin 100 μ L 0.4% OsO₄ at maximum speed for 2–3 min in an Eppendorf centrifuge. Place a drop on dental wax with 1–2 small slits. Hold the grid in water with 0.4% Photo-Flo and then place upright in the slit in OsO₄ for 10 min. Wash by running water with 0.4% Photo-Flo over the grid. Remove excess liquid with bibulous paper. Air-dry and store in a grid box. *Extreme caution: work in a fume hood so that the osmium vapor does not fix the cornea of the user's eyes.*

3.4.2. Analysis

1. Record the positions of the antigen as marked by the gold grains with a digitizer (**Fig. 19.4**). Summarize these positions for the length of the SC with computed standardized widths and display in graphic form.
2. For epitope mapping, immunogold localization on surface-spread SC gives an accurate and repeatable representation of protein epitope positions. In this analysis, the positions of the gold grains are computer digitized relative to the central and lateral elements and computed for standardized widths of SCs.

3.5. Shadow Casting

Palladium-gold wire is heat-evaporated by electric current in a vacuum at an angle of about 7° to a stationary or rotating platform holding the grids. This can be combined with immunogold-treated preparations.

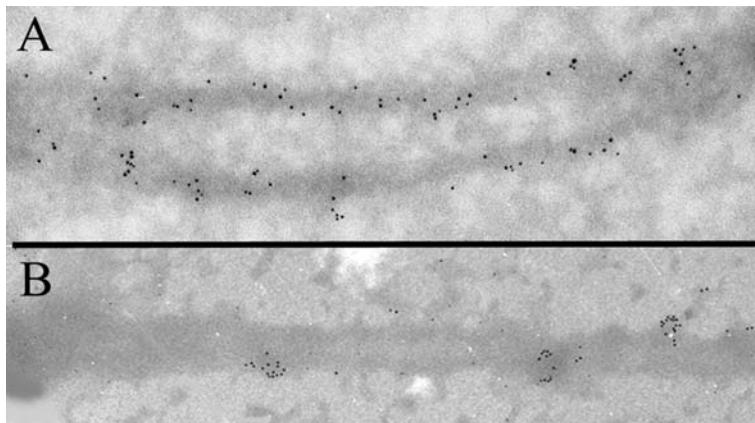


Fig. 19.4. Positions of gold grains on a synaptonemal complex. These can be recorded with a digitizer and graphed to show the relative distribution of specific antigens on the SC.

3.6. Silver Nitrate Visualization

For a quick assessment of chromosome core organization, one can stain surface-spread nuclei on a glass slide with silver, as follows.

1. Place a few drops of 45% silver nitrate onto surface-spread nuclei on a glass slide (*see Note 6*).
2. Cover the slide with a piece of 70-mesh nylon membrane and incubate at about 60°C until the cells turn pale brown in color. The cores and SCs can be seen with light or electron microscopy (Fig. 19.5A,B) (*see Note 7*).
3. If necessary, silver or gold grains can be enlarged with silver enhancement with a product such as IntenSE®M silver enhancement kit (GE Healthcare). With enhancement, one can use antibodies conjugated to very small gold grains (1 nm) which minimizes steric hindrance (Fig. 19.5C) (8).

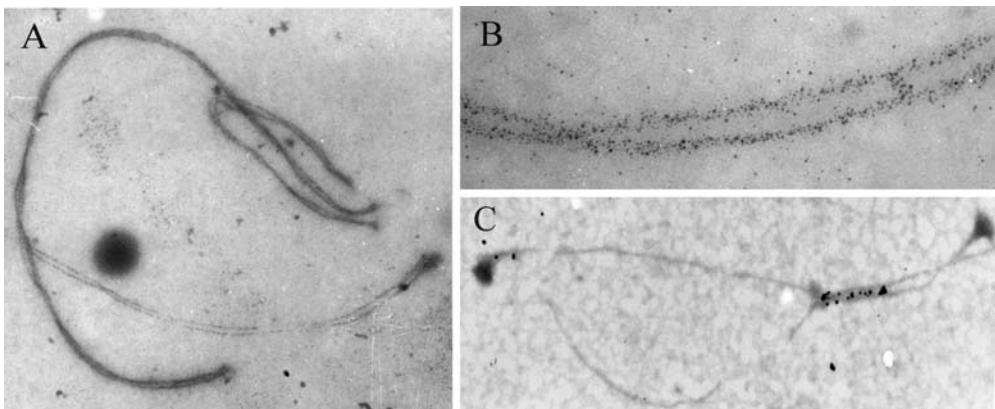


Fig. 19.5. (A, B) Silver stained whole-mount synaptonemal complexes and XY cores with silver-enhanced gold grains.

4. General Comments

The ultrastructure of meiotic chromosome components can be observed by electron microscopy of thin sections, by shadow casting (Fig. 19.6) and by whole-mount preparations (see Note 8). Resolution of EM is usually much greater than can be obtained from light or fluorescence microscopy. The trade-off is that fewer items can be examined within reasonable time limits. Visualization of EM images can be improved by various heavy metal stains and by immunogold labeling with antibodies. Depending on the technique, different aspects of chromosome components are evident.



Fig. 19.6. The image of a shadow casted SC is quite different from that seen with heavy metal staining. Instead of two-dimensional, sharply defined, thin cores, the cores/lateral elements are 80 nm wide tubular structures with a pronounced 40 nm wide central element (CE). Twists of the lateral elements are evident in the figure and the ovoid structure at the twist is probably a recombination nodule. This bulky image is probably more realistic considering the large numbers of proteins that have been reported to associate with the lateral elements. Apparently, uranyl, lead citrate, and silver nitrate bind to a subset of SC proteins.

Chromatin loops. Chromatin loops are attached to the cores. Organisms with a high DNA content tend to have longer chromatin loops (13). The cores/lateral elements can have a distinct periodic substructure in a number of fungi (14) and in *Locusta* (shown in Fig. 19.7).

The bouquet. Accurate and detailed three-dimensional SC organization within the meiotic prophase nucleus was obtained in the 1970's and 1980's from reconstructions of electron micrographs of serial sections through entire meiotic prophase nuclei (protist, yeasts, oomycetes, grasshoppers, rodents). Dieter von Wettstein and colleagues have reviewed in exhaustive detail the two decades of EM observations on serial reconstructions and surface-spread preparations (11). It is apparent that in most organisms both ends of the SCs are attached to the nuclear envelope. For *Locusta*, it was shown that the ends move along with nuclear envelope movements towards the activity center of the cytoplasm along with the centrioles, mitochondria, Golgi, and microtubules. The result is a fortuitous congregation of the SC ends at a restricted site on the nuclear surface. Subsequently, the centriolar

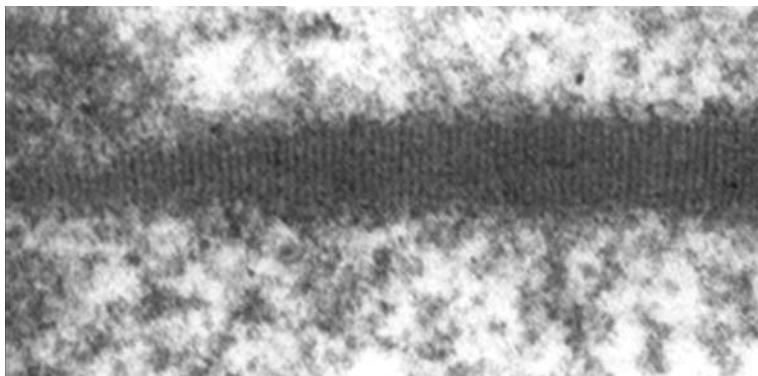


Fig. 19.7. Lateral elements of some insects and fungi have a distinct substructure.

pairs that are attached to the outer membrane of the nuclear envelope move to opposite sides of the nucleus, and some of the SCs ends move along with the centrioles giving proof of the nuclear envelope as the transport mechanism of SC ends. The movements are affected by inhibitors of microtubules. The polarization of SC ends is frequently referred to as a bouquet, which originally was shown to be the remnant of the previous nuclear division in somatic cells with centromeres at the poles and chromosome ends towards the equatorial plane—the so-called Rabl orientation (15). Meiotic chromosome polarization on the other hand is generated anew by nuclear envelope-mediated movement of chromosome ends. The particular benefit of electron microscopy in these studies is that the nuclear envelope is sharply defined, unlike in light- or fluorescent microscope images.

Exceptions to this pattern include the nematode *Ascaris suum* where only one SC end attaches to the nuclear envelope in oocytes and SCs in spermatocytes are not attached at either end (16). Apparently, bouquet formation is not a necessary requirement for SC formation in this case.

Chromosome core components. A number of chromosome core antigens are recognized by antibodies. For mammalian meiotic prophase cells, there are antibodies to the structural 30 kD and 190 kD proteins of the cores, to the 110 kD transverse filaments, to SYCE1 and SYCE2 (proteins of the central element), TEX12 and several cohesin subunits: SMC1, SMC1- β , SMC3, and STAG1. Antibodies recognize SC-associated recombination-related proteins SPO11, MRE11, RAD51, DMC1, RPA, BLM, MSH4/5, RAD1, MLH1, MLH3, and specialized functions ATR, TOPBP1 (**Fig. 19.4B**).

The localization of the various antigens can be demonstrated with immunogold preparations. In **Fig. 19.4A**, the 30/33 kD antigens of the chromosome cores are recognized by 10-nm-gold-conjugated secondary antibody. The positions of the gold grains

can be recorded with a digitizer and summarized for the length of the SC with computed standardized widths and shown in graphic form. For lateral element proteins, the majority of the gold grain distributions are over the lateral elements. For central element proteins, the majority of grains are between the lateral elements. These computations have been adopted for epitope mapping of central element proteins (8, 17, 18). The highly specific localization of gold grains to surface-spread yeast SC substructures confirms this as the preferred methodology for epitope mapping (18).

Colocalization. Groups of gold particles—so-called foci—are usually about 100 nm in diameter. The same foci when viewed with immunofluorescence measure about 300–400 nm in diameter. For this reason, precise co-localizations are best determined by electron microscopy to avoid artifactual overlap from fluorescence-enhanced signals. True co-localization is best demonstrated with different sized gold grains as shown for MLH1 and MLH3 in Fig. 19.3 (inset).

Modified SCs. As early as the 1960's, it became apparent that SCs do not necessarily exist only between homologously paired chromosomes. It was found that haploid plants—tomato, maize, and barley—that have only one set of chromosomes regularly have SCs in their pollen mother cells. Haploid yeast, when induced to sporulate, also develop SCs (19). A different kind of nonhomologous synapsis was reported in mouse spermatocytes where an inversion loop would gradually convert into a straight SC with what must be a nonhomologously paired segment. Similarly, short inversions in maize do not display inversion loops. The general conclusion appeared to be that initial synapsis is homology-based but later synapsis is homology-independent. But that conclusion was challenged by the observations on hexaploid wheat with three sets of distantly related genomes. Synapsis was frequently initiated between homeologous chromosomes but this was subsequently resolved and replaced by homologous SC formation (11). Apparently, in wheat, the second phase is based on homology. The general conclusion is that SCs are usually, but not always, evidence for homologous synapsis.

SC components have the capacity of auto-assembly even when not associated with chromosomes. A striking example is seen in the nematode *Ascaris suum* where stacks of poly SCs form in the cytoplasm of germ cells prior to transport into the nucleus (20). Stacks of poly SCs can be found in insect metaphase I chromosomes and in anaphase I cytoplasm, in protist meiotic nuclei, in meiotically compromised yeast nuclei, and can be induced in cultured mammalian COS-7 cells (21).

Particularly intriguing is the generation of poly SCs in post-meiotic prophase mosquito oocytes nuclei. After meiotic prophase is completed, the chromosomes form a karyosphere while the non-chromosomal portion of the nucleus expands to the full length of

the egg, some 1,000 μm . In *Aedes egypti*, the karyosphere generates massive amounts of SC components that surround the karyosphere with polycomplexes in the manner of a substitute nuclear envelope complete with nuclear pores (**Fig. 19.8**). *Culex*, on the other hand, generates massive amounts of transverse filaments that interconnect all SCs forming a simile of a nuclear envelope.

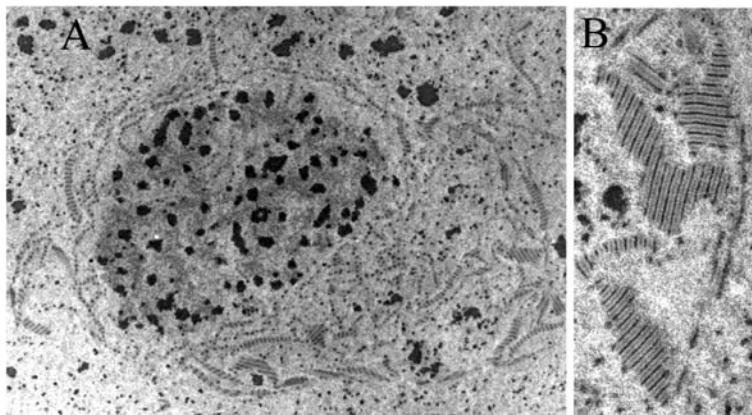


Fig. 19.8. Synaptonemal complex components can autoassemble into polycomplexes.

5. Notes



1. Before proceeding with collecting nuclei on the plastic-coated slides, make sure that the film floats off easily. If it does not, the slides may be too clean.
2. Make sure that the glass bottle holding the chloroform is completely dry before adding the chloroform. Any moisture in the bottle will result in small holes in the plastic. Before use, the chloroform solution may be filtered with a glass funnel and standard #1 filter paper to remove any floating debris. Do not use plastic ware.
3. The thickness of the film can be adjusted by changing the time that the slide is left in the chloroform fumes. If the film is golden colored, it is too thick and will absorb heat in the electron beam and rupture. To remedy such a situation, leave the slides in the chloroform fumes longer.
4. When serial sectioning, avoid aerosols such as perfumes that will disrupt ribbons of sections by altering the surface tension of the water surface.
5. When carrying out the initial dissection, make sure that all fat is removed from the tissue before releasing the meiotic cells. Failure to do so may result in the surface spread nuclei sinking to the bottom of the water bath.

6. Avoid skin contact with the silver nitrate solution. If you do get it on your skin, it can be removed with a bleach solution.
7. In shadow casting, the size of the grains is a function of the temperature of the wire. The specimen should be screened from the source of the grains until the wire has reached the optimum temperature, at which point the screen can be removed. The angle, current, and duration of time the specimen spends in the evacuator may have to be adjusted for optimal results. This will vary from machine to machine.
8. A common complaint when using the electron microscope is a feeling of nausea after a prolonged session. This is because the user views the image as though it was close to the person's face. One must learn to view the specimen as though it were at arm's length from the viewer.

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Chapter 20

Analyzing Mammalian Female Meiosis

Martha Susiarjo, Carmen Rubio, and Patricia Hunt

Abstract

The goal of this chapter is twofold: First, to acquaint the reader with the problems inherent in analyzing mammalian female meiosis and, second, to provide a step-by-step approach to mastering the necessary techniques. Although the methods presented are for use in the human and mouse, with subtle alterations the same techniques should be applicable to most mammalian species.

Key words: Meiosis, aneuploidy, chromosome, oogenesis, meiotic prophase, meiotic metaphase.

1. Introduction

By comparison with all other species, the human female is “meiotically challenged.” The incidence of genetically abnormal gametes is astonishingly high and strongly influenced by maternal age. Although the reason for the high incidence of meiotic errors remains unclear, recent data suggest that meiotic events that occur both during fetal development and in the adult ovary contribute to the effect (reviewed in 1). Despite the fact that meiosis in the female is vulnerable to error, most of what we know about the role of specific genes in the mammalian meiotic process is based on studies in the male. This is a reflection of the fact that the various stages of meiosis are considerably more accessible in the male. As shown schematically in Fig. 20.1, sex-specific differences in the time of onset and the duration of meiosis make studying the process very different in males and females. Because all stages of meiosis and of postmeiotic germ cells are present in the adult testis, material obtained from a small testicular biopsy

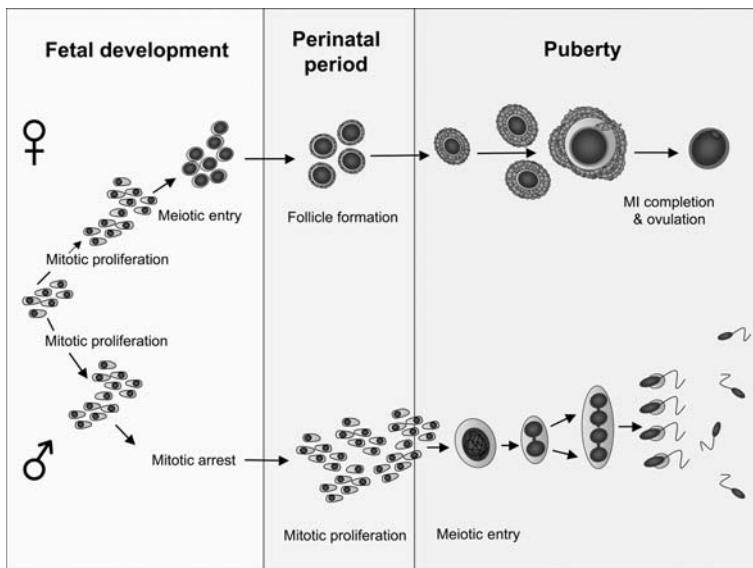


Fig. 20.1. Timeline of mammalian meiosis. The timing, onset, and duration of meiosis is sexually dimorphic in mammals. During fetal development, germ cells in the developing ovary undergo mitotic proliferation and enter prophase of meiosis. When oocytes reach the diplotene stage of prophase, they enter an extended period of meiotic arrest (dictyate), and remain in this arrested state until just prior to ovulation in the adult female. In the perinatal period (prior to birth in the human and just after birth in the mouse), the arrested oocytes become surrounded and enclosed by somatic cells, forming primordial follicles. With the onset of sexual maturity, groups of primordial follicles are recruited each cycle into the pool of growing oocytes. Although most growing oocytes undergo atresia, typically each cycle culminates with the ovulation of one (human) or a small group (mouse) of mature eggs. In contrast, germ cells in the fetal testis undergo a brief period of mitotic proliferation and then enter a period of mitotic arrest. When the male becomes sexually mature, spermatogonial stem cells resume mitotic proliferation and groups of cells begin to enter meiosis.

provides a means of detecting and quickly diagnosing meiotic aberrations in the male. In contrast, female meiosis is initiated during fetal development and characterized by two distinct arrest phases (Fig. 20.1). This, coupled with the fact that the first and second meiotic divisions normally occur in only one (human) or a small number (mouse) of oocytes at a time, makes meiotic studies of mammalian females far more difficult and time consuming than studies in the male. Although female meiotic studies may not be for the faint-hearted, improved methods for culturing oocytes and preimplantation embryos and the development of hormonal stimulation protocols that allow the simultaneous maturation of groups of oocytes have greatly facilitated female meiotic studies. The methodology detailed below presents a chronological approach to meiotic studies in the mammalian female.

2. Materials

2.1. Prophase Analysis

1. Hypo-extraction buffer (Make fresh and use within 2 h of DTT addition):

Tris-HCl (600 mM) pH 8.2	500 µL (30 mM)
Sucrose (500 mM)	1 mL (50 mM)
Trisodium citrate dihydrate (170 mM)	1 mL (17 mM)
EDTA (500 mM)	100 µL (5 mM)
Dithiothreitol (DTT) (500 mM)	50 µL (0.5 mM)
Phenylmethylsulphonylfluoride (PMSF) (100 mM)	50 µL (0.1 mM)
Water	7.3 mL
Adjust pH to 8.2–8.4 before use.	10 mL

2. 1% Paraformaldehyde (PFA): Add 0.25 g of PFA to 22.5 mL water. Add one drop of 1 N NaOH and place at 60°C for 20 min to dissolve. Cool to room temperature and adjust pH to 9.2 using 50 mM boric acid. Add 50 µL Triton X-100 and dissolve well. Adjust volume to 25 mL with water.
3. 10 × Antibody dilution buffer (ADB): 10 mL Normal Donkey Serum (Jackson Immunoresearch 017-000-121), 3 g bovine serum albumin, 50 µL Triton X-100, 90 mL 1 × PBS. Filter sterilize using a 0.45 µm filter and add 0.02 g sodium azide. Can be stored at 4°C for two weeks.
4. 1 × PBS: 8 g NaCl, 0.2 g KCl, 0.92 g Na₂HPO₄, 0.2 g KH₂PO₄. Adjust volume to 1 L with ddH₂O. Adjust pH to 7.4 with NaOH.
5. Humid chamber: Fill both sides of a large gel box with HOT tap water, leaving middle section dry for slides. Close chamber to accumulate condensation on the gel box lid (*this is important for good preparation*).
6. 0.4% Photo-Flo: 200 µL Photo-Flo (Fisher NC9296513) in 50 mL of ddH₂O.
7. Antibodies for mouse studies: Mouse monoclonal to SYCP3 (Novus NB100-2065), rabbit polyclonal to MLH1 (Calbiochem PC56). Secondary antibodies: Rhodamine-donkey anti-mouse (RDAM) (Jackson Immunoresearch 715-025-150), FITC-donkey anti-rabbit (FDAR) (Jackson Immunoresearch 711-095-152).
8. Antibodies for human studies: Rabbit polyclonal to SYCP3 (Novus NB300-232A), mouse monoclonal to MLH1 (BD Pharmingen 551091). Secondary antibodies: Rhodamine-

donkey anti-rabbit (RDAR) (Jackson Immunoresearch 711-025-152), FITC-donkey anti-mouse (FDAM) (Jackson Immunoresearch 711-095-150).

9. PN Buffer: 13.8 g NaH₂PO₄-H₂O (0.1 M), 14.2 g Na₂HPO₄(0.1 M), 50 µL Nonidet P-40 (0.05%). Adjust final volume to 1 L with ddH₂O. Adjust pH to 10.0 with NaOH.
10. DAPI stain: Make a stock solution of 1 mg/mL DAPI in PN buffer. Unless noted otherwise, typical working concentration is 1 µg/mL. Use 60 µL per slide.
11. Antifade.
12. Soft coverslips: Parafilm cut to appropriate size.
13. Watch glasses (Electron Microscopy Sciences 70543-30)

2.2. Mouse Oocyte Collection and Meiotic Maturation In Vitro

1. Collection Medium (CM): 45 mL Waymouth's medium with 1% penicillin-streptomycin solution, 5 mL fetal bovine serum. Store in 5 mL aliquots at 4°C. Prior to use, add 50 µL of 2.5 mg/mL sodium pyruvate and incubate for 20 min at 37°C in 5% CO₂ to equilibrate.
2. Mineral oil (Squib): Place 15 mL Waymouth's medium in a T-25 flask and add mineral oil to fill. Shake vigorously to mix, loosen cap to allow gas exchange, and place flask at 37°C in 5% CO₂. Allow oil to separate prior to use.

2.3. Stimulation with Exogenous Hormones

1. Pregnant mare serum gonadotropin (PMSG)
2. Human chorionic gonadotropin (hCG)

2.4. Conventional Air-Dried Chromosome Preparations

1. Slide preparation (*see Fig. 20.2A*).
2. Hypotonic solution: 0.9 g sodium citrate in 100 mL ddH₂O (0.9%).
3. Acidified water: Eight to ten drops of glacial acetic acid in 50 mL ddH₂O.
4. Carnoy's fixative: Three parts absolute methanol: one part glacial acetic acid. Make fresh immediately prior to use and store in airtight bottle.
5. Pulled pipettes: Using a fine flame (e.g., a methylated spirits lamp), pull 6 inch Pasteur pipettes. Very fine pipettes with a diameter of ~120–150 µm allow for maximal volume control and are excellent for water droplets and transferring oocytes to the slide. Slightly larger diameter pipettes work well for fixative.

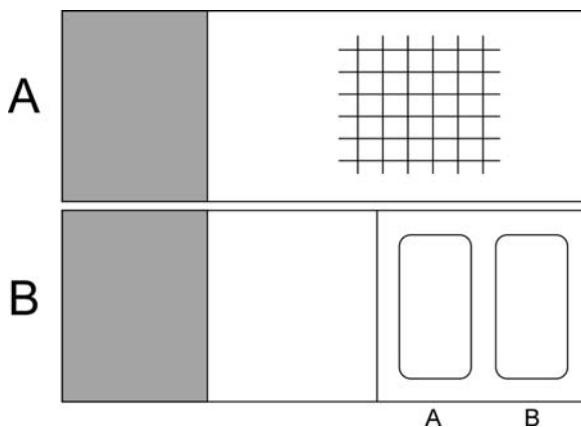


Fig. 20.2. Slide Preparation. **(A)** Conventional air-dried chromosome preparations. Using a diamond-tip pencil, lightly etch horizontal and vertical lines on the under side of a microscope slide as illustrated. Take care to *gently* score the slide – deeply scored lines make the slide prone to breakage. **(B)** Fixing intact eggs and embryos. Using rubber cement, make two chambers (**A** and **B**) to hold media drops for washing and clotting, respectively. *Two supplemental videos are available on the companion CD for this volume.*

2.5. Chromosome Preparations that Preserve Chromosome-Associated Proteins

1. 1% Paraformaldehyde (PFA) fixative: Add 0.25 g of PFA to 22.5 mL ddH₂O. Add one drop of 1 N NaOH and place at 60°C for 20 min to dissolve. Cool to room temperature and adjust pH to 9.2 using 50 mM boric acid. Add 35 µL Triton X-100 (0.15%) and dissolve well. Add 150 µL of 500 mM DTT (3 mM final). Adjust final volume to 25 mL with water.
2. 2% Pronase: Add 0.01 g of Pronase to 500 µL of gassed collection media (**Section 2.2, step 1**). Incubate to maintain temperature and pH until use.
3. Agarose dishes: Mix 0.5 g agarose with 25 mL 1 × TAE buffer. Carefully heat by microwaving to dissolve agarose. Pour into 35 mm tissue culture dishes, coating the bottom of each dish with ~2 mm of agarose. Cool, and store dishes at 4°C.

2.6. Fixing Intact Eggs and Embryos

1. Poly-L-lysine-coated slides: Dilute 5 mL poly-L-lysine in 50 mL ddH₂O in a Coplin jar. Add clean microscope slides and soak at room temperature for 30 min. Stand slides upright to drain on paper towels and air dry overnight. Prepare slides with separate chambers for washing and clotting eggs or embryos (**Fig. 20.2B** and **Supplementary Video 1**) (*see Note 1*).
2. Ringer's solution: 0.45 g NaCl, 0.021 g KCl, 0.0125 g CaCl₂. Bring volume to 50 mL with ddH₂O and filter sterilize.
3. 5 × Stabilization buffer (SB): 7.55 g (0.1 M) PIPES, 0.25 g (5 mM) MgCl₂, 0.235 g (2.5 mM) EGTA. Adjust final volume to 50 mL with ddH₂O. PIPES will not go into solution until pH of 6.1–7.5 is achieved. Stock solution is stable at 4°C for 1 year.

4. Fibrinogen: Store aliquots of 0.005 g fibrinogen at -20°C. For use, add 400 µL Ringer's solution to one aliquot and incubate at 37°C for 5–10 min. This working solution can be maintained at 37°C and used for 24 h.
5. Thrombin: Reconstitute 250 units of lyophilized thrombin with 1 mL of water. Adjust final activity to 100 units per mL by adding 1.5 mL 1 × PBS for a final volume of 2.5 mL (diluting in this manner provides a better osmotic balance for oocytes). Store in 20 µL aliquots at -20°C. For use, thaw an aliquot and maintain it on ice.
6. Microtubule Stabilization Buffer (MTSB): 2 mL 5 × SB stock (described above), 1 µL aprotinin, 10 µL of 1 MDTT, 5 mL D₂O (Sigma D-4501), 10 µL Taxol. Adjust final volume to 10 mL with ddH₂O.
7. Stabilizing fixative solution: 5 mL MTSB, 5 µL of Triton X-100, 270 µL of 37% formaldehyde.
8. 0.1% Normal goat serum (NGS) wash solution: 500 mL 1 × PBS (sterile), 500 µL of normal goat serum. Store at 4°C; use within 1 month.
9. 10% NGS wash solution: 500 µL Triton X-100, 50 mL normal goat serum, 0.1 g sodium azide. Bring volume to 500 mL with sterile 1 × PBS. Store at 4°C; use within 1 month.

3. Methods

The early stages of meiosis, when homologous chromosomes undergo synapsis and recombination, are critical for the orderly segregation of homologs at the first meiotic division. Advances in the fixation and immunostaining of prophase-stage cells have made it possible to directly analyze the processes of synapsis and recombination (**Section 3.1**). Subtle aberrations that result from mutations in the genes that mediate these events are readily detectable using this methodology, and recent studies have demonstrated defects associated with male infertility in humans (e.g., 2) and mice (reviewed in 3). Studies in the female, however, are complicated by the fact that meiotic prophase occurs during fetal development. Thus, by the time fertility defects become detectable in the mature female, it is no longer possible to determine if perturbations in prophase events are responsible. Obviously, in the mouse, where genetically identical mutants can be analyzed at different developmental stages to obtain a complete meiotic profile, this is not an issue. In the human, however, prophase studies are restricted to ovarian material obtained

from pregnancy terminations. Nevertheless, the data obtained from studies to date provide exciting new information about the early stages of human female meiosis (e.g., 4).

Analyzing the division phases of female meiosis is inherently difficult due to the small number of cells available. Indeed, in both the human and mouse these studies require expertise in handling and culturing of single cells (**Sections 3.2, 3.3**). Nevertheless, these divisions are “where the action is”; errors in chromosome segregation during the meiotic divisions in the female are the leading cause of human pregnancy failure. The techniques detailed below provide the necessary methodology, but patience and persistence is the key. Mastering the art of preparing chromosome preparations from single eggs (**Sections 3.4, 3.5**) or fixing intact eggs and embryos using fibrin clots (**Section 3.6**) is much like learning to ride a bicycle – almost no one masters it without practice. You simply have to break a lot of eggs!

3.1. Fixation of Fetal Ovaries for Analysis of Prophase

A number of different techniques have been described for the preparation of prophase-stage nuclei. The method detailed below is based on the technique developed by Antoine Peters (5) and is particularly useful for oocytes. It should be noted, however, that understanding and being able to recognize the substages of meiotic prophase requires careful study of cells at different stages. Although accurate guides for mastering spermatogenesis are readily available (e.g., 6, 7), a similar comprehensive guide to female meiotic prophase has not been published. Nevertheless, the Cohen laboratory has recently provided an excellent description of staging criteria for the human female with illustrations of the various prophase substages (4).

Because meiosis is initiated during fetal development, fetal ovaries provide the starting material for these studies. In the human, the first group of oocytes enter prophase at ~8 wk of gestation. Prior to 14 wk of gestation, early prophase (leptotene and zygotene) stages predominate (8, 9). By 20 wk, pachytene-stage cells are plentiful; at this stage the synaptonemal complex (SC), the protein scaffold that forms between homologous chromosomes, is fully formed. Because synapsis is complete and the sites of recombination are evident as foci of MLH1 or MLH3 staining along the SC, analysis of pachytene-stage oocytes provides a means of detecting synaptic defects and of assessing recombination levels.

In the mouse, timed matings are used to obtain fetuses at the appropriate developmental stage. Although the exact timing is strain dependent, generally leptotene and zygotene-stage cells predominate at 14–16 d of gestation (the day a copulation plug is found is counted as Day 0), pachytene-stage cells from 17 to 19 d, and diplotene-stage cells can be obtained right around the time of birth. Note that the plug date is not always a totally reliable indicator of gestational age as the actual age may vary by ±0.5 d. In addition, slight variation may be

observed among littermates. For accurate staging, detailed staging criteria should be utilized (see, e.g., [http://genex.hgu.mrc.ac.uk/
Databases/Anatomy/MAstaging.shtml](http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.shtml)).

3.1.1. Fixation of Prophase Oocytes

1. For optimal preparations, rapidly dissect ovaries from female mouse fetuses after the pregnant female is killed (*see Note 2*). Carefully remove adherent tissue, place ovaries in a small volume of PBS in watch glasses or separate wells of a multiwell plate, and maintain at 37°C.
2. Place each pair of ovaries in hypo-extraction buffer in a clean watch glass, taking care to immerse ovaries completely. Incubate for at least 15 min but no more than 30 min (mouse) or at least 45 min (human).
3. Pipette 40–50 µL of 100 mM sucrose onto a clean slide and transfer one pair of ovaries to the drop (for human samples, adjust volume to achieve appropriate cell density). Using two 26-gauge needles, tease the ovaries apart to release cells into the sucrose solution. With forceps, remove large pieces of ovary and carefully pipette sucrose solution to disperse cells.
4. Place several drops of PFA on a second slide, spread evenly over the slide surface, rapidly drain excess PFA (but do not wick), and pipette half of the sucrose mix onto the wet surface.
5. Carefully roll the slide to spread cells and place in the humid chamber (immediately close the chamber to preserve humidity). Repeat with a fresh slide and remaining sucrose solution (i.e., one pair of mouse ovaries should yield sufficient material for two slides).
6. Incubate the slides in the closed humid chamber overnight.
7. Remove chamber lid and allow slides to dry completely.
8. Wash slides in 0.4% Photo-Flo solution (0.04% for human samples) for 2 min, air dry, and store at –20°C (*see Note 3*).

3.1.2. Immunostaining to Visualize the SC and Sites of Recombination (see Note 4)

3.1.2.1. Day 1

1. Soak slides in 1 × ADB for 45 min.
2. Dilute MLH1 primary antibody 1:75 in 1 × ADB (*see Note 5*). Remove slide from ADB, drain, add 60 µL of antibody dilution, mount with a glass coverslip (*do not blot excess liquid*), and seal with rubber cement.
3. Incubate overnight at 37°C in a humid chamber.

3.1.2.2. Day 2

1. Soak slide in 1 × ADB. Carefully remove rubber cement and return to ADB, allowing coverslip to soak off.
2. Dilute SCP3 antibody 1:1,000 in 1 × ADB. Remove slide from ADB, drain, add 60 µL of diluted antibody, and cover with soft coverslip.

3. Incubate for 2 h at 37°C in humid chamber.
 4. Remove soft coverslip and wash slides in two changes of 1 × ADB (20 min each) on a shaker.
 5. Dilute secondary antibody to MLH (FDAR) 1:75 in 1 × ADB. Remove slide from ADB, drain, add 60 µL of antibody dilution, mount with glass coverslip (*do not blot excess liquid*) and seal with rubber cement.
 6. Place in humid chamber and incubate at 37°C overnight.
- 3.1.2.3. Day 3
1. Soak slide in 1 × ADB. Carefully remove rubber cement and return to ADB, allowing coverslip to soak off.
 2. Dilute secondary antibody to SCP3 (RDAM) 1:100 in 1 × ADB. Remove slide from ADB, drain, add 60 µL of diluted antibody, and cover with soft coverslip.
 3. Incubate for 1 h at 37°C in humid chamber.
 4. Remove soft coverslip, wash twice for 20 min each in 1 × PBS in the dark.
 5. Drain slide, add DAPI and antifade, mount with glass coverslip, blot excess liquid, and seal with rubber cement. Slides may be stored at 4°C or immediately analyzed using fluorescence microscopy.

3.2. Mouse Oocyte Collection and Meiotic Maturation In Vitro

Meiotically competent oocytes can be obtained by collecting oocytes from antral follicles at any time during the reproductive lifespan of the mouse. However, a large cohort of follicles initiates growth in the sexually immature female, hence the greatest number of meiotically competent oocytes can be obtained from 25- to 28-day-old females. We utilize the collection methodology described below to obtain large populations of semi-synchronized oocytes for studies of the meiotic divisions. Stimulation with pregnant mare's serum gonadotropin (**Section 3.3**) approximately 48 h prior to oocyte collection can be used to enhance the rate of polar body extrusion.

Considerable effort has been made to optimize culture conditions for both mouse and human oocytes (e.g., *10, 11*). Careful control of temperature and pH is critical. The methodology detailed below is useful for short-term cultures to obtain oocytes and eggs at different meiotic stages. However, if the ultimate goal is developmental studies of embryos or embryo transfer to obtain live mice, the reader is encouraged to seek additional information (*12*).

1. Remove ovaries and place in gassed collection medium (CM) (*see Note 6*).
2. Dissect all adherent tissue from ovaries, taking care to remove *all* fat.
3. Transfer ovaries to fresh CM and rupture antral follicles using 26 gauge needles.

4. Collect germinal vesicle (GV) stage oocytes, transfer to small (~20 µL) drops of CM, overlay with mineral oil, and incubate at 37°C in 5% CO₂ in air.
5. After 2 h in culture, score oocytes for GV breakdown (disappearance of the nuclear envelope) and remove any that remain at the GV stage. To obtain oocytes at prometaphase, metaphase, and anaphase of meiosis I, mouse oocytes are typically fixed after 2–4, 6–8, and 9–12 h in culture, respectively (note that the rate of cell cycle progression varies depending upon the strain of mice and whether exogenous hormone stimulation has been used). Overnight culture (16–18 h) can be used to obtain metaphase II arrested oocytes or to assess meiotic competence (i.e., the proportion of oocytes that remain at meiosis I vs. those that extrude a first polar body and arrest at metaphase II after overnight culture).

3.3. Stimulation with Exogenous Hormones

Stimulation with exogenous hormones provides a simple means of increasing the yield of ovulated eggs or preimplantation embryos. The response is both strain- and age- dependent but is best in sexually immature females between 3–5 wk of age [for additional details, *see*(13)]. The procedure involves two intraperitoneal injections: pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), which mimic the actions of FSH and LH, respectively. Injection timing is dependent upon the light/dark schedule of the room, but should be calculated so that the hCG injection precedes the endogenous LH surge by several hours. The schedule detailed below is based on a 6 am to 6 pm light cycle.

1. Day 1: Between 3 pm and 4 pm inject each female with 2.5–5 IU PMSG (*see Note 7*).
2. Day 3: 44–46 h after the PMSG injection, inject each female with 5 IU hCG.
3. For studies of preimplantation embryos, place each female with a stud male at the time of hCG injection, check the following morning for the presence of a copulation plug, and flush the oviducts (d 0–2) or uterine horns (d 3) to obtain embryos at the desired stage of development.

3.4. Conventional Air-Dried Chromosome Preparations

This technique [a modification of the method developed by Tarkowski (14)] can be used to obtain preparations of diakinesis-stage oocytes for the analysis of chiasmata, metaphase II arrested oocytes for the analysis of meiosis I nondisjunction, or pronuclear and early cleavage embryos to assess the genetic quality of embryos (**Supplementary Video 2**).

1. Move a small number (five to ten) of oocytes to hypotonic solution for 5–10 min.

2. Using a finely drawn pipette, place a small ($\sim 1 \mu\text{L}$) drop of acidified water on the upper surface of the etched slide so it lies within the marked region.
3. Quickly transfer one oocyte from hypotonic solution to the acidified water drop, taking care to transfer as little hypotonic solution as possible. Draw back a small amount of liquid until the oocyte appears to attach to the slide. The volume of liquid that remains is critical: the oocyte must make contact with and stick to the slide, but sufficient water must be retained around the oocyte to prevent drying prior to fixation.
4. Quickly change pipettes and add one drop of Carnoy's fixative directly to the oocyte. Pause to allow the fixative to disperse (see Note 8), then quickly add several additional drops of fixative.
5. Allow to air dry or, for improved spreading of chromosomes, *gently* blow on the slide.
6. Check chromosomes in a phase contrast microscope to assess spreading and repeat steps 2–5 with additional oocytes to accumulate a group of cells within the marked area of the slide.
7. Stain slides with DAPI and analyze by fluorescence microscopy to determine if the quality of the preparations is sufficient for subsequent fluorescence *in situ* hybridization (4) studies.

3.5. Chromosome Preparations that Preserve Chromosome-Associated Proteins

This technique was devised by Craig A. Hedges (15). Although difficult to master, it has the advantage of providing outstanding chromosome morphology while preserving chromosome-associated proteins. Further, because the eggs/embryos effectively “melt” onto the slide, it has two additional advantages over conventional cytogenetic preparations: (1) artifactual chromosome loss is less of a problem, (2) cells at anaphase and telophase can be readily distinguished from metaphase cells.

1. Move oocytes to 2% pronase solution for 2–5 min to remove the zona pellucida. Watch for loosening of the zona (see Note 9).
2. Move oocytes through two successive drops of culture medium to remove pronase. Transfer to culture medium on an agarose-coated dish (see Note 10).
3. Dip a microscope slide in PFA fixative (see Note 11), remove excess fixative by *gently* tapping slide on a paper towel, and rapidly deliver a small number (10–20) of oocytes along the length of the slide. Optimal preparations are obtained when oocytes are delivered in the smallest volume of medium possible to avoid diluting the PFA. Oocytes should burst within seconds of transfer and “melt” onto the slide.

4. Immediately transfer slide to a humid chamber and allow to slowly dry overnight.
5. After drying, soak slides in 0.4% Photo-Flo for 2 min and air dry.
6. As with conventional preparations, slides can be scanned by phase contrast microscopy to assess chromosome spreading and morphology, stained with a chromatin stain, and subjected to FISH. Unlike conventional preparations, these preparations are also suitable for immunostaining with antibodies to chromosome-associated proteins.

3.6. Fixing Intact Eggs and Embryos

This technique has the advantage of preserving the three-dimensional structure of oocytes (or preimplantation embryos) but maintaining the cell in a specified orientation, allowing sequential studies of the same oocyte (for example, immunofluorescence staining followed by FISH). It is not, however, suitable for all purposes and, for some localization studies, oocytes must be fixed free as described (**Section 3.6.2**). Alternatively, oocytes may be placed in fibrin clots *after* fixation and antibody staining to facilitate analysis or for subsequent FISH studies. The specifics of the fixation process (i.e., the choice of fixative, the duration of fixation, and whether oocytes can be fixed in clots or not) will vary depending upon the antibodies being used. The fixative method here is adapted from Messinger and Albertini, 1991 (16). For additional details on fixation *see* (17) and (18).

3.6.1. Fibrin Clots (Supplementary Video 1)

1. Place 1 μ L drops of fibrinogen on poly-L-lysine-coated slides and overlay with mineral oil to prevent evaporation (*see Note 12*). To remove media, wash oocytes in a fibrinogen drop in the wash chamber, and immediately transfer to a fibrinogen drop in the clot chamber, taking care to carry over as little volume as possible (*see Note 13*).
2. Add 1 μ L of thrombin to the fibrinogen drop and allow 30 s for a clot to form.
3. Remove oil by carefully washing slide with 2% Triton X-100 in PBS, immediately place slide in stabilizing fixative (at 37°C) and fix for 30 min.
4. Transfer slide to 0.1% NGS at 37°C for 15 min.
5. Transfer slide to 10% NGS at 37°C for 1 h. Slides can be stored in 10% NGS at 4°C prior to immunostaining.
6. Add 60 μ L diluted primary antibody to the slide (*see Note 14*), cover with a soft coverslip, and place in a humid chamber at 37°C for 1 h.
7. Remove the coverslip and wash the slide in 10% NGS at 37°C for 1 h.

8. Add 60 μ L of an appropriate secondary antibody to the slide, cover with a soft coverslip, and place in the humid chamber at 37°C for 1 h.
9. Remove the coverslip and wash the slide in 10% NGS for 1 h.
10. Add DAPI (400 ng/mL) for 2 min to stain the chromatin if desired. Drain the slide, cover with a glass coverslip using antifade, and seal with rubber cement.

3.6.2. Free Fixation

1. Collect and culture oocytes (**Section 3.2**).
2. Transfer oocytes to a watch glass containing stabilizing fixative (*see Note 15*) at 37°C for 30 min. Oocytes will float to the surface of the fix when added – take care to collect them at the bottom of the watch glass.
3. Transfer oocytes through three 5 min washes in 0.1% NGS at 37°C.
4. Transfer oocytes to 10% NGS at 37°C for 1 h (oocytes can be stored at 4°C at this point).
5. For immunostaining, dilute the desired primary antibody with 5% NGS, transfer oocytes to a watch glass containing the antibody dilution, and incubate in a humid chamber at 37°C for 1 h (incubation time may vary with antibody used).
6. Transfer oocytes through three 5 min washes of 10% NGS.
7. Dilute the appropriate secondary antibody with 5% NGS, transfer oocytes to a watch glass containing the antibody dilution, and incubate in a humid chamber at 37°C for 1 h.
8. Wash twice for 10 min each in PN buffer.
9. Transfer oocytes to 1 μ g/mL DAPI for 1 min, rinse in PN buffer.
10. Add a small drop of antifade to a clean microscope slide, transfer oocytes to the antifade drop, cover carefully with a glass coverslip and seal with rubber cement. Note: eggs may be placed in fibrin clots prior to DAPI staining and analysis. This provides a means of preserving the specimen for subsequent studies (e.g., FISH analysis).

4. Notes



1. Poly-L-lysine solution should *not* be stored for reuse.
2. Details on the dissection of fetuses from the uterus and removal of fetal gonads can be found in (19).

3. Certain epitopes (e.g., MLH1) are less stable; for optimal visualization, slides should be immunostained as soon as possible.
4. This 3 d staining protocol allows for simultaneous visualization of the SC and the sites of recombination (MLH1 or MLH3 foci) in pachytene cells. An accelerated protocol with a 2 h incubation in primary antibody followed by a 1 h incubation in secondary antibody provides SC staining suitable for visualizing all substages of meiotic prophase.
5. Antibodies vary from vendor to vendor and from lot to lot; thus, adjustment in dilutions may be necessary.
6. Minimizing fluctuations in temperature and pH is critical in the handling and culture of oocytes and embryos. To avoid pH fluctuation during collection, HEPES-buffered medium may be substituted, but oocytes should be transferred to bicarbonate-buffered medium for culturing.
7. Although most sources recommend 5 IU, lowering the dose to 2.5 IU improves stimulation of 3–5-week old females on some genetic backgrounds.
8. To prevent hydration, fixative should be kept tightly covered in its storage bottle. Methanol evaporation (evident as failure of the fixative drop to spread evenly) results in poor fixation. Thus, for optimal preparations, fresh fixative should be made frequently.
9. After removal of the zona pellucida, oocytes will be very sticky. To avoid loss, the use of siliconized pipettes is recommended.
10. To improve attachment of the oocytes to the slide, wash oocytes in culture medium without BSA or serum prior to fixation.
11. Modification of the pH of PFA fixative may be necessary for some antibodies. A pH of ~9.2 provides optimal chromosome morphology, however, the amount of PFA on the slide will influence morphology; too much will cause excessive spreading and chromosome loss, while too little will result in insufficient spreading. In addition, the delivery of oocytes and embryos in an excessive amount of medium will adversely affect both chromosome morphology and protein stabilization.
12. To contain oil, small chambers can be made on the slide using rubber cement (*see Section 2.6 and Supplementary Video 1*). Following fixation, rubber cement can be pulled from the slide without disturbing clots.

13. The proper ratio of fibrinogen to thrombin is critical for clot formation. Because fibrinogen and thrombin may vary from lot to lot, the appropriate ratio for clot formation should be established before using a new lot.
14. Use 5% NGS for dilution of all antibodies.
15. A modified version of this fixative that lacks the very expensive stabilizers (see below) can be used if the goal is simple visualization of chromosomes and meiotic spindles. This fixative is less expensive, but will not preserve cellular architecture as effectively. Simple fixative solution: 1 mL of 5 × SB stock (described in **Section 2.6**), 270 µL of 37% formaldehyde, 100 µL of Triton X-100, 3.63 mL of ddH₂O (5 mL final volume).

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Chapter 21

Cytological Analysis of Interference in Mouse Meiosis

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Abstract

In most eukaryotes, meiotic crossovers (COs) are non-randomly placed along the bivalents, such that the presence of a CO reduces the probability of additional COs nearby. This phenomenon, named CO interference, was originally defined genetically, but can also be analyzed cytologically by studying the chromosomal positions of protein complexes that are involved in CO formation, or by studying the positions of chiasmata. Here we focus on the cytological analysis of interference among protein complexes involved in meiotic recombination and CO formation in the mouse. During the pachytene stage of meiosis, these protein complexes can be visualized as immunofluorescent foci along synaptonemal complexes (SCs), which are linear protein structures that are formed along homologous chromosome pairs (bivalents) during meiotic prophase. We describe how to make cytological preparations that are suitable for the analysis of interference among these foci, and how to estimate the strength of interference among foci, using the gamma distribution as a mathematical model for focus/CO positioning.

Key words: Meiosis, interference, crossing over, immunofluorescence, mouse.

1. Introduction

1.1. Cytological CO Markers

In most eukaryotes, COs appear to inhibit the formation of additional COs in their vicinity on the same bivalent, which results in an even spacing of COs (reviewed by 1). This phenomenon, called (positive) CO interference, was originally noticed in genetic experiments in which recombination was analyzed across two adjacent or nearby chromosomal segments. If R_1 and R_2 represent the recombination fractions across the first and second segment and R_{12} the recombination fraction for the two segments simultaneously, R_{12} is expected to equal $R_1 R_2$ if COs are randomly placed. However, the ratio $R_{12}/R_1 R_2$ (called the coefficient of coincidence or CC) was usually found to be less than 1, which indicates

(positive CO) interference. It was furthermore noticed that CO interference acts in a distance-dependent manner, because—all other conditions being equal—CC increases and approaches 1 with increasing distance between the analyzed segments (reviewed in 1).

Although CC is a useful indicator of interference, it is problematic as a measure for the strength of interference (i.e., the extent to which a CO appears to suppress the formation of additional COs in its neighborhood), because the genetic size of the analyzed chromosomal segments co-determines the value of CC. With increasing segment lengths, R_1 and R_2 approach $\frac{1}{2}$ and R_{12} approaches $\frac{1}{4}$, so that CC approaches 1, even if there is interference. Mathematical models dealing with the exact CO positions are therefore preferable to CC for estimating the strength of interference, if these positions are precisely known (discussed in 2). In genetic studies, the exact positions of crossovers are often not known, but only the segments (delimited by genetic markers) within which crossover(s) must have occurred. In such studies crossover interference is necessarily analyzed in chromosomal segments. In cytological studies, in contrast, the occurrence of CO markers along the bivalents indicates the exact CO positions, so that mathematical point process models can be applied. This implies an important advantage of the cytological approach compared to the genetic analysis of CO interference. Other advantages are that (i) cytological CO markers provide genome-wide information about CO positions; (ii) polymorphic genetic markers are not required; (iii) completion of meiosis and the production of viable progeny are not essential; (iv) large numbers of meioses can be analyzed; and (v) the positions of the cytological CO markers along the bivalent reflect the effect of interference directly, because interference is exerted at the level of the bivalent (3). Genetic analyses on the other hand, deal with CO positions on chromatids. Because each CO involves only two non-sister chromatids, chosen at random from the four chromatids of the bivalent, the CO positions on a chromatid correspond with a random sample of 50% of the CO positions on the bivalent. Therefore, in genetic studies, the CO spacing on the bivalent has to be reconstructed from the CO positions on the chromatids (4, 5).

Ideally a cytological CO marker should unambiguously indicate all (prospective) CO positions and should not mark positions where COs will not be (have not been) formed. In the methods section we describe in detail how to pursue this goal. Three types of cytological CO markers are known, which each have their own advantages and disadvantages for cytological studies of interference, namely chiasmata, late recombination nodules (LN)s and immunofluorescent foci of proteins involved in CO formation (reviewed in 6).

Chiasmata show up at the end of meiotic prophase, in diakinesis, when chromosome condensation starts. Their main advantage is that they are direct cytological manifestations of crossing over: a

chiasma shows that a reciprocal exchange between non-sister chromatids of the bivalent has occurred. Their main disadvantages are that chromosome condensation limits the accuracy of chiasma localization along bivalents and hampers the separate discernment of closely spaced chiasmata. The number of observed chiasmata therefore provides a minimum estimate of the number of COs. Furthermore, bivalent configurations can be misinterpreted, and there is no guarantee that chiasmata stay in place.

LNs are spherical or oval electron-dense protein complexes that are associated with SCs during the pachytene stage of meiotic prophase, when homologous chromosomes are paired along their entire length. In the species where it has been analyzed, LNs correlate well with genetic exchanges and/or chiasmata, both with respect to numbers and positions (reviewed in 7). Because LNs are small (100–200 nm in diameter) compared to the SC lengths (tens of μ m in most eukaryotes), their positions along the bivalents can be determined with great precision, but this requires labor-intensive ultrastructural analysis of meiotic prophase cells (e.g. 8). Also, LNs are not easily recognized in all eukaryotes. Furthermore, there is no guarantee that all (apparent) LNs will yield COs: Certain structures, e.g., early recombination nodules (i.e. ultrastructurally recognizable protein complexes that are thought to be involved in early and intermediate steps in meiotic recombination) (7) may be mistaken for LNs in some species, or CO formation may occasionally fail, even though an LN has been formed. Nor is there a guarantee that all COs are marked by LNs. For instance, it is possible that the LNs are not present at prospective CO sites during the entire time window of observation. In theory it is also possible that there is more than one CO type, and that LNs do not mark all types, although there is no evidence for this in the species analyzed so far (7, 9). The usefulness of LNs as CO markers should therefore be validated in every species, e.g., by comparing the numbers of LNs per chromosome or chromosome arm with the numbers of genetic exchanges or chiasmata.

In comparison with LNs, immunofluorescent foci have the important advantage that they can be analyzed by light microscopy. Proteins involved in CO formation have been identified in several species, and two of these proteins, MLH1 and MLH3, have been demonstrated in LNs by immunogold labeling (10). Here we will focus on MLH1. Immunofluorescence double labeling of pachytene nuclei by anti-MLH1 and anti-SC antibodies reveals the SCs as thin lines carrying sharp, well-defined MLH1 foci, which can be accurately located along SCs (**Fig. 21.1**). However, there is no guarantee that all (apparent) MLH1 foci will yield COs, partly for similar reasons as mentioned above for LNs: CO formation may fail at sites marked by MLH1 foci (e.g. 11), and immunofluorescent background signals may be mistaken for MLH1 foci. There is also no guarantee that MLH1 foci mark all COs,

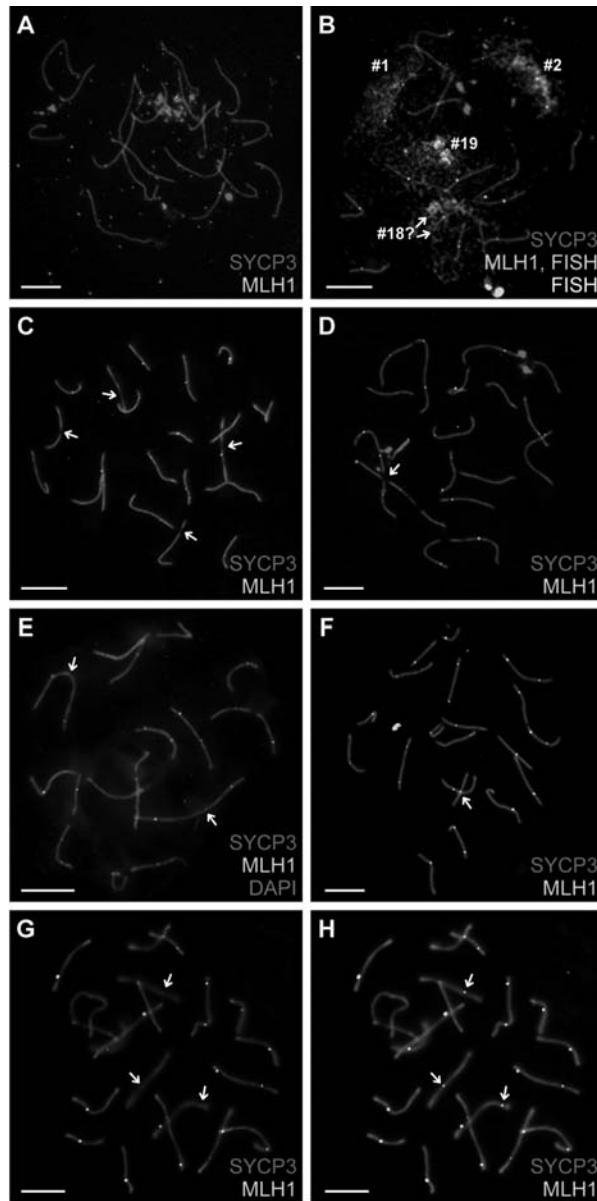


Fig. 21.1. Nuclei or synaptonemal complexes (SCs) that do not qualify for measurements. Shown are spread mouse oocytes (**A–F**) or spermatocytes (**G–H**) labeled for synaptonemal complex protein 3 (SYCP3) (red), and MLH1 (green) that are not useful for measurements of interfocus distances for the following reasons: **(A)** *The signal-to-background ratio is too low:* The signal-to-background ratio for the green MLH1 signal does not allow unambiguous identification of all MLH1 foci. **(B)** *Chromosomes cannot be identified:* After immunofluorescence labeling of SYCP3 and MLH1, this nucleus was subjected to FISH using probes against chromosomes 1 and 19 (green) and 2 and 18 (white). Chromosomes 1, 2, and 19 are readily identified, but chromosome 18 is not because both SCs indicated by arrows could be chromosome 18. **(C)** *SCs appear stretched:* Arrows indicate SCs that appear stretched. **(D)** *SCs are broken:* The arrow indicates a break in an SC. **(E)** *SCs cannot be traced from end to end:* After immunofluorescent labeling of SYCP3 (red) and MLH1 (green), this nucleus was stained with DAPI. The centromeric ends of SCs can be recognized by the intense DAPI staining. The arrows indicate positions where SCs appear to be fused at the centromeric end, making it difficult to trace the individual SCs from end to end. **(F)** *MLH1 foci cannot be assigned with certainty to a single SC:* The arrow indicates an MLH1 focus located at

because MLH1 may not be present at prospective CO sites during the entire time window of observation, and/or the immunofluorescence labeling of MLH1 may fail at some of the CO sites. In addition, it is possible that there is more than one CO type, and that MLH1 does not mark all types. This is not a merely theoretical possibility: In yeast, meiotic COs are formed by at least three different pathways, only one of which depends on Mlh1 (12); only the Mlh1-dependent COs, which comprise ~70% of all COs in yeast, are thought to display interference (13). In tomato, MLH1 marks specifically a subset of strongly interfering COs (14). *Arabidopsis* also has more than one CO type, and it seems likely that MLH1 is not involved in all types (reviewed in 15). For the MLH1-independent CO pathways, no immunofluorescence markers are available yet. Therefore, as we have argued above for LNs, the usefulness of MLH1 foci as CO markers should be validated in every species, e.g., by comparing the numbers of MLH1 foci per chromosome or chromosome arm with the numbers of genetic exchanges or chiasmata.

In mouse, chiasmata can be used for CO interference analysis, although the accuracy of chiasma localization is limited (16). LNs have been recognized in ultrastructural preparations of mouse spermatocytes, but it is as yet not feasible to unambiguously identify every individual LN in large numbers of mouse spermatocytes (17). MLH1 foci, on the other hand, can be easily identified and located along mouse SCs (18), and we will therefore describe here how to analyze CO interference in the mouse primarily by determining MLH1 focus positions along SCs. At the level of chromosomes, the numbers of MLH1 foci correlate well with those of chiasmata (16) and genetic exchanges (4). Although some few COs are formed in MLH1-deficient mice (19), there are reasons to believe (discussed in 20) that in wild-type mice all prospective COs are marked by MLH1 foci, which greatly facilitates the correlation of cytological measurements with genetic data. Another feature that facilitates cytological (and genetic) interference analysis in the mouse is that all chromosomes are acrocentric, so that there are no complications regarding interference across the centromere (discussed in 21). Furthermore, antibodies are available against several proteins that are thought to be involved in early and intermediate steps of mouse meiotic recombination. Because some of these proteins form foci along SCs, this allows the analysis of interference in stages of meiotic recombination that precede CO formation (2).

Fig. 21.1. (continued) the crossing of two SCs. It is uncertain to which of the two SCs the MLH1 focus belongs. (**G** and **H**) *The SCs and/or foci are not in focus:* (Panel **G**) shows the nucleus in a single focal plane, whereas (panel **H**) represents an overlay of two different focal planes of the same nucleus. The arrows indicate MLH1 foci that are visible in only one of the two focal planes. Foci on the same SC that are in different focal planes can occur in nuclei that are not well spread.

1.2. Estimating the Strength of Interference Using the Gamma Model

The gamma distribution has repeatedly emerged as a most useful model for estimating the strength of interference from the precise positions of chiasmata/COs/LNs/foci (discussed in 5, 22). It is commonly used for the analysis of distances between events along a linear axis, and is described in most textbooks on probability theory and stochastic processes. In the context of CO interference analysis, the gamma distribution describes the frequency distribution of inter-CO distances that one would get if (imaginary or real) CO precursors would be randomly placed along the bivalent, but only every n th precursor would yield a CO. Thus, after a CO has been generated, CO formation is suppressed at the next $n-1$ precursors. Consequently, the probability of additional COs in the vicinity of an existing one is reduced, which corresponds with the phenomenon of CO interference. Obviously, if n equals 1, COs will be randomly distributed along the SC, and there is no CO interference. When moving along a bivalent, the probability of *not* encountering a CO will then decrease exponentially with the traversed distance, and the inter-CO distances will thus display a negative exponential distribution. If λ represents the mean number of CO precursors (and thus COs in this particular case) per unit length, the probability density function (p.d.f.) of inter-CO distances x is:

$$f(x; \lambda) = \lambda e^{-\lambda x}, x > 0$$

On the other hand, if n is greater than 1, the inter-CO distances will equal the sum of n exponential variables, and the resulting frequency distribution of inter-CO distances is called a gamma-distribution. For a given integer value of n and on average λ CO precursors per unit length, the p.d.f. of inter-CO distances x is:

$$f(x|\lambda, n) = \frac{\lambda^n x^{n-1} e^{-\lambda x}}{(n-1)!}$$

(for a detailed explanation, see textbooks and Ref. 2). The greater n is above 1, the stronger CO suppression near existing COs is, and the stronger (positive CO) interference is. n is therefore called the interference parameter of the gamma model, and it can serve as a measure for the strength of interference.

If one assumes that the biological mechanism of interference conforms to the gamma model (i.e., the CO precursors are real, and there is a mechanism that counts them), n can only be a positive integer. However, if one only wants to use the gamma model as a device for estimating the strength of interference, it is not necessary to assume this. To indicate this, we will further denote the interference parameter as ν , which represents positive,

but not necessarily integer values, as distinct from n , which represents integer values only. If the interference parameter is not necessarily an integer, a more general form of the gamma p.d.f. applies:

$$f(x|\lambda, \nu) = \frac{\lambda^\nu x^{\nu-1} e^{-\lambda x}}{\Gamma(\nu)}, \nu > 0$$

[$\Gamma()$ is the so-called gamma-function. It can be seen as a normalizing constant that ensures that for a given ν value the probabilities of all inter-CO distances add up to unity.] Thus, one can obtain an estimate of the strength of interference by determining for which ν value the observed inter-CO distances fit best to this p.d.f. This can be done by maximum likelihood estimation, using standard statistics software.

Figure 21.2A shows the shape of the gamma distribution for various ν values. For a given average inter-CO distance, this shape is narrower for higher ν values, implying that the stronger interference is, the more evenly COs are spaced, which represents another aspect of interference. ν is thus a measure for the evenness

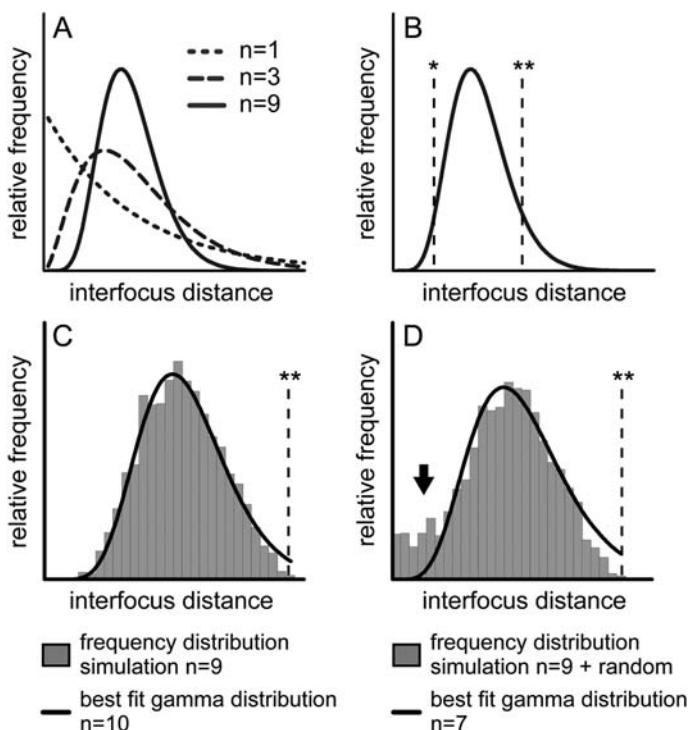


Fig. 21.2. The gamma distribution as a tool for measuring the strength of interference. (A) Shape of gamma distributions for different n (or ν) values. The average inter-focus distance is the same for all distributions shown. As n (or ν) increases, the very short and very long distances become sparser, and the distributions become narrower and more symmetrical. (B) The range of interfocus distances that can be observed is limited: the

of CO spacing, but *not* for the average inter-CO distance. Because prospective CO positions have probably been determined at the time when MLH1 foci are formed, the above consideration applies also to MLH1 foci.

Several factors other than interference may influence the estimate of ν :

1. The range of observable interfocus distances is limited: Although very long or short interfocus distances are scarce at high ν values, the theoretically possible interfocus distances range from zero to infinity for all ν values (Fig. 21.2A). The observable interfocus distances, however, range between the lower limit of resolution of the light microscope ($\sim 0.2 \mu\text{m}$) and the length of the SC. As is illustrated in Fig. 21.2B and C, this leads to a narrower distribution of interfocus distances and, consequently, to a higher ν estimate (see below and Ref. 2).
2. If there were no other factors that determine focus (or CO) positions than an interference mechanism that corresponds to the gamma model, foci are expected to be uniformly distributed along the SCs (i.e., the average focus density will be the same for all positions along the SC), but this is not observed: In most or all eukaryotes, including mouse (2, 18), the pericentromeric region is virtually focus-free. Because all mouse chromosomes are acrocentric, this further reduces the maximum observable interfocus distances in this species. In species with non-acrocentric chromosomes, this paucity of COs in the pericentromeric region creates an additional complication, because transcentromeric interfocus distances are not only determined by the interference mechanism, but also by factors that specifically exclude foci from this region; this results in underestimation of ν (spacing becomes more uneven). Regions where the focus density is higher-than average have also been reported, for instance the

←

Fig. 21.2. (continued) lower limit is the smallest interfocus distance that can be discerned by light microscopy ($\sim 0.2 \mu\text{m}$, indicated by *), and the upper limit is the length of the SC (indicated by **). This limitation leads to overestimation of n (or ν). (C) Simulation showing the effect of the limited range of observable interfocus distances on the estimate of n (or ν). In the simulation, the interfocus distances were gamma distributed with interference parameter $n=9$. Then all distances greater than $0.2 \mu\text{m}$ and smaller than one SC-length (indicated by **) were selected and fitted to the gamma distribution. The best fit was obtained for $n=10$. (D) Simulation showing that background foci result in underestimation of n (or ν). In the simulation, the distances between real MLH1 foci were gamma-distributed with interference parameter $n=9$. In addition, some background foci (10% of the number of MLH1 foci) were interspersed at random among the MLH1 foci. Subsequently, all observable interfocus distances (MLH1-MLH1, MLH1-background, and background-background) were determined, and fitted to the gamma distribution. The best fit was obtained for $n=7$, with a bad quality of the fit. Note that small interfocus distances are extremely rare if $n=9$ (panel C), but that they do occur (arrow in D) if there are randomly interspersed background foci.

centromere-distal subtelomeric region in the mouse, particularly in male meiosis (2, 18). The effect of this on the ν estimate depends on how this preferential subtelomeric positioning has arisen (e.g. by a higher density of focus precursors? Or by preferential placement of the first CO in this region?), but this is unknown.

3. In various species, evidence exists for a mechanism other than interference that ensures at least one CO per bivalent (the “obligate CO”) (discussed in 23), which is likely marked by MLH1 (20). The mechanism that ensures at least one CO/MLH1 focus has not been elucidated, and it is therefore uncertain how it influences the inter-CO and inter-focus distances and the estimate of ν .
4. Background signals can be mistaken for foci. If the average inter-focus distance is of the same order as the SC length and interference is strong, as is the case for MLH1 foci in the mouse, this has a substantial effect on the estimate of ν (**Fig. 21.2D**). It is therefore important to keep the background level as low as possible, and to make sure that the MLH1 signal is sufficiently strong so that foci can be distinguished unambiguously from background signals.
5. Foci can be missed because the immunofluorescence labeling fails. Additionally, as foci are tiny structures, they can escape notice if they were not in the focal plane during microscopic observation (**Fig. 21.1G and H**). Cytological interference analysis requires therefore entirely flat spreads of meiotic nuclei. Missing a focus has little effect on the ν estimates for mouse MLH1 foci, because it usually does not lead to observation of a single long interfocus distance where two short distances should have been observed, but rather leads to loss of an interfocus distance. If the average interfocus distance is substantially smaller than the length of the SC, as is the case for mouse MSH4 foci (6), missing foci will result in underestimation of ν because the spacing will appear to become more uneven.

1.3. Cytological versus Genetic CO Interference

Even if there is a 1:1 correlation between the occurrence of an MLH1 focus and the formation of a CO, the ν estimates based on inter-MLH1 distances are only equivalent to the ν estimates based on genetic distances if the MLH1 focus map and the genetic map are entirely colinear (discussed in 24). In male mouse meiosis they are not colinear, because MLH1 foci are not uniformly distributed along the SCs in spermatocytes (2, 18), whereas COs are uniformly distributed along the genetic maps, because the genetic distances are expressed in centiMorgans, and are therefore proportional to the number of COs. For male meiosis, it is thus possible that ν estimates based on inter-MLH1 distances differ from estimates

based on genetic distances, but this issue has not been analyzed. In female mouse meiosis, MLH1 foci are nearly uniformly distributed along the SCs (2). Furthermore, genetic high-density maps of polymorphic markers have allowed the estimation of ν for each chromosome from segregation data reflecting female meiosis (4). The resulting ν estimates were close to the ν estimates based on inter-MLH1 distances (2), which supports the assumption of a 1:1 correlation between MLH1 foci and COs in mouse meiosis.

2. Materials (see Note 1)

2.1. Spreading Spermatocytes from Testicular Tubules

2.1.1. Materials and Equipment

1. Male mouse, ~30-days old or adult (*see Notes 2 and 3*).
 2. Scissors and forceps for dissecting the mouse testis.
 3. Petri dish.
 4. Micropipettes 1–20 μ L, 5–50 μ L, and 50–200 μ L.
 5. Clean slides: Keep plain glass slides (Menzel) for at least 20 min in a slide rack in 100% ethanol. Submerge rack with slides in a beaker containing 0.2- μ m filtered water (*see Note 4*) and boil them for 25 min. Then drain and air dry the slides while protecting them from dust, and store them in a dust-proof box. The cleaned slides can be used for months.
 6. Watchmaker's forceps (e.g. Baltzer no. 7).
 7. Moisture-saturated box or large Petri dish (e.g., 15 \times 15 cm): choose a box or Petri dish with an entirely flat bottom. Cover the bottom with filter paper, saturate the filter paper with water, flatten the filter paper, and put two or four skewers on it that will serve as supports for the slides. Close the box at least one hour before use.
 8. Coplin jars.
 9. Phase-contrast microscope equipped with dry 25 \times and/or 40 \times objective lenses.
 10. Aluminum foil.
- 2.1.2. Solutions*
1. 1% (w/v) paraformaldehyde (PFA), pH 9.2, 0.15% (v/v) Triton X-100, freshly prepared. Add 1 g of PFA (Merck no. 4005) to 80 mL water, add two drops of 1 N NaOH, heat to 60°C while stirring until the solution is clear, and cool the solution quickly to 20°C on a mixture of water and ice. Using a micropipette, adjust the pH to 9.2 by adding small droplets (5–25 μ L) of 1 N or 0.1 N NaOH. Add water to a final volume of 100 mL and filter the solution through a 0.2- μ m filter. Add 150 μ L of Triton X-100 and let it disperse. Use within 8 h.

2. Hypotonic buffer: 30 mM Tris-HCl pH 8.2, 17 mM sodium citrate, 5 mM EDTA, 50 mM sucrose. Add dithiothreitol (DTT) from a 0.5 M stock solution to a final concentration of 5 mM. Prepare fresh and use the solution within 2 h after addition of DTT.
3. 100 mM sucrose. Filter-sterilize and store at room temperature.
4. Phosphate-buffered saline (PBS): 140 mM NaCl, 1.9 mM NaH₂PO₄, 8.9 mM Na₂HPO₄; check pH=7.3–7.4. Filter-sterilize and store at room temperature.
5. Water filtered through a 0.2-μm filter.
6. 0.4% (v/v) Photoflo (Kodak) in filtered water.

2.2. Spreading Spermatocytes from a Cell Suspension

2.2.1. Preparing a Cell Suspension from one or two Mouse Testes (see Note 5)

2.2.1.1. Materials and Equipment

2.2.1.2. Solutions at Room Temperature

2.2.1.3. Solutions on Ice

2.2.2. Preparing Spreads from a Cell Suspension

2.2.2.1. Materials and Equipment

1. Male mouse, ~30-days old or adult (*see Notes 2 and 3*).
2. Scissors and forceps for dissecting the mouse testis.
3. Conical plastic 15 mL centrifuge tubes.
4. Plastic Pasteur pipettes.
5. Small plastic funnel.
6. 70 μm mesh nylon filter.
7. Water bath (shaking) at 32°C.
8. Hematocytometer.
9. Table-top centrifuge
1. Testis cell isolation medium (TIM): 104 mM NaCl, 45 mM KCl, 1.2 mM MgSO₄, 0.6 mM KH₂PO₄, 0.1% (w/v) glucose, 6 mM sodium lactate, 1 mM sodium pyruvate; adjust the pH to 7.3 with 1 N HCl and filter-sterilize. Store at room temperature.
1. Collagenase (Sigma cat. no. C 0130): 20 mg/mL, freshly dissolved in TIM.
2. Trypsin TRL, Worthington 3703: 7 mg/mL, freshly dissolved in TIM.
3. Trypsin inhibitor, Sigma T9003: 20 mg/mL, freshly dissolved in TIM.
4. DNase I, Boehringer/Roche 104159: 400 μg/mL in TIM; can be stored at –20°C for months.
1. Micropipettes 1–20 μL and 5–50 μL.
2. Clean slides: *see Section 2.1.1*.
3. Moisture-saturated box or large Petri dish (e.g., 15 × 15 cm): *see Section 2.1.1*.
4. Coplin jars.

5. Phase-contrast microscope equipped with dry 25 × and/or 40 × objective lenses.
6. Aluminum foil.

2.2.2.2. Solutions

See Section 2.1.2

2.3. Spreading Oocytes

2.3.1. Materials and Equipment

1. Pregnant mouse (*see Note 6*).
2. Scissors and forceps for dissecting mouse fetuses.
3. Petri dishes.
4. Dissection microscope (magnification 10–100 ×), preferably with glass fiber illumination.
5. Clean slides (*see Section 2.2.1*).
6. Phase-contrast microscope.
7. Preparation needles.
8. Micropipettes 1–20 µL, 5–50 µL, and 50–200 µL.
9. Moisture-saturated box or large Petri dish (*see Section 2.1.1*).
10. Incubator at 37°C.
11. Coplin jars.

2.3.2. Solutions

1. 2% (w/v) paraformaldehyde (PFA), pH 9.0, 0.15% (v/v) Triton X-100, freshly prepared. Add 2 g of PFA (Merck no. 4005) to 80 mL water, add two drops of 1 N NaOH, heat to 60°C while stirring until the solution is clear, and cool the solution quickly to 20°C on a mixture of water and ice. Using a micropipette, adjust the pH to 9.0 by adding small droplets (5–25 µL) of 1 N or 0.1 N NaOH. Add water to a final volume of 100 mL and filter the solution through a 0.2-µm filter. Add 150 µL of Triton X-100 and let it disperse. Use within 8 h.
2. Hypotonic buffer (*see Section 2.1.2*).
3. PBS (*see Section 2.1.2*).
4. Water filtered through a 0.2-µm filter.

2.4. Immunocytochemical Labeling

2.4.1. Materials and Equipment

1. Affinity-purified (*see Note 7*) or monoclonal antibodies against a focus component (e.g., MLH1) and against a component of the axial elements of SCs (e.g., SYCP3), elicited in different species.
2. Appropriate secondary antibodies conjugated to different fluorescent labels.
3. Moisture-saturated box or large Petri dish (*see Section 2.1.1*).
4. Coplin jars.
5. Colorless nail polish.

2.4.2. Solutions

1. PBS (*see Section 2.1.2*).
2. Blocking solution: 5% (w/v) non-fat dry milk, 5% (v/v) goat serum (*see Note 8*), 0.01% (w/v) NaN_3 (*see Note 9*) in PBS; adjust the pH to 7.4 with 1 N NaOH, and store at -20°C . Before use, add phenyl methyl sulfonyl fluoride (PMSF) from a 1 M stock solution in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM (*see Note 9*), and centrifuge before use for 30 min at $\sim 16,000g$ (*see Note 10*).
3. FITC (fluorescein isothiocyanate) buffer: 150 mM NaCl, 50 mM NaHCO_3 , adjusted to pH 9.5 with NaOH; store at room temperature, and filter before use through a 0.2 μm filter.
4. Mounting medium with 4',6-diamidino-2-phenylindole (DAPI): Add 10 μL DAPI stock solution (1 mg/mL) to 990 μL Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA), and mix. Store the solution in the dark at 4°C and use within 2 weeks.

2.5. Acquiring and Processing Images

1. Fluorescence microscope, equipped with appropriate combinations of excitation and emission filters for the separate detection of FITC, Texas Red (TR), and DAPI signals in the same preparation, and preferably equipped with a CCD camera (*see Note 11*) and a computer-controlled motorized stage (*see Note 12*).
2. Image-processing software, e.g., Adobe Photoshop, or Image J (*see Note 13*).
3. Calibration slide.

2.6. Chromosome Painting of Immunofluorescence-labeled Slides**2.6.1. Materials and Equipment**

1. Chromosome paint probes (*see Note 14*).
2. Graded ethanol series in Coplin jars (70–90% and 100% ethanol).
3. Kimwipes.
4. Photo-paper glue (rubber cement).
5. Scalpel.
6. Coplin jars.
7. Incubator or heating plate at 37°C .
8. Heating plate at 65°C (optional).
9. Waterbaths at 37°C , 45°C , 65°C , and 80°C .
10. Moisture-saturated box or large Petri dish (*see Section 2.1.1*).
11. Avidin-Texas Red (TR) conjugate (e.g. Jackson Immuno Research).
12. Biotinylated anti-avidin (e.g. Jackson ImmunoResearch).

2.6.2. Solutions

1. PBS (*see Section 2.1.2*).
2. 20 × SSC: 3 M NaCl, 0.3 M sodium citrate, adjust pH to 7.0 with a few drops of 1 N HCl. Autoclave and store at room temperature.
3. 2 × SSC and 1 × SSC, both prepared from a 20 × SSC stock solution. Autoclave and store at room temperature.
4. Deionized formamide: stir 2 g of mixed bed resin (AG 501-X8, Bio Rad) with 50 mL of formamide for 2 h at room temperature. Remove the resin beads and store the deionized formamide in single use aliquots at –20°C. It can be used for months.
5. Denaturation solution: 70% (v/v) deionized formamide, 2 × SSC. Prepare by mixing 7 vol of deionized formamide, 1 vol of 20 × SSC and 2 vol of water. Prepare fresh.
6. Stringency wash buffer: 50% (v/v) deionized formamide, 1 × SSC, freshly prepared.
7. Detergent wash buffer: 4 × SSC; 0.05% (v/v) Tween-20.
8. Blocking solution: 5% (w/v) non-fat dry milk, 5% (v/v) goat serum (*see Note 8*), 0.01% (w/v) NaN₃ (*see Note 9*) in detergent wash buffer. Store in single use aliquots at –20°C. It can be used for months. Before use, add PMSF from a 1 M stock solution in DMSO (dimethyl sulfoxide) to a final concentration of 1 mM (*see Note 9*), and centrifuge for 30 min at ~16,000*g* (*see Note 10*); use within 2 h.
9. Filtered water.
10. FITC (fluorescein isothiocyanate) buffer: (*see Section 2.4.2*).
11. Mounting medium with DAPI (*see Section 2.4.2*).
12. 0.01% (w/v) pepsin (e.g. Sigma-Aldrich; cat. nr. P7000) in 10 mM HCl, freshly prepared from a 100 mM or 1 M HCl stock.

**2.7. Measuring
Interfocus Distances**

Personal computer and measuring software (*see Note 15*).

**2.8. Analysis of the
Interfocus Distances**

Personal computer and statistics software (*see Notes 16 and 43*).

3. Methods

The procedures described below are optimized for cytological analysis of interference. For reasons explained in the introduction, it is important (a) that the spread preparations of oocytes and spermatocytes are entirely flat, (b) that the background of the

immunofluorescent labeling of foci is minimal, particularly background dots, which can be mistaken for foci, (c) that stretching or breakage of SCs is avoided, and (d) that the signal-to-background ratio of the labeling of the foci is sufficiently high, so that all foci on an SC can be unambiguously distinguished from background.

3.1. Spreading Spermatocytes from Testicular Tubules (see Note 5)

1. Kill the mouse by cervical dislocation, and remove and decapsulate the testes.
2. Transfer the seminiferous tubules to a Petri dish with PBS until further use (*see Note 17*).
3. Place a small amount of tubules for ~30–60 min in hypotonic buffer (*see Note 18*).
4. Place a 20-μL droplet of 100 mM sucrose on a clean slide.
5. Transfer the swollen tubules from the hypotonic buffer to the droplet of 100 mM sucrose.
6. Disrupt the tubules between the tips of two fine watchmaker forceps.
7. Make a slightly cloudy cell suspension of the disrupted tubules (*see Note 19*), using a 1–10 μL pipette.
8. Remove big chunks of tubule-remnants from the suspension (*see Note 20*).
9. Dip a clean slide into the PFA solution. Tilt the slide, so that a drop of PFA solution forms at one of its corners.
10. Place the cell-suspension into the PFA drop, and disperse the PFA drop with cells slowly over the slide by swerving the slide gently.
11. Place the slide in a closed, moisture-saturated box for ~2 h.
12. Put the lid ajar for ~30 min (*see Note 21*).
13. Remove lid completely and allow slides to dry, which takes about 30 min (*see Note 21*).
14. Rinse the slides once by dipping them in filtered water (*see Note 1*), dip them twice in 0.4% Photoflo, and then rinse them with 0.4% Photoflo, using a pipette.
15. Air dry the slides in a slide rack at room temperature, while protecting them from dust.
16. Wrap in aluminum foil, and store at –80°C, where they are stable for months.

3.2. Spreading Spermatocytes from a Cell Suspension

3.2.1. Preparing a Cell Suspension from one or two Mouse Testes (see Note 5)

1. Isolate the testes, remove the capsules, and transfer the testes to a 15 mL tube containing 2 mL TIM.
2. Add 200 μL collagenase solution.
3. Incubate the tube for 55 min at 32°C in a shaking water bath (*see Note 22*).

4. Add TIM to a final volume of 15 mL; spin the tubules down for a few seconds at 60*g*. Remove the supernatant. Do this three times (*see Note 23*).
5. Resuspend the tubules in 2 mL TIM, add 200 µL trypsin solution and 20 µL DNase I solution, and incubate for 15 min at 32°C in a shaking water bath (*see Note 24*).
6. Add 500 µL trypsin inhibitor solution and 50 µL DNase I solution, and mix.
7. Pipette the trypsin-digested tubules/cells up and down for about 2 min, using a plastic Pasteur pipette with a wide mouth. You should get a single-cell suspension, which you can check using a hematocytometer.
8. Put a small plastic funnel onto a 15 mL tube, and put the 70 µm mesh nylon filter into the funnel. Pre-wet the nylon filter with TIM, and sieve the cell suspension through the nylon filter to remove the last cell clumps.
9. Add TIM to a final volume of 15 mL, mix well, and take a 50-µL sample for counting (*see Note 25*). Spin the remainder of the suspension for 5 min at room temp at 60*g*.
10. Remove the supernatant and add 15 µL DNase I solution to the cell pellet. Tap the cell pellet loose, and resuspend the cells in 15 mL TIM (*see Note 26*). Spin for 5 min at room temp at 60*g*.
11. Remove the supernatant and add 15 µL DNase I solution to the cell pellet. Tap the cell pellet loose, and resuspend the cells in 15 mL PBS. Spin for 5 min at 60*g* at room temp.
12. Remove the supernatant and add 15 µL DNase I solution to the cell pellet. Tap the cell pellet loose. For spreading by the dry-down technique, resuspend the cells in hypotonic buffer to a final concentration of ~3 × 10⁷ cells/mL.

3.2.2. Preparing Spreads from a Cell Suspension (see Note 5)

1. Start from a cell suspension prepared according to **Section 3.2.1**, with ~3 × 10⁷ cells/mL in PBS (*see Note 19*).
2. Mix equal volumes of this cell-suspension and hypotonic buffer and incubate the cells for ~30–60 min at room temperature (*see Note 18*). You will need 10 µL of cell suspension + 10 µL of hypotonic buffer per slide.
3. Add an equal volume of 100 mM sucrose to the mixture prepared in step 2.
4. Dip a clean slide into the PFA solution. Tilt the slide so that a drop of PFA solution forms at one of its corners.
5. Place 40 µL of the cell-suspension prepared in step 3 into the PFA drop, and disperse the PFA drop with cells slowly over the slide by swerving the slide gently.
6. Continue with steps 11–16 of **Section 3.1**.

3.3. Spreading Oocytes

1. Kill the pregnant mouse by cervical dislocation, and remove and decapitate the fetuses.
2. Remove the ovaries from the female fetuses and store them in fresh PBS at room temperature until further use (*see Note 17*).
3. Place the ovary in hypotonic buffer for 15 min.
4. Place a clean slide with a droplet (~25 µL) of 100 mM sucrose under the dissection microscope.
5. Transfer an ovary to the droplet of 100 mM sucrose and decapsulate the ovary.
6. Using the dissection needles, puncture and gently shake the ovary in the sucrose for 5 min (*see Note 27*).
7. Remove as much debris as possible from the drop of 100 mM sucrose (*see Note 20*).
8. Transfer the slide to a moisture-saturated box and wait for 5 min.
9. Add an equal amount of 2% PFA (~25 µL) to the drop of 100 mM sucrose, and close the moisture-saturated box for ~1 h.
10. Remove the lid and wait for ~30 min.
11. Transfer the slide to a 37°C incubator and allow the sucrose/PFA mixture to dry, until it has the consistency of a toffee (*see Note 28*). This takes about 2 h.
12. Rinse the slides three times by dipping and washing them in filtered water, or until the dried sucrose/PFA mixture is no longer visible (*see Note 29*), dip them twice in 0.4% Photoflo, and then rinse them with 0.4% Photoflo, using a pipette.
13. Air dry the slides in a slide rack at room temperature, while protecting them from dust.
14. Wrap the slides in aluminum foil, and store them at -80°C. The antigenicity of SCs and foci is stable for months.

**3.4. Immunocytochemical Labeling
(*see Note 30*)**

1. If the slides have been stored at -80°C, allow them to come to room temperature while still in the aluminum foil (*see Note 31*).
2. Wash the slides three times for 5 min in PBS.
3. Cover each slide with 500 µL blocking buffer and incubate them for 30 min in a closed, moisture-saturated box at room temperature.
4. Per slide, prepare 100 µL of a suitable dilution of the primary antibody in blocking solution (*see Note 32*) and centrifuge the dilution for 30 min at ~16,000g at 4°C (*see Note 33*).

5. Add 100 µL of the supernatant from step 4 (i.e., the diluted and centrifuged primary antibody) to each slide, and cover the slide with a coverslip.
6. Transfer the slides to a closed moisture-saturated box and incubate successively for 30 min at 37°C, overnight at 4°C, 15 min at room temperature, and 30 min at 37°C (*see Note 34*).
7. Wash the slides three times for 5 min in PBS.
8. Cover each slide with 500 µL blocking solution and incubate for 30 min in a closed, moisture-saturated box at room temperature.
9. Per slide, prepare 100 µL of the secondary antibody diluted in blocking solution (*see Note 32*) and centrifuge the dilution for 30 min at ~16,000g at 4°C.
10. Add 100 µL of the secondary antibody diluted in blocking solution to each slide, and cover with a coverslip.
11. Transfer the slides to a closed, moisture-saturated box and incubate slides for 1 h at 37°C.
12. Wash the slides three times for 5 min in PBS.
13. Dip the slides once in filtered water and once in FITC-buffer.
14. Mount the slides in 5 µL Vectashield + DAPI (*see Notes 35 and 36*), and seal the cover slide with nail polish.
15. If the slides are not viewed on the same day, store them at -20°C (*see Note 37*).

3.5. Acquiring and Processing Images

1. If the slides have been stored at -20°C, allow them to come to room temperature before applying the immersion oil.
2. Micrograph the immunofluorescent-labeled nuclei using the fluorescence microscope and CCD camera.
3. For each micrograph, note down which set of lenses (objective etc.) has been used.
4. If the chromosomes are to be identified using FISH (*see Section 3.6*), note the positions of the micrographed nuclei (*see Note 12*).
5. For each set of lenses used, micrograph the calibration slide. This information is needed later for the measurements.
6. Process and overlay the FITC, TR, and DAPI images of each nucleus, leaving the resolution of the images unaltered.

3.6. Chromosome Painting of Immunofluorescence-Labeled Slides

1. Remove the immersion oil from the slide by carefully wiping the slide with a tissue with 70% ethanol (*see Note 38*).
2. Remove the nail polish from the slide by scratching around the coverslip with a scalpel.

3. Wash in PBS until the coverslip comes off.
4. Wash twice for 5 min in PBS.
5. Dehydrate the slide by washing successively for 2×2 min in 70% ethanol, 2×2 min in 90% ethanol and 5 min in 100% ethanol.
6. Air dry the slide.
7. Incubate the slide for 15 min in 0.01% pepsin in 10 mM HCl at 37°C.
8. Wash the slide three times for 5 min in $2 \times$ SSC.
9. Dehydrate and air dry the slide (*see* step 5 and 6, this section).
10. Age the slide overnight at room temperature or for 90 min at 65°C.
11. Denature the chromosome paint probe: warm the probe (*see Note 14*) to 37°C, vortex and spin down. Use 3 µL probe and add hybridization buffer to a final volume of 15 µL. Incubate the mixture for 10 min at 65°C.
12. Spin the denatured probe-mix down and incubate it for 30–60 min at 37°C.
13. Denature the slide for 2 min at 80°C in the prewarmed denaturation solution.
14. Quench the slide in ice-cold 70% ethanol.
15. Dehydrate the slide (*see* step 5 and 6, this section).
16. Apply 15 µL of denatured probe mix per slide, and cover it with a coverslip.
17. Remove air bubbles and seal the edges of the coverslip with photo-paper glue (rubber cement).
18. Incubate the slide in a sealed, humidified box at 37°C for 18–45 h.
19. Prewarm the following to 45°C in a water bath at least 30 min before starting the posthybridization washes of the slides (the temperature of the solutions in the Coplin jars should be 45°C; check this):
 - a) two Coplin jars with stringency wash solution
 - b) two Coplin jars with $1 \times$ SCC
 - c) one Coplin jar with detergent wash solution
20. Incubate the slide for 5 min in $2 \times$ SSC at room temperature to soften the glue that seals the edges of the coverslips.
21. Remove the rubber cement glue (try to peel it off) and wash the slide in $2 \times$ SSC at room temperature until the coverslip comes off.
22. Wash the slide two times for 5 min in the prewarmed stringency wash buffer at 45°C.

23. Wash the slide two times for 5 min in the prewarmed 1 × SSC at 45°C.
24. Wash the slide 5 min in the prewarmed detergent wash buffer.
25. Wash the slide two times for 5 min in the detergent wash buffer at room temperature.
26. For FITC-labeled probes, continue with step 35; for biotin-labeled probes, prepare 200 µL avidin-TR diluted in blocking solution (*see Note 39*) per slide and centrifuge for 30 min at ~16,000g at 4°C.
27. Apply 100 µL of the avidin-TR diluted in blocking solution to each slide and cover it with a coverslip.
28. Incubate the slide for 20 min at 37°C in a prewarmed, moisture-saturated box.
29. Remove the coverslip and wash the slide three times for 5 min in detergent wash buffer at room temperature.
30. Per slide, prepare 100 µL biotinylated anti-avidin antibody diluted in blocking solution (*see Note 32*) and centrifuge for 30 min at ~16,000g at 4°C.
31. Apply the biotinylated anti-avidin diluted in blocking solution to the slide and cover it with a coverslip.
32. Incubate the slide for 20 min at 37°C in a prewarmed, moisture-saturated box.
33. Remove the coverslip and wash the slide three times for 5 min in detergent wash buffer at room temperature.
34. Repeat steps 27–29 of this section to amplify the Texas Red signal.
35. Dip the slides once in filtered water and once in FITC-buffer.
36. Mount the slides in 5 µL mounting medium with DAPI and seal the edges of the coverslip with nail polish.
37. Micrograph the nuclei that were previously micrographed, the positions of which were marked.
38. Using image-processing software, process and overlay the FITC, TR and DAPI images of each nucleus; use the DAPI signals as guides when aligning the FISH image with the immunofluorescence images.

3.7. Measuring Inter-focus Distances

The next steps can be performed using Adobe Photoshop or another image-processing software package:

1. Identify and mark the SCs of the FISH-labeled chromosomes.
2. Identify and mark the centromeric ends of the SCs (*see Notes 35 and 36.*)

3. Select only bivalents (SCs) that fulfill the following requirements (**Fig. 21.1** shows several examples of nuclei that are not suitable for measurements):
 - a) the SCs and all foci should be in focus (*see Section 1, and Notes 18, 19, and 40*).
 - b) the SCs should be clearly traceable from one end to the other (*see Note 36*).
 - c) the SCs should be intact and not appear to be stretched (*see Note 40*).
 - d) the signal-to-background ratio of foci and SCs has to be sufficiently high, so that all foci on an SC can be recognized unambiguously (*see Notes 4, 7, 10, 20, 21, 29, 30, 32–34, and 37*).
 - e) For a given SC, it should be unambiguously clear which foci should be assigned to it (*see Fig. 21.1F*).
4. For each set of lenses used for micrographing the cells, measure the known distances on the relevant micrograph of the calibration slide. Calculate the number of pixels per μm for each set of lenses.
5. Using the measuring software (*see Note 15*), measure the length (in pixels) of the selected chromosomes.
6. Determine the positions of the foci by measuring their distance (in pixels) to the centromere.
7. Use the information from step 4 of this section to calculate the length of the SCs in μm , and the positions of the foci, expressed as μm from the centromeric end.

3.8. Analysis of the Interfocus Distances

1. For each chromosome, calculate the inter-focus distances as fractions of the length of the SC (*see Note 41*).
2. Import the interfocus distances, expressed as fractions of the SC length into the GENSTAT program or an equivalent statistics program.
3. Obtain a provisional estimate of ν by fitting the interfocus distances to the gamma distribution (*see Note 42*).
4. Apply a correction of the provisional estimate of ν for the limited range of interfocus distances that can be observed (*see Note 43*).
5. It is advisable to compare in a graph the relative frequencies of interfocus distances with the relative frequencies expected based on the corrected ν value; this can, for instance, provide an indication that background dots may have been mistaken for foci (*see Fig. 21.2D*).

4. Notes



1. “Water” means deionized water with a resistance of 7 MΩ·cm or more.
2. For wild type, ~30-day-old mice are optimal for spermatocyte spreads, because they do not have many spermatozoa yet, the percentage of meiotic prophase cells in their testes is high, and all spermatocyte stages are represented. When testis cells spread on the slide, they tend to adhere to the tails of the spermatozoa, which causes cell clumping. Particularly if large numbers of cells have to be analyzed, it is important to avoid this. If only small numbers of cells have to be studied, it is also possible to start from adult mice.
3. For mice having a knockout mutation in a meiosis-related gene, it is important to test various ages. Several mutations cause apoptosis in mid-pachytene of male meiosis. Because this ultimately leads to depopulation of testicular tubules, the first round of spermatogenesis should be analyzed. For spermatocytes, ~20-day-old knockout mice are usually optimal.
4. Solutions that come in contact with cytological preparations are filtered through 0.2 µm filters, if possible. This helps reduce the number of background dots that could be mistaken as foci.
5. Suspensions of testicular cells usually give a higher yield of analyzable cells per testis than testicular tubules, and large numbers of slides can be prepared more efficiently from a cell suspension (*see Section 3.2.2*) than from testicular tubules (*see Section 3.2.1*). If only a few slides are required, it is more efficient to start from testicular tubules.
6. In female mice, meiotic prophase takes place during fetal development. The pachytene stage is passed through at day 17–19 post fertilization.
7. Affinity-purification of polyclonal antibodies is usually essential for keeping the background sufficiently low.
8. If one of the primary antibodies has been elicited in goat, use serum from a species other than goat for blocking, or the secondary antibody will recognize the blocking serum.
9. NaN₃ (azide) and PMSF (a protease inhibitor) are added to protect the spreads from bacterial contamination and proteolytic breakdown during prolonged incubation steps (*see Note 34*). PMSF is not stable in aqueous solutions and is therefore added just before use.
10. The blocking solution cannot be filtered through 0.2 µm filters, because the milk powder will clog the filter. Therefore, this solution is centrifuged to remove aggregates of denatured

protein and other particles (if any). Aggregates of denatured protein might stick to antibodies, and give rise to focus-like dots. Because such aggregates are formed continuously, they should be removed by centrifugation just before the blocking solution is used.

11. Only CCD cameras are sufficiently sensitive for recording all focus signals.
12. The cells will be micrographed twice, first for recording the immunofluorescent and DAPI signals, and later for recording the FISH (fluorescent *in situ* hybridization) signals. A motorized stage greatly facilitates retrieval of the cells. Alternatively, the rulers on the microscope can be used to note the positions of the nuclei.
13. Image J is available at <http://rsb.info.nih.gov/ij/>
14. We used STARFISH probes (Cambio, Cambridge, UK), labeled with biotin or FITC. Probes for different chromosomes can be applied simultaneously to the same slide if they carry different labels, and/or if they bind to chromosomes of largely different sizes.
15. We used the public domain image analysis program Object Image, which is an extended version of NIH Image by N. Vischer (University of Amsterdam) and is available at <http://simon.bio.uva.nl/object-image.html>. For measurements of the lengths of SCs and AEs and the positions of foci on SCs, we prepared a macro for the Object Image program, which allows batch measurement of SC lengths and positions of MLH1 foci and centromeres for several nuclei. The macro is available from F.G.P. Lhuissier (franck.lhuissier@keygene.com). Another program for length measurements is Micro-Measure, available at <http://www.colostate.edu/Dept/Biology/MicroMeasure.html>.
16. For fitting the inter-focus distances to the gamma distribution, we used the GENSTAT software package version 8 (VSN International, Hemel Hempstead, UK), which is Windows-compatible.
17. Tubules/ovaries can be kept for a short period of time in PBS at room temperature. However, process them as quickly as possible, or the quality of the spreads will fall off.
18. The time in hypotonic buffer is critical for the quality of the spread. The longer the cells spend in this buffer, the more they swell, until they finally burst. If cells have spent too little time in the hypotonic buffer, they will not flatten completely when applied to the slides. If they have spent too long in this buffer, they burst, and will not spread at all. If the tubules become fuzzy and whitish, the cells have almost certainly been too long in this buffer. Spermatocytes from mice having a

knockout mutation in some meiosis-related gene tend to burst more quickly in hypotonic buffer than spermatocytes from wild-type mice. It is therefore advisable to test a time series for the incubation of tubules in hypotonic buffer, and judge the spreads using a phase-contrast microscope. If the SCs have a high contrast, the cells are not well spread.

19. A high cell concentration inhibits the effective spreading of the nuclear contents and should be avoided. Reduce the amount of tubules or lower the cell concentration in the cell-suspension if the number of cells on the slides is too high.
20. Excess debris on the slide will cause the nuclei on the slide to stick to the debris, rupture and stick to other nuclei and debris, resulting in the formation of large clumps of unusable nuclei. Therefore remove as much debris as possible.
21. This is another critical step in the spreading procedure. If the slides dry too quickly, the cells do not spread well, which can result in a reduced accessibility of SCs and foci for antibodies.
22. The collagenase digests the connective tissue between the tubules, and the testis falls apart into tubules if you gently shake the tube. If this does not happen, add more collagenase and prolong the incubation.
23. The interstitial cells between the tubules are removed in this step.
24. Trypsin digests the connections between spermatogenic cells. If the cells are digested for too long, they will lyse; if they are not digested long enough, they remain associated in cell clumps. The DNase I will digest the DNA that is released by cells (if any) that already lyse during the 15 min incubation. DNA is very sticky, and will cause clumping of the cells, which is bad for the spreading procedure.
25. The two testes of an adult wild-type mouse yield about 10^8 cells.
26. If necessary, the cells can be kept for a short period of time at this step. However, process the cells further (*see Section 3.2.2*) as quickly as possible.
27. The combined puncturing and shaking will release the oocytes from the ovary. Avoid fragmenting the ovary while releasing the oocytes, because this will result in much debris on the slide.
28. The sucrose/PFA mixture should not be wet or sticky, but not too dry either. Pressing your finger onto the slide, should leave a clear fingerprint. If the slides get too dry, it will be harder to rinse the excess sucrose/PFA mixture from the slide.

29. Insufficient removal of PFA from the slide can reduce the antigenicity of target proteins and cause increased background levels in the immunofluorescent labeling, so make sure the slides are well rinsed before air-drying them.
30. During the entire immunofluorescent labeling procedure, the slides must not dry up, because this virtually precludes labeling of MLH1.
31. If the aluminum foil is removed before the slides have assumed room temperature, ice crystals may form on the preparation and damage the cells.
32. The optimal dilution depends on the antibody. Therefore, test a dilution-series for each antibody.
33. The diluted serum is centrifuged just before use, to remove aggregates of denatured antibodies (if any), because they might give rise to focus-like dots.
34. The optimal incubation times and temperatures depend on the antibody and the antigen. For instance, for both affinity-purified and monoclonal anti-MLH1 antibodies, the best signal-to-background ratio is obtained if the slides are incubated in the anti-MLH1 antibodies for, successively, 30 min at 37°C, ~64 h at 4°C, 15 min at room temperature, and 30 min at 37°C. Presumably the MLH1 antigens are difficult to access for antibodies. We inserted the prolonged incubation step at 4°C to allow the antibodies to diffuse to their target while keeping deterioration of the structure of the cells and bacterial growth on the slide to a minimum. However, for antibodies against other focus components, increasing the incubation time resulted in an increase of the background. For very strong antibodies against axial element components of the SC, an incubation of 1 h at room temperature can be sufficient. It is advisable to test several incubation times and temperatures for each antibody and judge the signal-to-background ratio before performing immunocytochemical labeling for measurements. As a rule, if the optimal incubation time is long (e.g. overnight or longer), incubate the slides most of the time at 4°C to avoid bacterial growth on the slide; *see also Note 9.*
35. The DAPI stain allows easy identification of the centromere, since the centromeric heterochromatin is generally intensely stained by DAPI. The DAPI signal is also needed for overlaying the images from the immunocytochemical labeling and the FISH.
36. The position of the centromere is not required for the analysis of the level of interference between the foci, since only the distances between the foci are required for this type of

analysis. However, if the distribution of foci along the chromosomes is also analyzed, the position of the centromere has to be known.

37. The best results are obtained if the slides are viewed immediately after labeling. If the slides are stored at -20°C , the immunofluorescent signal drops off initially, and then stabilizes at a lower level.
38. The ethanol will also soften the nail polish used to seal the edges of the coverslip onto the slide, so be careful: applying too much pressure or getting too much ethanol on the nail polish whilst wiping will cause the cover slip to slide and possibly damage the nuclei on the slide. Also, avoid allowing immersion oil to come into contact with the mounting medium, or the oil will disperse in fine droplets over the slide.
39. The optimal dilution varies for each avidin-TR conjugate. It is therefore advisable to test a dilution series for each conjugate.
40. If the SCs or parts of the SCs within a nucleus are not in the same focal plane, the nuclei are probably not well spread. If the SCs appear stretched, the nuclei are probably overspread. To solve these problems, vary in the spreading procedure (*see Sections 3.1 and 3.2*) the incubation time in hypotonic buffer, the amount of PFA on the slide, or the concentration of cells in the cell suspension.
41. Ideally, the interfocus distances should be expressed in the unit of length that is relevant to the interference mechanism. For instance, if the interference mechanism counts focus-precursors, the interfocus distances should be expressed in numbers of skipped precursors. If one would express the interfocus distances in μm of SC length, local variation in the precursor density (precursors/ μm SC length) would influence the ν estimate besides the interference mechanism. However, as long as the interference-relevant unit of length is not known, we express the interfocus distances as percentage of the length of the SC, so that at least one source of variation other than the interference mechanism (namely variation in cell spreading) is eliminated.
42. In GENSTAT one obtains an estimate of two parameters, k and b . k represents the interference parameter ν , and b is the number of (hypothetical or real) focus precursors per length unit (*see Section 1*). GENSTAT also calculates the deviance, which, together with the number of degrees of freedom, is a measure for the quality of the fit. One can derive a p value from it (e.g., using the CHIDIST function in Microsoft Excel), which represents the probability of an as-bad or worse fit, if the inter-focus distances are gamma-distributed.

43. The ν estimate provided by GENSTAT is a provisional one, because it does not take into account the limited range of observable inter-focus distances (see Section 1). The effect of this limitation on the ν estimate can be simulated for integer values of ν , using a macro in Microsoft Excel. This macro can be obtained from C. Heyting (c.heyting10@hetnet.nl) and is provided on the companion CD for this volume. For a given average number of foci per SC and a given integer ν value (the “input” ν value), the macro generates focus positions along an imaginary, long chain of linked-up SCs, then calculates the interfocus distances, and subsequently selects all intra-SC interfocus distances that can be discerned by light microscopy. In GENSTAT, these selected simulated distances can be fitted to the gamma distribution, to obtain an “output” ν value, which will be slightly higher than the input ν value (see Section 1). This simulation should be carried out for integer ν values that are close to the provisional ν estimate, until two output ν values are obtained that flank the provisional ν estimate. One can then estimate by interpolation the input ν value that corresponds to the provisional ν estimate. This input ν value is the corrected ν estimate.

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Chapter 22

Analysis of Telomere Dynamics in Mouse Spermatogenesis

Harry Scherthan

Abstract

A complex meiotic differentiation program generates genetically diverse haploid cells (gametes or spores) to compensate for the genome doubling that occurs at fertilization. To this end, homologous chromosomes must undergo pairing and recombination before they become partitioned in haploid sets by two consecutive meiotic divisions. Chromosome ends (telomeres) contain a protective complex that is crucial for genomic stability. In meiosis, telomeres become key players in the chromosome pairing process during prophase to the first meiotic division. At the onset of prophase I, telomeres attach to the nuclear envelope, about which they move and transiently cluster in a limited sector of the nuclear periphery. The dynamic clustering of telomeres (bouquet formation) occurs at the onset of the zygotene substage and supports homologue recognition, pairing and telomere DNA metabolism. The following chapter outlines the protocols that have been useful in studies on telomere dynamics and the frequency of earliest prophase I stages in testis suspensions of the mouse, and may be useful to address similar questions in particular mouse mutants that become increasingly available.

Key words: Telomere, spermatogenesis, FISH, DNA labeling, microscopy, mouse mutants.

1. Introduction

Meiosis lies at the heart of a complex cellular differentiation program that generates genetically diverse haploid gametes (or spores) and copes with the genome doubling at fertilization. Two successive meiotic divisions that lack an intervening DNA replication mediate this genome reduction. During the extended prophase to the first meiotic division, homologous chromosomes tightly pair and (in most species) recombine, which facilitates reductional chromosome segregation at MI. Without an intervening S phase, a subsequent mitosis-like division generates half the chromosome number in gametes or spores.

Telomeres of mammalian chromosomes contain 2–50 kb of $(T_2AG_3)_n$ DNA repeats (1, 2) that are complexed with telomere repeat binding factors (TRF1 and TRF2) and associated proteins, thereby forming a protective complex at the chromosome end that confers genomic stability (for a review see Ref. 3). During first meiotic prophase, telomeres are key players in the chromosome pairing process that facilitates homologue segregation. To this end, telomeres depart from their three-dimensionally scattered premeiotic (somatic) distribution (4) at the onset of prophase I and attach to the inner nuclear membrane whereabouts they move to cluster in a limited sector as defined by the adjacent centrosome (5). The dynamic rearrangement of telomeres and their clustering during a minimal time window at the onset of the zygotene stage of prophase I is known as bouquet formation, and is thought to contribute to homologue recognition, pairing, and telomere DNA metabolism (for reviews see Refs. 6–8).

Because of the vital functions telomeres perform in meiotic prophase and given the numerous mutants available in the mouse, it may be of interest to detect and study the dynamic redistribution of telomeres in mammalian meiosis and in spermatogenesis in particular. To this end, telomere dynamics can be deduced from the frequency of different distribution patterns observed in a cell type, e.g., a clustered or dispersed pattern among spermatocyte nuclei. Using such an approach, we observed that telomere dynamics are different among mammals, in male and female meioses and in different mutants (9–11).

In the male mouse, telomere clustering is a short-lived stage at the leptotene/zygotene transition (12) that only leaves a few such cells ($\sim 0.5\%$) detectable (11). Nevertheless, the universal occurrence of telomere clustering is highlighted by mouse mutants. Telomere dynamics are for instance altered in male *Atm*^{-/-} or *H2ax*^{-/-} mutants such that the bouquet frequency is increased up to 20-fold (11, 13, 14). Numerous protocols and methods are available to delineate telomeric T_2AG_3 repeats *in situ*. In our hands, long telomeric G- and C-strand oligonucleotides or peptide nucleic acid (PNA) probes have proven effective in telomere detection by fluorescence *in situ* hybridization (FISH). Here, I outline hapten labeling and telomere FISH protocols that have been successfully applied to study the dynamics of telomeres and the frequency of earliest prophase I stages in testis suspensions of the mouse (14–17).

2. Materials

2.1. General Lab Equipment for Molecular Cytogenetic Procedures

Water bath (preferably with shaking), incubator, Coplin jars with lids, glass slides, micropipettes, sterile pipette tips, benchtop- and microcentrifuge, scalpels, forceps, phase-contrast and fluorescence microscope.

2.2. Oligonucleotide Probes and Labeling

1. Telomere probes are obtained from a commercial source as 5'-(T₂AG₃)₇ and 5'-(C₃TA₂)₇, deoxyoligonucleotides homologous to the G-rich and C-rich strand of the vertebrate telomere sequence, respectively (1). Centromeric satellite DNA may be outlined using a directly labeled oligonucleotide to major satellite DNA (12). Oligonucleotides are preferably labeled by 3'-tailing using terminal transferase (see Note 1).
2. 0.4 mM Biotin-11-dUTP (e.g., Invitrogen, Gaithersburg).
3. 1 mM dATP: prepare from a 100 mM stock.
4. Terminal deoxynucleotidyl transferase (TdT).
5. Cacodylate buffer (5 ×): 1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/mL BSA, pH 6.6. This buffer usually is provided by the enzyme manufacturer. Cacodylate is a toxic chemical—always handle with care.
6. 25 mM CoCl₂.
7. TE buffer: 1 mM EDTA, 10 mM Tris-HCl pH 7.4.
8. Graded ethanol, 70% ethanol.
9. Double distilled (dd.) or MilliQ water.

2.3. Dot Blot Test

1. Nylon membrane or nitrocellulose.
2. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP): Dissolve at 50 mg/mL in dimethylformamide, store at -20°C.
3. Nitroblue tetrazolium (NBT): Prepare stock at 75 mg/mL in 70% dimethylformamide, store at -20°C.
4. AP1 buffer: 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5.
5. AP2 buffer: 0.15 M NaCl, 50 mM MgCl₂, 50 mM Tris-HCl, pH 9.5.
6. Substrate solution: mix 4.4 µL of NBT, 3.3 µL BCIP, and 1 mL of AP2. Wear gloves when handling dye- or substrate solution.
7. Bovine serum albumin (BSA, fraction V).
8. Blocking buffer: 1% BSA in AP1.
9. Streptavidin-alkaline phosphatase.

2.4. Tissue Collection and Storage

1. Scalpels, forceps, and fine surgical scissors.
2. 2-methyl-butane (isopentane).
3. Dry Ice.

2.5. Reagents for Testis Suspension

The analysis of three-dimensional telomere distribution should preferably be carried out in structurally preserved interphase nuclei of cell suspensions, which have the advantage that they can be

prepared from any tissue and render nuclei usually free of neighboring cells and cytoplasm. Thereby, a large number of nuclei can be inspected, facilitating scoring of rare events (*see Note 2*).

1. Acid-free 37% formaldehyde. Formaldehyde is toxic—handle always with care!
2. Fixative: 4% formaldehyde, PBS, 0.1 M sucrose, pH 7.4.
3. MEM medium.
4. Proteinase inhibitor cocktail tablets.
5. Ethanol cleaned, aminosilane-coated glass slides (Superfrost plus, Menzel).
6. Fume hood.
7. 70 µm nylon mesh (e.g., Nybolt).
8. Tween-20, 0.1% in deionized water.
9. Small Petri dishes and scalpels.
10. Ice in styrofoam box.

2.6. Pretreatment

1. Pepsin (3,200–4,000 U/mg protein).
2. Pepsin stock solution: 10 mg/mL pepsin in deionized H₂O. Prepare fresh from powder prior to use. Storage at –20°C is not recommended, since enzyme activity will drop with time.
3. 1 M NaSCN stock in deionized water. This solution is stable at room temperature for several months when stored in an amber bottle.
4. 10 mg/mL RNase A stock solution in 1 × SSC. Inactivate contaminating enzymes by heating the stock solution for 7 min to 90°C. Store at –20°C. Working solution is 0.2 µg/mL in 1 × SSC.
5. Coplin jars with lids.

2.7. Fluorescence *In Situ* Hybridization

1. 20 × SSC: 3 M NaCl, 0.3 M Na₃citrate, pH 7.0. Store at room temperature. Make up dilute SSC solutions from this stock and discard after use.
2. Formamide, research grade. A small quantity of deionized formamide is required for the hybridization solution. Prepare small aliquots by filling the tip (~300 µL) of a 1.5 mL micro-fuge tube with ion exchange resin (20–50 mesh; Biorad). Add 1 mL of formamide, mix and store at –20°C. Note: formamide is a harmful chemical and should be handled with care (*see Note 3*).
3. 70% Formamide, 30% 2 × SSC, pH 7.0. This solution can be stored for several weeks in the refrigerator.

4. Oligonucleotides specific for telomere and centromeric satellite DNA (12). Alternatively, telomeres can be marked with PNA probes directly labeled with fluorochromes (e.g., FITC or Cy3; these can be obtained from commercial suppliers [e.g. DAKO]) (see Note 4).
5. Carrier DNA from *E. coli* sheared by sonication to 300–1,500 bp.
6. Coverslips, 22 × 50 mm and 13 × 13 mm.
7. Coplin jars with covers.
8. Hybridization solution: 50% (v/v) formamide (see Note 5), 10% of 20 × SSC, *E. coli* carrier DNA at a final concentration of 1 µg/µL, oligonucleotide DNA probe at 1 ng/µL final concentration (see Note 6). The amount of slides to be hybridized determines the volume to be prepared: use 10 µL per 22 × 50 mm cover slip. Use deionized water to adjust volume.
9. Heating block or hot plate, capable of reaching >80°C.
10. Moist chamber to prevent slides from drying out. This can be a lidded plastic box with wet paper and glass rods to raise slides above the moist surface.

2.8. Posthybridization Washes and Signal Detection

1. Avidin-FITC (e.g., ExtrAvidin-fluorescein).
2. Tween-20.
3. Bicarbonate Tween Gelatin (BTG) buffer: 0.15 M NaHCO₃, 0.1% Tween-20, 0.1% fish gelatin, pH 8.3. Make up fresh every day; pH adjustment is not required.
4. 0.05 × SSC.
5. BSA (fraction V).
6. Biotinylated goat-anti-avidin antibodies (Vector Labs, Burlingame).
7. Water bath, preferably with shaking. This is used to adjust the temperature of the solutions in Coplin jars. Always measure temperatures inside Coplin jars.

2.9. Specimen Counter Staining

1. 1 mg/mL DAPI (4',6-Diamidino-2'-phenylindole dihydrochloride) in sterile water. Store at -20°C.
2. Antifade solution: Vectashield (Vector Labs) is recommended as mounting medium for fluorescence microscopy, since it efficiently reduces fading of fluorochromes during microscopic analysis (18)(see Note 7).
3. For counter staining of DNA, DAPI is added to the antifade solution to a final concentration of 0.5 µg/mL. Alternatively, a ready-made solution can be commercially obtained (e.g. Vector labs).

3. Methods

This section describes experimental details for tissue handling, preparations of cell suspension, probe labeling and FISH with oligonucleotide probes and their use in analysis of mammalian telomere dynamics.

3.1. Tissue Collection and Storage

Collect testis tissue samples from 4 wk old mice (*see Note 8*).

1. Remove testicles immediately after sacrificing the animal.
2. Instantly transfer testicles to an Eppendorf tube containing 2-methyl-butane, prechilled on dry ice.
3. Leave some 2-methyl-butane in the tube to prevent freezer burn of specimens, seal the tube, and store tissues below -70°C until use (*see Note 9*).

3.2. Preparation of Nuclear Suspension from Testes

1. Obtain fresh or frozen tissue samples (*see Section 3.1.*).
2. Cut a small piece of tissue from a testicle and transfer to approx. 0.5 mL of ice-cold MEM containing 0.5% mammalian protease inhibitor at the rim of a small plastic Petri dish on ice (*see Note 10*).
3. Mince testes with scalpels until medium turns turbid. Add more medium when the sample turns too dense.
4. Remove larger tissue fragments with fine forceps.
5. Collect cell suspension with a syringe and force through a 70 µm Nylon mesh into an Eppendorf tube on ice.
6. Transfer 50 µL of the cell suspension to an ethanol-cleaned glass slide.
7. Mix with 150 µL of ice-cold fixative by tilting slides.
8. Examine a drop of suspension by phase-contrast microscopy at low power. Dilute suspension with MEM if it appears too dense. Large cells should be seen freely floating without touching each other.
9. Allow the solution to sit for 30 min at 4°C, e.g., in a cold room or fridge (*see Note 10*). Thereafter, transfer preparations to a chemical fume hood at room temperature until they are completely dry.
10. Seal slides in plastic boxes and store at -20°C until use.

3.3. Probe Labeling

Enzymatic 3' tailing of oligonucleotides has proven an effective method for the generation of rapidly penetrating FISH probes.

1. Label each oligonucleotide in a separate reaction containing:
15 pmol oligonucleotide (approx. 160 ng of a 42mer)
1 nmol biotin-11-dUTP and 1.5 nmol dATP (*see Note 11*)

- 10 µL 5 × cacodylate buffer
 0.5 µL of 25 mM CoCl₂
 50 units of terminal transferase
 add dd. H₂O to a final volume of 50 µL.
2. Mix and incubate at 37°C for 3 h to overnight.
 3. Set 1 µL of each reaction aside for dot blot test (see Section 3.4.).
 4. Place tubes on ice.
 5. Add 3 vol of ethanol and 20 µg of *E. coli* carrier DNA.
 6. Precipitate labeled oligonucleotides together with *E. coli*-carrier DNA at -20°C for 30 min (up to overnight). Additional salt is not required for efficient precipitation.
 7. Spin for 30 min at high speed.
 8. Discard supernatant and wash pellet once with 70% ethanol.
 9. Air-dry pellet by placing the open tube for 5–10 min in a drying block or incubator at 65°C.
 10. Dissolve pellet in 16 µL TE buffer.

3.4. Dot Blot Test for Efficacy of Probe Labeling

This test is applied to determine the efficiency of the labeling reaction (see Note 12).

1. Spot six drops of 9 µL 6 × SSC on a piece of Parafilm.
2. Add 1 µL of the labeled oligonucleotides (equivalent to 3.2 ng of unlabeled oligomers, see Section 3.3 step 3) to the first drop, mix by repeated pipetting in and out of the drop.
3. Transfer 1 µL of this drop to the next and mix by repeated pipetting.
4. Repeat step 3 for the remaining four drops.
5. Cut a small piece of nitrocellulose (or nylon) membrane.
6. Remove 1 µL from the drop with lowest probe concentration and spot it onto the nitrocellulose membrane. Repeat this step for drops with increasing probe concentrations.
7. Air dry completely.
8. Place the membrane in a small plastic jar, cover with blocking buffer and incubate for 5 min at 50°C.
9. Pour off blocking buffer and submerge filter in AP1 buffer containing streptavidin alkaline-phosphatase conjugate at 0.5 µg/mL. Incubate for 10 min at room temperature with agitation.
10. Discard solution and wash 3 × 2 min with large volumes of AP1 and 3 min with AP2.
11. Place filter in an appropriately sized plastic bag and add 1 mL of substrate solution.

12. Seal bag and allow the color reaction to proceed in the dark. The dot with the highest oligonucleotide amount (equivalent to 0.32 ng) should become clearly visible within 2–5 min, dots 4 and 5 should be visible after 20–30 min, while the dot with the lowest amount will remain invisible in most cases.
13. Remove the filter from the bag and stop the reaction by a brief wash in 70% ethanol. Air dry.

3.5. Pretreatment and Denaturation of Testes Suspensions

This section describes a procedure to achieve an efficient penetration of the probe molecules to their target regions in formaldehyde-fixed nuclear suspensions.

1. Submerge preparations (*see Section 3.2.*) in a Coplin jar containing 0.1% Tween-20 and incubate for 5–10 min under agitation at room temperature to remove sugar and fixative.
2. Wash briefly in deionized water and drain off excess fluid.
3. Apply 100 µL 1 M NaSCN and cover with a 22 × 60 mm coverslip (*see Note 13*).
4. Incubate at 70°C for 30 min in a humid chamber.
5. Dip-wash preparations in H₂O to remove coverslips and NaSCN solution.
6. Wash 5 min with PBS, 0.1% glycine to saturate any remaining aldehyde groups.
7. Dip-wash preparations in 1 × SSC.
8. Apply 100 µL RNase working solution and cover with a 22 × 60 mm coverslip.
9. Incubate for ≥120 min in a humid chamber at 37°C.
10. Dip-wash preparations in 1 × SSC to remove coverslips.
11. Dip wash in distilled H₂O, shake off excess liquid and cover slides with 100 µL 70% formamide, 2 × SSC (*see Note 3*).
12. Mount a 22 × 60 mm coverslip and place slides for 5 min on a heating block at 75°C for denaturation (*see Note 14*).
13. Remove slides from hot surface with forceps.
14. Wash off coverslips and denaturation solution with a jet of ice-cold deionized water.
15. Dry slides with an air jet, e.g. from a rubber blow ball (*see Note 15*).

3.6. Fluorescence In Situ Hybridization

1. Denature hybridization solution for 5 min at 93°C (*see Note 16*).
2. Cool microfuge tube containing hybridization solution for 3 min in ice water or cooling block.

3. Apply an appropriate volume to a region of the previously denatured slide that has the requisite density of interphase nuclei (3 µL is sufficient for a 13 × 13 mm coverslip).
4. Mount coverslip and seal with rubber cement (*see Note 17*).
5. Place slides for 4–16 h in a moist chamber or incubator at 37°C (*see Note 18*).
6. After hybridization, peel off rubber cement with forceps.
7. Submerge slides in 0.05 × SSC at room temperature until coverslips float off.
8. Discard SSC solution and wash preparations three times for 5 min in 0.05 × SSC at 37°C (*see Note 3*).

3.7. Signal Detection

The following procedure is a fluorescent detection method which creates green signals at the hybridization site (*see Note 19*). Preparations that were hybridized with directly fluorochrome-labeled probes are instead dip-washed in BTG buffer at this stage and then embedded.

1. Transfer slides to a Coplin jar containing 0.5% BSA in BTG buffer at 37°C and equilibrate for 5 min.
2. Remove slides from Coplin jar and drain excess fluid. Preparations should never be allowed to dry during this and subsequent steps.
3. Apply 100 µL BTG buffer containing 2.5 µg/mL avidin-FITC and cover with a large coverslip.
4. Incubate for 1 h at 37°C in a moist chamber.
5. Shake gently or submerge slides in BTG buffer to remove coverslips.
6. Wash slides three times for 3 min in BTG buffer at 37°C.
7. Apply 100 µL BTG buffer containing 2.5 µg/mL biotinylated goat anti-avidin antibody, cover with coverslip, and incubate for 30 min at 37°C in a moist chamber.
8. Wash three times for 3 min in BTG buffer at 37°C.
9. Repeat step 3 and incubate for a final 30 min at 37°C.
10. Repeat step 8, remove slides from Coplin jar and drain excess fluid.
11. Apply 18 µL antifade solution containing DAPI as DNA-specific counter stain and mount a 22 × 60 mm coverslip (*see Note 20*).
12. Cover slide with a Kimwipe and streak with gentle pressure along center of slide to remove excess liquid and trapped air bubbles. Inspect under fluorescence microscope *see Note 21*) and scan preparations for different cell types (*see Fig. 22.1*).

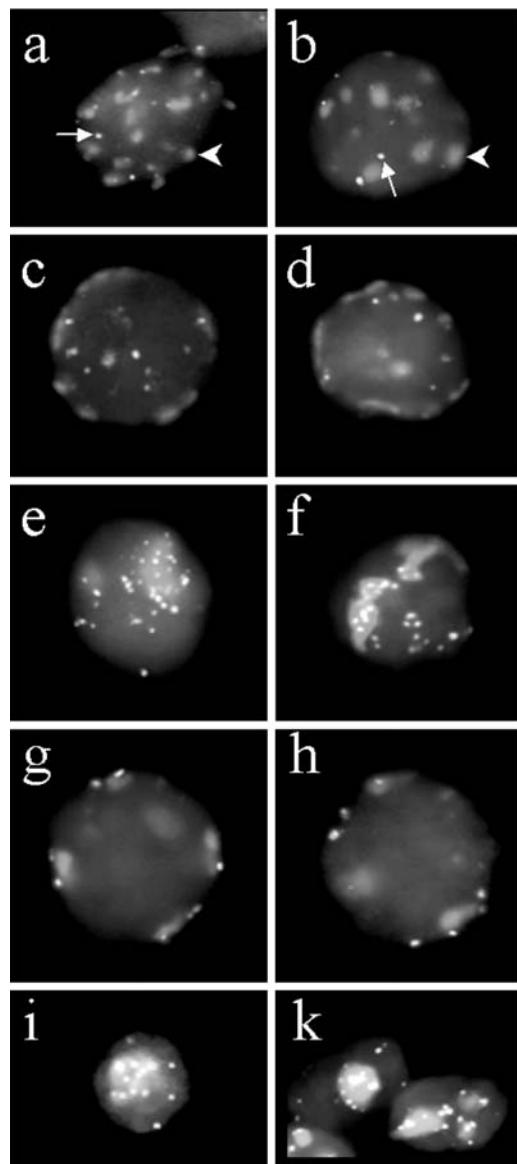


Fig. 22.1 Nuclei of different spermatogenic cell types of a mouse testes suspension stained by FISH with a biotinylated $(T_2AG_3)_7/(C_3TA_2)_7$ oligonucleotide probe for telomeres (small whitish dots, detected with Avidin-FITC; examples are arrowed in panels **a** and **b**), and for pericentric major satellite DNA (seen as the large, light-gray signal areas, arrowheads in panels **a** and **b**) by FISH with a Cy5-labelled 42mer oligomer. Different spermatogenic cell types exhibit characteristic telomere/satellite DNA distribution patterns of these chromosomal regions (dark-gray: DNA stain [DAPI]). (a) Nucleus of a spermatogonium with a scattered (somatic) telomere distribution. (b) Nucleus with a somatic telomere and major satellite distribution. (c, d) Nuclei of so-called “mid-preleptotene” spermatocytes (equivalent to postreplicative B-type spermatogonia) that can be identified by a peripheral shell of major satellite DNA and scattered telomeres. This cell type is furthermore characterized by a diffuse nucleolar distribution of the SYCP3 axial element protein (12). (e) Telomere clustering in an early bouquet stage nucleus as indicated by accumulation of many small telomere signals in a limited region of the top of

3.8. Evaluation

A testis suspension will contain a number of cells that stem from somatic tissues (i.e., myoid and endothelial cells, Leydig cells, Sertoli cells) as well as cells from all stages of spermatogenesis (i.e., all types of spermatogonia, spermatocytes I and II, metaphase cells, round and elongating spermatids, and developing spermatozoa) (for details see Ref. 19). The relative amount of spermatogenic cell types depends on the developmental stage of the testis (20, 21) or the mutant condition under scrutiny. In the case of numerical analysis of bouquet-stage spermatocytes and prophase I, it is advisable to determine the frequency of bouquet spermatocytes among total spermatocytes. Discrimination of spermatocytes from other cell types can be done by co-staining of telomeres and prophase-specific proteins (cohesins, axial element or SC-specific proteins) or protein modifications (e.g., γ -H2AX; (15, 22)). Furthermore, the conspicuous patterns of telomere and pericentric heterochromatin distribution and size of spermatoocytes also allows one to discriminate them from premeiotic and spermigenic cell types (Fig. 22.1) (11, 12). For numerical evaluation of the frequency of bouquet spermatocytes according to telomere and pericentric major satellite FISH patterns, it is suggested to simultaneously score so-called “mid-preleptotene” spermatocytes that are identified by a peripheral layer of major satellite DNA and intranuclear telomeres (12). This allows one to evaluate delayed entry into prophase I in addition to or as an alternative to tissue-section analysis. Since bouquet-stage spermatocytes are rarely encountered in male *Mus musculus* meiosis, a large number of spermatocytes (usually $>4,000$) has to be inspected to obtain a sufficient number of “events” (i.e., >5) to make a comparison by Chi-square statistics meaningful (11). The latter may be enhanced when the contingency tables of Crow and Gardner (23) are used in cases where only a low number of specific nuclei have been observed.

For setting up a reference value it is advisable to determine the bouquet frequency in several individuals of a particular strain and, whenever possible, to use nonmutant individuals of the same litter for direct comparisons. All preparations should be evaluated

Fig. 22.1 (continued) this nucleus. Major satellite forms two diffuse chromocenters, one below the telomere cluster. The focal plane of this image is at the *top* of the nucleus. (f) Relaxed telomere grouping in a more advanced spermatocyte nucleus as indicated by accumulation of large distal telomere signals towards the *lower right* of this nucleus, while pericentric satellite DNA forms two irregular masses surrounding clustered proximal telomeres. The focal plane of these images is at the *top* of the nucleus. (g, h) Telomeres and a few pericentric satellite DNA clusters are exclusively located at the periphery of these pachytene spermatocyte nuclei. The focal plane of these images is at the nuclear equator. (i) Association of most telomere signal with a single chromocenter characterizes round spermatid nuclei. (k) A similar pattern is maintained in elongating spermatid nuclei. For details on staging of the cells see (11, 12). A color version of this figure is provided on the companion CD for this volume.

by the same trained user(s) to minimize differences due to individual variations in microscopic analysis. Furthermore, in mutant conditions where spermatocytes are eliminated by checkpoint functions during the pachytene stage, subsequent stages will be missing, which will lead to the artificial inflation of the frequency of bouquet cells among total spermatocytes. To compensate for this error the investigator should determine the substage where the mutant meiosis arrests. This can be done using protein or chromosomal markers for stage-specific hallmarks of prophase I, e.g., SYCP3, histone H1t, XMR, γ -H2AX expression, or sex body formation (11, 24, 25). The frequencies determined in the mutant (or wild type) are then corrected for the percentage of cells missing before making comparisons to the wild-type situation (11).

4. Notes



1. By virtue of their small size (<50 bp) telomere deoxyoligonucleotides have proven useful FISH probes for metaphase and interphase telomeres. Ready-to-use telomere FISH kits are commercially available. Short PNA oligonucleotide probes to telomere repeats have yielded superior detection efficiencies in a short time (16) but are expensive and, due to high formamide requirements, sometimes difficult to combine with other probes. If telomere probes are the only FISH probes to be applied, then PNA probes are the first choice for the speed and ease of handling.
2. The analysis of bouquet-stage frequency in testes tissue sections is not recommended to the untrained user, as this is challenging—the telomere cluster has to lie perpendicular or on top of the nucleus so that it can be recognized by the novice investigator. The same applies to nuclei in suspensions, but in this case the signal pattern can be evaluated without overlap from neighboring nuclei that are densely stacking in testis tissue sections (12). Finally, analysis of spread preparations for telomere dynamics is not recommended, because spreading usually dissolves the telomere cluster of leptotene/zygotene spermatocytes.
3. Formamide-containing wash solutions can be replaced effectively with dilute salt solutions, thereby reducing the handling of formamide to the few μ L required for preparation of hybridization and denaturation solution.

4. PNA probes render quantitative FISH signals and are easy to use. However, they cannot be easily combined with DNA probes as PNA probes require 70% formamide in hybridization solution to suppress high background staining.
5. The formamide concentration has to be lowered to 30% when DNA telomere oligonucleotides are combined with major satellite DNA-specific ones as this satellite DNA has a high AT content.
6. Oligonucleotide probes do not need dextran sulfate, which is used to speed up hybridization reaction in the case of genomic DNA probes.
7. Vectashield may be diluted by adding an equal volume of antifade solution II. The latter may be used to replace commercial ones when needed. Make up antifade solution II by mixing 245 mg of Diazabicyclo-222-octane (DABCO), 800 μ L of sterile H₂O, 200 μ L of 1 M NaHCO₃(pH 8.3), and 9 mL of high-grade glycerol (86%). The compounds of solution II should be added in the order listed above, with the NaHCO₃ solution to be added in small quantities, since it may effervesce. DABCO is corrosive—always handle with care and wear protective equipment.
8. It may be of interest to collect and preserve a number of tissue samples and store them for later analysis. It is recommended to obtain testes from mice of 4 wks *post partum*, which have fully developed spermatogenesis. This excludes potential variations of bouquet frequencies that may result from an altered onset of the first synchronous meiotic wave, e.g., in a mutant condition, which would increase the frequency of early prophase I substages relative to total spermatocyte numbers and complicate comparisons with the wild type or other conditions.
9. According to my experience, tissues stored in this way will perform well in FISH and even immunostaining procedures, even after years of storage.
10. When possible, this work should be carried out in a cold room to prevent adverse effects of elevated temperature on nuclear three-dimensional structure.
11. Enzymatic 3' end-labeling of oligonucleotides is compatible with a variety of hapten-modified nucleotides (e.g., digoxigenin or fluorochromes). The sensitivity of oligonucleotide probes may be increased by an optimal spacing of haptens within the nucleotide tail, which improves the access of detection agents (fluorochrome-conjugated avidin or antibodies). This is achieved by diluting the hapten-labeled nucleotides with dATP. Note that optimal spacing of the haptens

- modified nucleotides depends on the size of the haptens. For example, a molar ratio of approx. 1/6 has been found optimal for digoxigenin-11-dUTP/dATP, while the optimal ratio was 1/1.5 in case of biotin-11-dUTP/dATP (26).
12. For the incorporation of haptens other than biotin, these have to be monitored by applying appropriate alkaline phosphatase-conjugated antibodies against the haptens of choice (e.g., digoxigenin) in the dot blot test.
 13. Successful detection of telomeric regions depends on the access of the DNA probe to its target sequence, particularly in preparations fixed with cross-linking agents like formaldehyde. If the hybridization reaction renders no signals it is often sufficient to open up fixed proteins by incubation with a 1 M solution of the chaotropic chemical sodium thiocyanate (29).
 14. During denaturation, coverslips may tend to stick to the slide. This is due to evaporation from the rim of the coverslip, which can be prevented by applying a few µL of denaturation solution to its rim every 3–4 min.
 15. Often, ethanol dehydration of preparations is performed after denaturation. This is actually not required. It is sufficient to wash down the coverslip and hybridization solution with a jet of ice-cold deionized water from a wash bottle and air-dry the preparations with an air jet.
 16. Probe and target DNAs can be denatured simultaneously. However, separate denaturation is recommended, especially when the hybridization solution contains dextran sulfate. This polymer creates shearing forces during thermal denaturation, which can cause inferior chromosome and nuclear morphology. Furthermore, background noise is reduced by the additional wash steps. Preparations fixed with a cross-linking fixative such as formaldehyde require higher denaturation temperature and more time as compared to preparations obtained by standard methanol/acetic acid fixation protocols (27, 28). If a denaturation step does not yield FISH signals it is recommended to repeat the denaturation/FISH from **Section 3.4.**, step 10 onwards. This will open up chromatin for hybridization (29).
 17. Sealing of coverslips for hybridization is only required for longer hybridization times (overnight or longer). In the absence of rubber cement, coverslips may be sealed with bicycle-tube repair liquid.
 18. Although oligonucleotide telomere probes may produce positive signals already after 1–2 h of hybridization [PNA telomere oligonucleotide probes create sufficiently intense signals after even 30 min of hybridization (30)], standard

DNA probes require longer hybridization times which can result in more intense signals (31). Finally, under certain conditions, overnight incubations are convenient.

19. When telomere oligonucleotide probes are combined with other probes, multiple color detection protocols have to be followed to differentially delineate the chromosomal targets (28, 29).
20. Mounted slides will maintain their fluorescence up to several months or even years when stored at 4° or -20°C. Reduced fluorescence after such storage can sometimes be revived by washing the slides in BTG buffer and applying new antifade.
21. Preparations are inspected using an epifluorescence microscope equipped with appropriate filter sets for the visualization of green, red, and blue fluorescence. A double-band pass filter for simultaneous inspection of red and green fluorescence (Chroma Technologies) is recommended for scoring directly down the microscope. Images are usually recorded with digital image acquisition systems. It may be useful to record at least three different focal planes of representative nuclei to document the three-dimensional distribution of chromosomal regions under investigation. Laser scanning or deconvolution microscope systems offer recording of image stacks and three-dimensional reconstruction. However, this is time consuming and not always necessary or possible.

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Chapter 23

Immunofluorescence Analysis of Human Spermatocytes

Evelyn Ko and Renée H. Martin

Abstract

New immunofluorescence techniques allow visual identification of human cells in various stages of meiotic prophase. Antibodies to the synaptonemal complex, the centromere and sites of recombination allow these stages of meiotic prophase to be identified. The progress of chromosome synapsis, recombination and associated phenomena such as interference can be studied in normal men, translocation heterozygotes and men with infertility problems. This has greatly stimulated research in human meiosis, leading to many exciting studies on the mechanisms underlying recombination and the generation of chromosome abnormalities.

Key words: Meiotic recombination, pachytene spermatocytes, synaptonemal complex analysis, MLH1, human spermatogenesis, meiotic chromosomes.

1. Introduction

Meiotic recombination is of central importance for pairing of homologous chromosomes and the accurate segregation of chromosomes into normal haploid gametes. Cytological analysis of recombination (crossing-over) has been possible through the study of diakinesis preparations of human meiocytes (1–3). This approach enabled Hultén and her colleagues to examine the frequency and distribution of chiasmata in humans for the first time. However, diakinesis chromosomes are highly condensed, making it difficult to locate chiasmata precisely. Also, cells are available only in small numbers.

Happily, the introduction of new immunofluorescence techniques to identify a number of mammalian orthologues of recombination proteins of lower organisms has revitalized human

meiotic research. Components of the synaptonemal complex (SC) can be identified by immunofluorescence. The SC is the proteinaceous structure linking homologous chromosomes in prophase of meiosis I. The centromere can be localized by use of CREST antibodies and sites of crossover recombination can be marked by MLH1, a DNA mismatch repair protein (4, 5). Use of this immunocytogenetic technique has produced clear meiotic spreads that have provided information on the frequency and distribution of meiotic recombination in humans (4–9), the variation in recombination frequencies among individuals (5, 10, 11), precise recombination maps for individual chromosomes (12, 13), examination of the process of interference in humans (5, 8, 12, 13), effects of gaps on the recombination frequency (14), abnormalities of recombination in infertile men with azoospermia (15–20), pairing and recombination in translocation heterozygotes (21–23), and the time course of proteins involved in recombination (24).

The detailed methods described in this chapter are modifications of the first methods described by Hultén and Barlow (1). We hope that this expanded protocol will help others find success in this exciting area of reproductive genetics.

2. Materials

2.1. Spermatocyte Recovery from Testicular Biopsy Samples (Notes 1, 2)

1. 600 mM Tris-HCl, pH 8.2: Dissolve 36.34 g Tris in approximately 400 mL of water (*see Note 3*), adjust pH to 8.2 with HCl, using a probe that can measure the pH of Tris accurately, and bring the vol to 500 mL. Autoclave. Prepare 500 µL aliquots (*see Note 4*); store aliquots and stock bottle at room temperature.
2. 500 mM sucrose: Dissolve 17.115 g in water and bring solution to a total vol of 500 mL. Autoclave. Prepare 1 mL aliquots and 100 µL aliquots (*see Note 4*); store aliquots and stock bottle at 4°C.
3. 170 mM citric acid: Dissolve 25 g trisodium citrate dihydrate in water and bring solution to a total vol of 500 mL. Autoclave. Prepare 1 mL aliquots (*see Note 4*); store aliquots and stock bottle at room temperature.
4. 500 mM EDTA: Dissolve 93.06 g disodium EDTA in approximately 400 mL of water, adjust pH to 8 with NaOH pellets, bring the vol of the solution to 500 mL. Autoclave. Prepare 100 µL aliquots (*see Note 4*); store aliquots and stock bottle at room temperature.

5. 500 mM dithiothreitol (DTT): Dissolve 7.71 g in water and bring to a total vol of 10 mL. Prepare 50 µL aliquots, then divide the remaining solution into 1 mL aliquots and store at -20°C (*see Notes 4 & 5*).
6. 50 mM phenylmethylsulfonyl fluoride (PMSF): Because of the toxicity of PMSF, work in a fume hood and do not weigh this reagent. Simply add 14.35 mL of isopropanol to the contents of the 250 mg bottle that it comes in, prepare 100 µL aliquots, and store at -20°C (*see Notes 4 & 6*).
7. 50 mM boric acid: Dissolve 0.31 g in water, and bring solution to a total vol of 100 mL. Store at room temperature.
8. Preweigh 0.25 g paraformaldehyde (PFA) aliquots into 50 mL conical centrifuge tubes (with screw-cap lids).
9. Triton X-100.
10. 10 N NaOH: you will need to prepare a 1 N solution, and it may be necessary to prepare a 0.5 N solution as well (*see Note 7*).
11. Humidified chamber (*see Note 8*).
12. Fine-tipped dissection forceps (standard tip Dumoxel Dumont forceps).
13. Glass embryo dishes, or other small volume dish with concave bottom for dissection (e.g., Electron Microscopy Sciences 70543-30).
14. Microscope slides, 25 × 75 mm, with frosted end for labeling. Preclean slides: dip for several seconds in a Coplin jar of methanol to which a drop of 1 N HCl has been added, then remove slide and air-dry at room temperature. With a diamond marker, etch a vertical line on the underside of the slide approximately 0.5 cm from the frosted area, and 0.5 cm from the other end of the slide. Store in covered slide boxes until needed (no time limit).
15. Microcover glasses, 40 × 22 × 0.1 mm.
16. Also needed: Pasteur pipette and bulb, 15 mL tube with screw-cap lid.

2.2.

Immunocytochemistry on Fixed Spermatocytes

1. Phosphate-buffered saline (PBS): dissolve 32 g NaCl, 0.8 g KCl, 10.88 g Na₂HPO₄·7H₂O, and 0.96 g KH₂PO₄ in 3,600 mL of water. Adjust pH to 7.4 with HCl, bring total vol to 4 L with water, autoclave, and store at room temperature.
2. Normal donkey serum (Jackson Immunoresearch 017-000-121): to prepare 100% normal donkey serum, reconstitute lyophilized serum in original bottle by injecting 10 mL of water. Prepare 4 mL aliquots (*see Note 9*) and store at -20°C.

3. On the first day that you will need it (*see Note 10*), prepare 10× modified antibody dilution buffer (ADB; *see Note 11*): to 4 mL of 100% normal donkey serum, add PBS to a total vol of 10 mL, then add 5 µL of Triton X-100 and mix well. Store at 4°C for a maximum of 2 wk. Just before use, dilute 3.3 mL of 10× ADB to a total vol of 33 mL with PBS to prepare 1× ADB.
4. Immediately before use, prepare 0.04% Photo-Flo: add 20 µL of Photo-Flo 200 solution (Kodak 146 4510) to 50 mL of water for each Coplin jar of 0.04% Photo-Flo needed (*see Note 12*).
5. Just before use, dilute primary antibodies (*see Notes 13, 14* and **Table 23.2** for antibody selection and dilution): MLH1, SYN1, SCP3, and CREST (calcinosis, Reynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia).
6. Rubber cement to seal cover slips to the slide.
7. Just before use, dilute secondary antibodies to 0.015 mg/mL (*see Note 15* and **Table 23.3**): donkey anti-rabbit Alexa 488 (green), donkey anti-goat Alexa 555 (red), donkey anti-mouse Cy3 (red), and donkey anti-human AMCA (blue)
8. 20× SSC: Dissolve 87.65 g NaCl and 44.1 g trisodium citrate dihydrate in 400 mL of water, adjust the pH to 7.0 with 10 N NaOH, and bring final vol to 500 mL with water. Autoclave, and store at room temperature.
9. 2× SSC/0.1% NP-40: Dilute 50 mL of 20× SSC to 500 mL final volume with water. Autoclave. Add 0.5 mL of Nonidet P-40 (NP-40; *see Note 16*), mix well, allow bubbles to subside before using, and store at room temperature.
10. Also needed: numerous Coplin jars for washes, forceps for removing slides from Coplin jars.

2.3. Visualization of Synaptonemal Complexes

1. Antifade stock solution (AF): Dissolve 5 mg of p-phenylenediamine in 10 mL of 2× SSC/0.1% NP-40, add 10 mL of glycerol, mix well, prepare 1 mL aliquots, and store at -20°C (*see Note 17*).
2. A field finder slide (Gurley Precision Instruments 7100) is necessary to pinpoint spread locations, in order to revisit individual spreads at a later date.
3. A fluorescence microscope fitted with filters for rhodamine, FITC, and DAPI and a cooled CCD camera, along with software to capture the images. We use Zeiss AxioPlan and AxioPhot microscopes, SenSys cameras, and Applied Imaging's CytoVision software. An eyepiece with a cross in the center is essential for marking spread location with the field finder slide.

4. To aid in noting spread locations, sheets with depictions of the appearance of one unlabeled square of the field finder slide's grid should be prepared in advance – we have 15 such grid pictures per page (5 rows, 3 columns), so that the appropriate grid label plus a dot, indicating where the center of the SC falls within the grid, may be marked for each spread during scanning (*see Note 18*).

3. Methods

3.1. Testicular Biopsy Sample Dissection and Slide Preparation

1. Depending on whether testicular biopsy specimens arrive at a prearranged time, or without warning, the next five steps should be carried out either immediately before or immediately following arrival of tissue samples. Biopsy tissues should be kept in a microcentrifuge tube of PBS on ice from the time of surgery (*see Note 19*) until step 7, below. The day when the specimen is obtained is considered day 1 of this protocol.
2. Add 22.4 mL of water to preweighed PFA in tube, add 1 drop of 1 N NaOH and incubate for 20–60 min at 37°C to dissolve the PFA.
3. In a 15 mL tube with a screw cap, prepare 10 mL of hypoextraction buffer and use within 2 h of DTT addition (**Table 23.1**). When solution has been prepared, use 0.5 N NaOH to adjust pH to 8.2–8.4. The pH is critical (*see Note 20*).

Table 23.1
Hypoextraction buffer: stock solutions, volumes, and final concentrations

Stock solution	Volume	Final concentration
600 mM Tris	500 µL	30 mM
500 mM sucrose	1 mL	50 mM
170 mM citric acid	1 mL	17 mM
500 mM EDTA	100 µL	5 mM
500 mM DTT	50 µL	2.5 mM
50 mM PMSF	100 µL	0.5 mM
Water	7.25 mL	NA

4. Prepare 100 mM sucrose working solution: add 400 μ L water to one 100 μ L aliquot of 500 mM sucrose (*see Note 21*).
5. Ensure that the PFA is completely dissolved, and cool to room temperature.
6. Pour a small amount (1–2 mL depending on dish size) of hypoextraction buffer into two embryo dishes.
7. Start stopwatch counting up, to measure the total amount of time that tissue is in hypoextraction buffer (*see Note 22*).
8. Using forceps, remove biopsy tissue from tube, touch lightly to a paper towel to remove adhering PBS, and rinse off in first dish of hypoextraction buffer.
9. Transfer tissue to second dish of hypoextraction buffer.
10. While observing under a dissection microscope at low (~6–10 \times) power, hold tissue in place with one pair of forceps. Holding second pair of forceps closed, insert tips between tubules and allow tips to spread open, gently separating the tubules from one another. Repeat until testicular tubules are approximately two-dimensional, with a little space between adjacent tubules so that all tubules are exposed to the hypoextraction buffer (*see Note 23*).
11. Incubate tubules in the hypoextraction buffer at room temperature for 60 min total (including the time spent in steps 8–10).
12. During the incubation time, label precleaned slides with the experiment number and slide number.
13. During the incubation, adjust the pH of room-temperature PFA to 9.2 with 50 mM boric acid (*see Note 24*). Add 35–50 μ L of Triton X-100 (measure 35 μ L; because this viscous solution adheres tenaciously to the pipette tip, the actual amount delivered may be up to 50 μ L) and mix well. This step is critical, as Triton X breaks the surface tension of the PFA and allows it to coat the slide evenly.
14. Ensure that the humidified chamber is at hand, on a level surface.
15. At the end of the hypoextraction incubation period, work quickly! Place 20 μ L of 100 mM sucrose solution on a pre-cleaned microscope slide (you need to keep one Pipetman (set at 20 μ L) with a clean tip for adding more sucrose solution as needed in later steps).
16. Remove a portion of the testicular tubule tissue from the hypoextraction buffer (*see Note 25*), touch to the side of the dish and then lightly to a piece of paper towel to remove hypoextraction buffer clinging to the tissue, and transfer to the sucrose drop on the microscope slide, on the dissecting microscope stage.

17. Monitoring the process under higher power ($\sim 15\text{--}30 \times$) on the dissecting microscope, hold the tubule tissue in a tight bundle with forceps in the left hand, and rip away tissue bits from this blob with forceps held in the right hand, rupturing the tubules as much as possible. When the tissue has all been pulled out of the left forceps, gather all the pieces together again into the left forceps, repeating the process until all the tissue has been shredded into tiny bits OR until a large number of pachytene cells have been released (these appear as cloudiness in the sucrose).
18. Pick up the slide, and tilt it slightly so that the shredded tubules are toward you. With the tip of a 20 μL Pipetman (set at 10 μL), push the tissue bits up and out of the way, allowing gravity to permit the sucrose containing the pachytene cells to separate cleanly from the tissue pieces.
19. Draw 10 μL of sucrose/pachytene cells into the pipette tip, and place close by.
20. Add 20 μL of 100 mM sucrose to the microscope slide with the tissue fragments, and place back on the dissecting microscope stage away from the lighted area (to avoid undue drying). Try to check this tissue slide from time to time as you are preparing the cell slide (steps 21–24), and add 20 μL aliquots of sucrose as necessary to keep tissue pieces from drying out.
21. Hold a precleaned slide tilted very slightly down to the right, and using a Pasteur pipette, expel a small puddle of PFA (~ 1.2 cm in diameter) about 0.5 cm to the right of the frosted area on the slide.
22. With gravity assisting, use the side of the Pasteur pipette to spread the PFA across and to the end of the slide. Try to keep a fairly thick “layer” of PFA on the slide, as it improves the quality of the pachytene extensions. Briefly touch the end of the slide, then the lower right corner of the slide to a paper towel, then wipe the last 0.5 cm on the right of the slide dry with a paper tissue so that cells won’t spread to the end of the slide.
23. Working very quickly, with the slide tilted slightly down to the left, expel the cells (*from* step 19) and an air bubble onto the right edge of the PFA layer on the slide. The cells and air bubble will roll to the left edge of the PFA. Rapidly tilt the slide forward and back (“up” and “down”) while gradually tilting the slide down to the right to spread the cells over the slide as evenly as possible. Track the movement of the cells by watching the air bubble. If the bubble has not yet popped by

the time it reaches the right edge of the PFA, tilt slide slowly back to the left while continuing rapid forward-and-back tilting. Stop when the bubble pops, or when cells are spread evenly.

24. Once cells are spread, immediately place the slide flat in the prepared humidified chamber.
25. Repeat steps 17–24, re-shredding the same tissue (as there are generally plenty of pachytene cells still to be released), making a 2nd slide.
26. Repeat steps 16–25 for the other half of the tissue (small sample), or for each pencil-eraser-sized portion (large sample), to prepare at least four slides. If desired, particularly with small samples, new tissue can be added to, and shredded along with the already-shredded tubules, to maximize cell recovery.
27. Allow slides to dry overnight in the humid chamber at room temperature (*see Note 26*).

3.2.

Immunocytochemistry on Fixed Spermatocytes

1. On day 2 (the day following specimen receipt), prepare 10 × ADB if needed.
2. Dilute 3.3 mL of 10 × ADB to a total vol of 33 mL with PBS.
3. Place humid chamber into a 37°C incubator to warm up.
4. Prepare one Coplin jar of 0.04% Photo-Flo for every four slides to be washed.
5. Open humidified box, and finish drying slides flat at room temperature and atmospheric humidity, if necessary.
6. Spacing well apart for good circulation, transfer slides in groups of four to Coplin jars containing 0.04% Photo-Flo and wash for 4 min (with side-to-side agitation throughout) to remove PFA and proteinaceous matter.
7. Air dry slides for 10 min. At this point, the pachytene cells appear large and puffy under phase contrast (*see Note 27*).
8. Soak air-dried slides in a Coplin jar of 1 × ADB for 30 min, agitating every 5 min to circulate the solution around the slides.
9. As the slides are soaking, dilute the primary antibodies in PBS (*see Table 23.2* and *Note 14*).
10. Remove slides one at a time from the ADB solution; ensure that they do not dry out. Drain and briefly blot first the end of the slide and then one corner of the end on a paper towel, wipe the back and the last 0.5 cm of the slide dry with a paper tissue, add 57 µL of antibody cocktail to each slide, apply a 22 × 40 × 0.1 mm cover glass (*see Note 28*), and secure it with a narrow seal of rubber cement (*see Note 29*).

Table 23.2
Primary antibody cocktail: antibody and PBS volumes for 2, 4, 6, or 8 slides

Antibody (Ab)	# slides	µL Ab	# slides	µL PBS
MLH1	2	1.25		
	4	2.5		
	6	3.75		
	8	5.0		
SCP3	2	0.5		
	4	1.0		
	6	1.5	2	121.9
	8	2.0	4	243.8
SYN1	2	0.125	6	365.6
	4	0.25	8	487.5
	6	0.375		
	8	0.5		
CREST	2	1.25		
	4	2.5		
	6	3.75		
	8	5.0		

11. Place slides into the humid chamber and incubate overnight at 37°C.
12. On day 3, remove the rubber cement from the slides, then remove the cover glasses by soaking the slides in PBS for 20 min.
13. Wash the slides for 48 h at 4°C in PBS (*see Note 30*).
14. On day 5, prepare the secondary antibody cocktail (*see Table 23.3 and Note 15*), add 57 µL of the antibody cocktail to each slide, apply a cover glass, and incubate slides in the humid chamber at 37°C for 90 min.
15. Remove cover glass, and in the dark, wash slides in three changes of PBS for 10 min, 20 min, and 30 min, respectively, with side-to-side agitation of the slides every 5 min.
16. Drain slides, give a quick rinse with 2 × SSC/0.1% NP-40, apply 20 µL antifade and cover glass, blot gently to remove excess antifade, and secure cover glass with a narrow edge of rubber cement.

3.3. Visualization of Synaptonemal Complexes

1. Scan slides under fluorescence (we find it best to scan for the red synaptonemal complexes, using a 40 × objective). When an SC spread has been located, switch to the 100 × objective and center the spread in the field.

Table 23.3
Secondary antibody cocktail: antibody and PBS volumes for 2, 4, 6, or 8 slides.

Antibody (Ab)	# slides	µL Ab	# slides	µL PBS
Donkey anti-rabbit Alexa 488	2	0.9		
	4	1.9		
	6	2.8		
	8	3.75		
Donkey anti-goat Alexa 555	2	0.9		
	4	1.9		
	6	2.8	2	120.7
	8	3.75	4	241.2
Donkey anti-mouse Cy3	2	1.25		
	4	2.5	6	361.9
	6	3.75	8	482.5
	8	5		
Donkey anti-human AMCA	2	1.25		
	4	2.5		
	6	3.75		
	8	5		

2. Capture green, red, and blue images using a cooled CCD camera and image-capturing software.
3. Change lighting to brightfield, and carefully replace the SC slide with the field finder slide (avoid moving the stage).
4. On the prepared sheets, identify the cell number above the grid picture, mark the finder slide's grid number on the grid picture, and place a dot on the grid picture to mark location of the center of the spread (the center of the cross in the field of view).
5. Carefully replace the SC slide on the stage, switch back to fluorescence, locate, capture, and mark the location of the remaining SCs on the slide as per steps 1–4 above.

4. Notes



1. Proper caution should be exercised when dealing with any chemicals: read labels and Material Safety Data Sheets (MSDS), wear appropriate protective gear (gloves, lab coat, safety goggles) as specified by the MSDS, and work in a fume hood when indicated. PMSF is particularly toxic.

2. Stock solutions should be prepared in advance. When prepared and stored as directed – and if care is taken to avoid contamination of the stock bottles – these stock solutions will last indefinitely.
3. All solutions should be prepared using purified water, i.e., water that has a resistivity of 2.5 meg-ohms/cm² (conductivity of 0.4 micro-siemens/cm²), and which has passed through a filter that removes organic content.
4. Each aliquot should ideally contain a few microliters more than stated, to allow pipetting of the exact volume needed for the working solution without worrying about losing small amounts that typically cling to the outside of the pipette tip. Preparing aliquots in advance allows the rapid preparation of working solutions, and minimizes contamination of the main stock solution. Further, if microcentrifuge tubes for the different stock solutions are color-coded, it can be determined at a glance if all components are at hand when preparing working solutions, time spent labeling tubes is kept to a minimum, and the color-coded tubes can be collected as they are emptied, then refilled and reused.
5. To avoid damaging DTT by repeated freeze-thawing, thaw a single 1 mL aliquot as needed, distribute the entire amount into 50 µL aliquots, and re-freeze the aliquots.
6. PMSF is sparingly soluble in water. Because isopropanol is used as solvent, the solution does not solidify at -20°C.
7. Sodium hydroxide at this concentration is extremely caustic – ensure that you wear adequate protective clothing, gloves, and eye protection when working with it. To prepare a 1 N solution from the 10 N solution: to a 100 mL measuring cylinder containing 45 mL of water, slowly add 5 mL of 10 N NaOH. The mixing of NaOH and water will give off quite a lot of heat, so exercise caution. *IMPORTANT: ensure that you add NaOH to the water and NOT the other way around! Do NOT add water to sodium hydroxide, as the heat generated when the water touches the base will cause uncontrollable and dangerous spattering, and probable injury.* Prepare 0.5 N NaOH from the 1 N solution: to a measuring cylinder containing water, slowly add an equal vol of 1 N NaOH (e.g., add 10 mL of 1 N NaOH to 10 mL water in a 50 mL cylinder). Again, as above, remember to add the NaOH to the water, and not the reverse.
8. You may use any container that is not too deep and is large enough to hold the prepared slides without crowding, equipped with a wetted piece of filter paper or similar on the bottom to provide humidity, and with some way of elevating

the slides off the wet floor of the container. In our lab, we have been using Nalgene reusable plastic utility boxes (Nalgene # 5700 0500, Fisher Scientific 03-484C), into each of which is placed a very wet piece of filter paper topped with 1-mL glass pipettes (heated and bent into a U-shape) to keep the slides from contact with the wet filter paper on the bottom of the box.

9. If two bottles of normal donkey serum are reconstituted at the same time, the math works out better: Five aliquots of 4 mL each can be prepared.
10. In order to minimize waste, do not prepare $10 \times$ ADB any earlier than necessary, as it can be stored for a maximum of only 2 wk at 4°C , then must be discarded and new solution prepared.
11. Originally, ADB (actually used as a blocking buffer) contained 0.3% bovine serum albumin (BSA), but with some antibodies we were finding unacceptable levels of background. Accordingly, we eliminated the BSA from our ADB and increased the concentration of donkey serum from 1% to 4%, and the background problem disappeared.
12. You will need one Coplin jar of 0.04% Photo-Flo for every four slides to be washed. Placement of only four slides per 50 mL Coplin jar ensures good circulation of Photo-Flo around each slide, and therefore good cleaning. Do not reuse the Photo-Flo after four slides have been washed, or all the protein material that has been removed from the first four slides will be deposited on the next group of slides as you put them into the Coplin jar!
13. We have found that it is unnecessary to use ADB for antibody dilution, and now use PBS for the dilutions. However, if you prefer to dilute antibodies in $1 \times$ ADB, depending on the number of slides prepared from the testicular tissue, set aside a volume of $1 \times$ ADB equal to the volume of PBS indicated in **Table 23.2** (also see **Note 14**.)
14. MLH1 marks recombination sites, SYN1 and SCP3 delineate the components of the synaptonemal complex, and CREST shows the centromere. For our purposes, we have been using MLH1 (Oncogene PC56-100UG, rabbit host), SCP3 (mouse host), SYN1 (goat host), and CREST (human host). The three antibodies which do not have a source listed above were gifted to us, but SYN1 (sc-20836, goat host) and SCP3 (ab12452 [10G11], mouse host) have been commercially available from Santa Cruz Biotechnologies (<http://www.scbt.com/>), and ABCAM (<http://www.abcam.com/index.html?pageconfig=browse>), respectively, and may also be available from other sources. (When you are looking for

antibody sources in your area, it may be helpful to know that SYN1 is synonymous with SCP1, and that SCP3 is sometimes listed as COR1). I have not been able to locate a commercial source for CREST antibody; ours was a gift from Dr. Marvin Fritzler of the University of Calgary.

It is important to remember, when deciding which antibody combination to use, that you want three different fluorescent colors when the secondary antibodies have been applied: one color for MLH1, a second color for SYN1 and SCP3, and a third color for CREST. Therefore, you must have at least three different hosts for the primary antibodies (it is feasible to have a single mouse or goat host for both SYN1 and SCP3 – we used two different hosts because of antibody availability). Different combinations of hosts/antibodies than what we have used may work better for you, depending on availability of primary antibodies in your locale, so do not feel bound by our antibody/host combinations.

Allow for at least 60 µL of antibody cocktail per slide when determining how much cocktail to prepare. Using the indicated volumes in **Table 23.2** (a sample dilution table for illustration purposes, showing the antibodies which we use as an example) will give final volumes of approximately 125, 250, 375, and 500 µL for 2, 4, 6, and 8 slides, respectively. The literature in commercially purchased primary antibodies should indicate the recommended dilution for that antibody. The recommended final concentrations of our four primary antibodies were: MLH1 – 1:100, SCP3 – 1:250, SYN1 – 1:1,000, CREST – 1:100, and all are combined into a single cocktail.

15. We have chosen green for MLH1, red for both the SYN1 and SCP3, and blue for CREST signals. Depending on the number of slides prepared from the testicular sample, consult **Table 23.3** to determine the volumes of antibodies and PBS needed to prepare the secondary antibody cocktail (antibodies at 0.015 mg/mL).
16. Nonidet P-40, a nonionic, nondenaturing detergent, is no longer commercially available except in Japan. Unless you already have this reagent in your laboratory, you can substitute Igepal® CA-630 (Sigma 56741), which is chemically indistinguishable from NP-40 according to Sigma-Aldrich literature. The detergent is very viscous and therefore difficult to measure and transfer accurately. A simple way to overcome this difficulty is to use a 100–1,000 µL pipette tip whose end has been cut off to create an opening that is approximately 0.5 cm in diameter.

17. For short-term storage of up to six months, antifade may be kept at 4°C (typically, we keep a single aliquot at 4°C as we are using it, and the remainder at -20°C until needed). From time to time, it may be necessary to centrifuge the AF solution, as a precipitate appears at both refrigerator and freezer temperatures. If inadvertently introduced onto a slide and viewed under the microscope, these precipitate particles are huge and black – and obliterate view of specimens completely when present. To remove precipitate from AF solution, centrifuge the AF tube for 5–10 min (we use maximum speed on our minicentrifuge for about 5 min), then carefully remove the supernatant by pipette to a clean microcentrifuge tube, taking care to transfer no solid material (which is not firmly pelleted and is easy to disrupt). Repeat whenever particles can be seen in the AF solution; effectiveness of the antifade does not appear to diminish even after removing precipitate several times.
18. An example of a filled grid is shown in **Fig. 23.1** (an empty grid picture has only the outside box with ticked intervals and the center cross). In order to more accurately transfer the location of the dot (which marks the location of the crosshair in the center of the field of view under the 100 × objective), two lines marking the coordinates of the dot on the grid lines are first hand-drawn on to the empty grid picture, and then the dot is drawn (usually in red ink, to make it easier to see) at the point where the lines would intersect. Finally, the letters and numbers identifying that particular square on the field finder slide are added.
19. We have carried out tests which have determined that keeping testicular specimens on ice for up to 2 d (as for overnight air freight shipment) is a viable alternative to processing samples immediately, and does not alter MLH1 ascertainment or frequency (25).

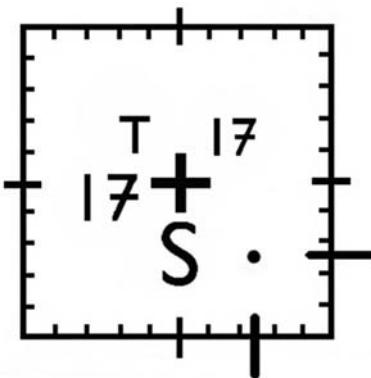


Fig. 23.1 Filled grid picture. See text for details (**Section 3.3** and **Note 18**).

20. Exercise caution when adding 0.5 N NaOH: depending on your water, the initial pH of this solution may need virtually no adjustment (as is the case in our laboratory), or may need 1 mL or more of NaOH.
21. This solution is used for the shredding of testicular tissue and the recovery of pachytene cells for slide preparation.
22. Make a note of the length of time that the tissue remains in the hypoextraction buffer for each slide; it will give you an indication of what amount of time in hypoextraction buffer gives the best results, and whether you need to speed up the shredding and slide-making process. For example, if you notice that the pachytene spread quality deteriorates for later slides, it may be a good idea to utilize a second person for slide preparation, to effectively halve the time that the tissue is left waiting in the hypoextraction buffer after the initial 60 min incubation.
23. Make every effort to avoid puncture of the tubules, which results in the loss of cells. Do not spend an inordinate amount of time in spreading the tubules apart – allowing hypoextraction buffer access to all portions of the tubules is all that is necessary, and there are other procedures that need to be undertaken during the time that the tubules are incubating in the hypoextraction buffer.
24. While stirring PFA in the tube with a mini stirbar, monitor the pH as ten drops of boric acid are added. The amount of boric acid needed to adjust the PFA's pH to 9.2 varies between laboratories: some labs need only ten drops, but we need much more – about 2 mL.
25. The size of the tissue piece transferred will depend on the size of the testicular biopsy specimen received. When we have a large testicular sample, we transfer a piece of tissue approximately the size of a pencil eraser to the sucrose. With a small testicular sample (typically the size of a pencil lead), we transfer half of the specimen.
26. Slides sometimes appear to be dry as soon as 2–4 h after they are placed in the humid chamber, but at this point, pachytene cells are still small and will not be usable, so don't skimp on the drying time. Conversely, we sometimes find that even after overnight drying in the humid chamber, the slides still have a wet surface. When this happens, we open the chamber and allow the slides to finish drying at atmospheric humidity.
27. Process slides immediately for best SYN1/SCP3 results (i.e. so that synaptonemal complexes can be visualized).

28. To apply the cover glass, hold the slide/antibody cocktail slightly tilted down to the right and slightly tilted toward you, so that the cocktail pools in the lower right corner of the slide. Holding the cover glass by the right end of the long edges using thumb and forefinger, tilt it a bit more toward you than the angle of the slide with about 1/3 of the cover glass extended beyond the end of the slide. Allow the lower edge of the cover glass to barely touch the antibody cocktail along the near edge of the slide, then drop the cover glass flat onto the end of the slide. At this point, the cocktail will extend across the width of the slide and the cover glass will probably be drawn completely onto the slide by capillary action. You want the cover glass to reside between the two etched lines. If the cover glass is not drawn completely onto the slide, take hold of the overhanging end of the cover glass and tug it gently *off* the end of the slide, release, and it should snap into place. Repeat this tug-and-snap if necessary (it usually isn't).
29. There is a lot of liquid under the cover glass, and therefore a lot of potential for the cover glass to roll around and out of place when applying rubber cement. To forestall this problem, wearing latex gloves, temporarily hold the slide by its long edges with the thumb and index finger of the right hand. Carefully and gently (without too much pressure, as you do not want the antibody cocktail to escape from under the cover glass), bend the index finger of the left hand into position under the slide to support it, and apply the left thumb to the top of the left edge of the cover glass, about 0.5 cm from the frosted area on the slide (approximately at the etched marking). Holding the cover glass/antibody cocktail in place, apply rubber cement, slightly rotating the slide away from each edge of the cover slip in turn as rubber cement is being applied. Thus, the slide is tilted slightly towards yourself when rubber cement is being applied to the far edge of the cover glass, slightly down to the left when it is being applied to the right edge of the cover glass, slightly away from yourself when applying it to the close edge of the cover glass, and finally, slightly down to the right when applying rubber cement to the left edge of the cover glass (at this point, it is safe to lift your thumb, as the three rubber-cemented edges will hold the cover glass in place). If you are using Fixogum, apply a thin, narrow edge of rubber cement directly from the tube. If your rubber cement comes in a large container, use a 3 mL or 5 mL syringe (no needle) to apply a thin, narrow edging of rubber cement. A narrow edge of rubber cement is desirable since there are cells right up to the edge of the slide, and you want to damage/lose as few of these peripheral cells as possible.

30. If time is critically short, then this wash time can be reduced in two ways: either wash overnight at 4°C, or, as a last resort, wash for 20 min at room temperature. Both of these washing methods are less desirable than the 48 h wash at 4°C, as the resultant background is significantly worse.

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Chapter 24

Cytological Techniques to Study Human Female Meiotic Prophase

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Abstract

Most of the human aneuploidies have a maternal origin. This feature makes the study of human female meiosis a fundamental topic to understand the reasons leading to this important social problem. Unfortunately, due to sample collection difficulties, not many studies have been performed on human female meiotic prophase. In this chapter we present a comprehensive collection of protocols that allows the study of human female meiotic prophase through different technical approaches using both spread and structurally preserved oocytes.

Key words: Human oocytes I, immunofluorescence, fluorescent *in situ* hybridization, spreads, structurally preserved nuclear preparations.

1. Introduction

Most of the aneuploidies detected in human newborns originate during oogenesis, in particular due to meiotic errors (1). So far, no clear reasons have been found to explain this important gender difference. Thus, a detailed study of the human female meiotic prophase may lead to a better understanding of this problem.

The few studies performed so far in human oocytes have shown some gender differences already described by indirect studies or in other mammalian species. For example, in the human oocytes, synaptonemal complex length is greater than in the spermatocytes (2). Similarly, the total number of crossovers is also increased in the female meiocytes as compared to the males (3, 4). In addition, nuclear dynamics seem to be different in the female in that bouquet topology lasts longer in females than in males (5).

Moreover, other sex differences have been observed recently. Human oocytes present an extreme heterogeneity which has not been observed in the males (6) and maybe more importantly, the double-strand break repair process seems to be delayed in the oocytes (5). The pairing process of the homologous chromosomes has also been studied in the human euploid (7, 8) and aneuploid oocytes (9–11), but all the data obtained so far seem to indicate that this process is not responsible for the excess of unbalanced human oocytes produced by oogenesis.

In this chapter, we present a compendium of detailed cytological protocols which have been developed to use fluorescent *in situ* hybridization and/or immunofluorescence on spread cells or structurally preserved preparations from human oocytes to enable the study of human female meiotic prophase I.

2. Materials

2.1. Collection of the Ovaries

1. 1% Penicillin/streptomycin in PBS (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 0.137 M NaCl, 2.7 mM KCl).

2.2. Processing the Sample in the Lab and Extraction of the Oocytes

1. Chilled PBS.
2. 2-Methylbutane (*see Note 1*).
3. Insect mounting pins (*see Note 2*).
4. Stereo microscope.

2.3. Oocyte-Spreading Technique for Cytogenetical Analysis

1. 0.88% (w/v) KCl in tridistilled water.
2. Fixative solution containing methanol and glacial acetic acid (ratio 3:1) (*see Note 3*).
3. Coplin jars.

2.4. Fluorescent *In Situ* Hybridization on Methanol: Acetic Acid Fixed Preparations

1. 47 mM magnesium chloride in PBS.
2. 50 mM magnesium chloride, 1% (v/v) formaldehyde in PBS.
3. PBS.
4. 70% (v/v) Formamide in 2 × SSC (30 mM sodium chloride and 30 mM sodium citrate in tridistilled water) pH 7.0–7.2 and 50% (v/v) formamide in 2 × SSC, pH 7.0–7.2 (*see Note 4*).
5. 70%, 85%, and 100% (all of them v/v) ethanol in water (*see Note 5*).
6. Whole Chromosome Probe (WCP) 13 Cy3 labeled (Cambio, Cambridge, UK).

7. Locus Specific Identification probe (LSI) for chromosome 13q14 (FITC labeled) and 21q22 (Cy3 labeled) (Appligene Oncor, Heidelberg, Germany).
8. 0.05% (v/v) Tween 20 in 4 × SSC.
9. Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 0.1 µg/mL of DAPI (4,6-diamidino-2-phenylindole).
10. Rubber cement.
11. 22 × 50 mm and 22 × 22 mm coverslips.
12. Coplin jars.

2.5. Structurally Preserved Nuclear Preparations

1. 2% (v/v) Acid-free formaldehyde (Merck, Darmstadt, Germany), 0.05% (v/v) Triton X-100 in PBS (*see Note 6*).
2. 0.5% (w/v) Glycine in PBS.
3. PBTG: 0.2% bovine serum albumin, 0.2% gelatin, 0.05% Tween 20 in PBS.
4. Superfrost Plus slides (Microm, Walldorf, Germany).
5. Coplin jars.

2.6. Oocyte-Spreading Technique for Immunofluorescent Analysis Starting from Frozen Sample

1. PBS.
2. 1% Lipsol (Barloworld Scientific, Staffordshire, UK) in tridestilled water.
3. 1% (w/v) Paraformaldehyde, 5 mM sodium borate, 0.15% (v/v) Triton X-100 in tridestilled water, pH 9.8.
4. 1% (v/v) Agepon (Agfa, Barcelona, Spain) in tridestilled water.
5. Coplin jars.

2.7. Oocyte-Spreading Technique for Immunofluorescent Analysis Starting from Fresh Sample

1. 0.1 M sucrose in tridestilled water.
2. 9% (v/v) Formaldehyde in tridestilled water.
3. 1% (v/v) Agepon (Agfa, Barcelona, Spain) in tridestilled water.
4. Coplin jars.

2.8. Immuno-fluorescence

1. PBTG.
2. 1% Formaldehyde in PBS.
3. 0.5% Glycine in PBS.
4. Vectashield mounting medium containing 0.1 µg/mL of DAPI.
5. Parafilm coverslips: cut a few 22 × 50 mm pieces of parafilm to use as coverslips to do the immunostaining.
6. 22 × 50 mm coverslips.
7. Coplin jars.

2.9. FISH on Immunostained Slides

1. 70% (v/v) Formamide in 2 × SSC (30 mM sodium chloride and 30 mM sodium citrate in tridistilled water), pH 7.0–7.2 (*see Note 4*).
2. 1 M sodium thiocyanate.
3. 70%, 85%, and 100% (all of them v/v) ethanol in water (*see Note 5*).
4. 0.05 × SSC.
5. Vectashield mounting medium containing 0.1 µg/mL of DAPI.
6. 22 × 50 mm and 22 × 22 mm coverslips.
7. Coplin jars.

2.10. Embedding of the Ovary in Paraffin

1. 4% (v/v) Formaldehyde in PBS.
2. 1 × SSC.
3. 70%, 80%, 90%, and 100% (all of them v/v) ethanol in water.
4. Ethanol: pentane (ratio 1:1) solution.
5. 50% and 100% (v/v) Paraplast in pentane.

2.11. FISH on Paraffin Sections

1. Xylene.
2. 2-Propanol.
3. 55%, 80%, and 100% (all of them v/v) ethanol in water.
4. 1 M sodium thiocyanate.
5. 1 × SSC and 0.05 × SSC.
6. 1 mg/mL pepsin in 0.01 M HCl, pH 2.
7. 1% Formaldehyde in PBS.
8. 0.5% Glycine in PBS.
9. Vectashield mounting medium containing 0.1 µg/mL of DAPI.
10. Rubber cement.
11. 1 cm-diameter round coverslips and 22 × 50 mm coverslips.
12. Coplin jars.

3. Methods

3.1. Collection of the Ovaries

Time lapsed between expulsion of the fetus and collection of the ovaries is critical to obtain good-quality cytological preparations. Unfortunately, most of the anatomical pathology units we have worked with have long queues of samples to be processed, thus making sample collection by pathologists almost useless. Therefore, whenever it has been possible, we have collected the sample

ourselves in order to make the time between fetus expulsion and sample collection as short as possible. Normally this time was less than 45 min (*see Note 7*).

1. Make an incision 2 cm up from the iliac crest to access the abdominal cavity.
2. Identify and extract the ovaries which should be located where the incision has been performed. At this stage the ovaries are quite soft and fragile and they look like white ovoid organs.
3. Store the ovaries in cold 1% penicillin-streptomycin in PBS to transport them to the laboratory where they are going to be processed.
4. Take notes about sample age, developmental stage (*see Note 8*), and anything that can be considered relevant.

3.2. Sample Processing in the Lab and Extraction of the Oocytes

1. Wash the ovaries with PBS in a Petri dish.
2. Remove the adjacent tissues under the stereo microscope, trying not to damage the ovaries.
3. Transfer one of the ovaries to a 50 mL tube containing 2-methyl butane at -80°C until it freezes. Then, fish it out with tweezers, place it into a clean cryotube and store it at -80°C until needed.
4. Transfer the other ovary into a clean Petri dish containing 3 mL of filtered PBS.
5. Prick the ovary with the insect mounting pins (*see Note 9*) under the stereo microscope. When the ovary is pricked, a solution blows out of the ovary. Under the stereo microscope it can be observed that this solution contains cells.
6. Collect the cell suspension into a 15 mL centrifuge tube.
7. Add 3 mL more of fresh PBS and repeat the pricking until no more cells come out of the ovary.
8. Collect the PBS into the 15 mL centrifuge tube.
9. Spin down the cell suspension for 7 min at $600g$.
10. Discard the supernatant.
11. Resuspend the cell pellet gently with a vortex using approximately 1 mL of filtered PBS (*see Note 10*).

3.3. Oocyte-Spreading Technique for Cytogenetical Analysis Using FISH

1. Pre-warm the hypotonic solution containing potassium chloride at 37°C .
2. Label the slides and put them into the cytocentrifuge chambers.
3. Using glass Pasteur pipettes, add eight drops of the hypotonic solution and one drop of the cell suspension obtained in Section 3.2 to the chamber tube.

4. Incubate for 30 min at room temperature.
5. Spin the slides at 115g for 15 min.
6. Incubate for 10 min at room temperature.
7. Remove the slides from the cytopspin chambers and incubate for 10 min longer at room temperature.
8. Submerge the slides in fresh methanol:acetic acid fixative for 10 min.
9. Transfer the slides into another Coplin jar containing fresh methanol:acetic acid fixative and let them fix for 10 min.
10. Let the slides air dry.
11. Store the slides at -20°C. These slides can be used for at least one year.

3.4. Fluorescent In Situ Hybridization (FISH) on Methanol: Acetic Acid Fixed Preparations

In this protocol, all the washing and fixations are performed in Coplin jars, unless specifically mentioned.

1. Thaw slide at room temperature and mark the location of the sample with a diamond pen on the back side of the slide.
2. Pre-warm the 70% formamide, 30% 2 × SSC at 70–71°C.
3. Wash the slide in 47 mM magnesium chloride in PBS for 2.5 min, shaking.
4. Fix the slide with 50 mM magnesium chloride, 1% formaldehyde in PBS for 5 min, shaking.
5. Wash the slide in PBS for 2.5 min, shaking.
6. Let the slide air dry.
7. Denature 10 µL of WCP and 10 µL of the LSI probe for 10 min at 75°C.
8. Denature the slide in 70% formamide in 2 × SSC at 69°C for approximately 11 min (*see Note 11*).
9. Stop denaturation and dehydrate the slide by washing it in cold 70%, 85%, and 100% ethanol solutions for 1 min each.
10. Air dry the slide.
11. Apply 20 µL of the denatured probe to the slide, add a 22 × 22 mm square coverslip and seal the edges with rubber cement, avoiding leaving any air bubble between the slide and the coverslip.
12. Let hybridization occur for 16–48 h in a dark humid chamber at 37°C.
13. Pre-warm 2 × SSC and 0.05% Tween 20 in 4 × SSC solutions at 45°C.
14. Peel the rubber cement off and remove the coverslip carefully, trying not to damage the specimen (*see Note 12*).
15. Wash the slide in 2 × SSC at 45°C for 5 min.

16. Wash the slide in 0.05% Tween 20 in 4 × SSC at 45°C for 5 min.
17. Wash the slide in 0.05% Tween 20 in 4 × SSC at room temperature for 5 min.
18. Let the slide air dry.
19. Apply 20 µL of a DAPI solution in Vectashield on a 22 × 50 mm coverslip.
20. Add the coverslip on top of the slide and remove the excess of mounting medium.
21. Analyze the slide using a fluorescence microscope.

3.5. Structurally Preserved Nuclear Preparations

This technique allows the acquisition of structurally preserved nuclear preparations, which enable the three-dimensional study of human oocytes at meiotic prophase (*see Supplementary Movie 1* [available on the companion CD for this volume] for an example). In this protocol, all the washing and fixation steps are performed in Coplin jars, unless specifically mentioned.

1. Prepare fixative solution and chill it on ice.
2. Cut a tenth of the frozen ovary (**Section 3.2**, step 3) and mince it with a scalpel on top of a Super Frost slide until no big clumps can be seen.
3. Add 60 µL of 2% formaldehyde, 0.05% Triton X-100 in PBS onto the slide, mix it and extend the solution along the slide carefully with the tip of the micropipette.
4. Let sit for 15 min at 4°C.
5. Air dry, at room temperature, until it is almost completely dry (*see Note 13*).
6. Wash the slide for 10 min in 0.5% glycine in PBS shaking.
7. Block the slide in PBTG for 10 min, shaking.
8. Proceed to immunostaining (**Section 3.8**) or store at –20°C in 80% glycerol in PBS. These slides can be used at least up to one year after fixation.

3.6. Oocyte-Spreading Technique for Immunofluorescent Analysis Starting from Frozen Sample

This protocol has been developed based on an existing technique described elsewhere used to spread mouse spermatocytes (12). This procedure allows the spreading of oocytes for IF and FISH analysis starting from frozen ovaries. In this protocol, all the washing and fixations are performed in Coplin jars, unless specifically mentioned.

1. Prepare the fixative solution and chill it on ice.
2. Cut a tenth of the frozen ovary (**Section 3.2**, step 3) and mince it on top of a Superfrost slide with a scalpel until no big clumps can be seen. Add 20 µL of chilled PBS if necessary in order to avoid drying.

3. Transfer 5 µL of the cell suspension to other slides. One tenth of the ovary should give enough material to prepare three or four slides.
4. Place the slides in a humid chamber and add 80 µL of 1% Lipsol in water.
5. Let the slides sit for 12 min in the humid chamber.
6. Add 90 µL of fixative and let them sit for 2 h in a humid chamber.
7. After this time, open the humid chamber and let the slides dry until they are almost completely dried (*see Note 13*).
8. Wet the slides by washing them four times, one min each in 1% Agepon in water.
9. Proceed to immunostaining (**Section 3.8**) or store at –20°C. These slides can be used at least up to one year after fixation.

3.7. Oocyte-Spreading Technique for Immunofluorescent Analysis Starting from Fresh Sample

This protocol is based on a previously published one (13) and can be used with the same purpose as the one described in **Section 3.6**. In this protocol, all the washing and fixations are performed in Coplin jars, unless specifically mentioned.

1. Pre-warm the sucrose hypotonic solution at 37°C and prepare the fixative solution and chill it on ice.
2. Put the Superfrost slides into the cytospin chambers.
3. Add 0.5 mL of the hypotonic solution and one drop of the cell suspension obtained in **Section 3.2** to the chamber tube.
4. Spin them at 115g for 15 min.
5. Remove the slides from the cytospin chambers and let them sit in a humid chamber for 2 h.
6. Add 0.6 mL of fixative solution and let the slides sit in a humid chamber for 10 min.
7. Open the humid chamber and let the slides air dry overnight.
8. Wet the slides by washing them four times for one min each in 1% Agepon in water.
9. Proceed to perform immunostaining (**Section 3.8**) or store them at –20°C. These slides can be used at least up to one year after fixation.

3.8. Immuno-fluorescent Staining

In this protocol, all the washing and fixations are performed in Coplin jars, unless specifically mentioned.

1. If the slide has been previously frozen, first thaw the slide, wash it two times in PBS for 5 min, shaking, and block it in PTBG for 10 min, shaking.
2. Apply 100 µL of the primary antibody diluted in PTBG over the slide, apply a parafilm coverslip on top and leave it overnight at 4°C in a humid chamber.

3. Wash the slides three times in PTBG at 37°C for 3 min, shaking.
4. Apply 100 µL secondary antibody diluted in PTBG, using a parafilm coverslip for 1 hour at 37°C in a humid chamber.
5. Wash the slides three times in PTBG for 3 min, shaking.
6. Fix the slide in 1% formaldehyde in PBS for 10 min in order to maintain the fluorescent signal in its proper location.
7. Wash the slide in 0.5% glycine in PBS for 5 min, shaking.
8. Wash the slide 10 min in PTBG, shaking.
9. Place a 22 × 50 mm coverslip on the bench and apply 20 µL of a DAPI solution in Vectashield on top.
10. Put the slide face down on top of the coverslip, then turn the slide over. The coverslip will now be attached to the slide by capillary action. Remove excess mounting medium.
11. Analyze the slide using a fluorescence microscope.

3.9. FISH on Immunostained Slides

This protocol has been developed to be applied on slides that were stained following the previous protocol (**Section 3.8**). If no fixation of the fluorescent signal has been performed during the staining, the denaturing conditions described below may be too strong and should be readjusted. In this protocol, all the washing and fixations are performed in Coplin jars, unless specifically mentioned.

1. Pre-warm 70% formamide, 30% 2 × SSC solution at 70°C.
2. Wash the slide 2 min in distilled water.
3. Let the slide air dry.
4. Denature the slide in 70% formamide 2 × SSC at 70°C for 5 min.
5. Wash the slide 1 min in distilled water.
6. Apply 100 µL of 1 M NaSCN for 3 h in a humid chamber at 65°C in order to destabilize peptide sulfur bonds.
7. Let the slide air dry.
8. Denature the slide again in 70% formamide 2 × SSC at 70°C for 5 more min.
9. Meanwhile, denature the probe following vendor recommendations.
10. Stop denaturation and dehydrate the slide by washing it in cold 70%, 85%, and 100% ethanol for 1 min each.
11. Apply the denatured probe with a 22 × 22 mm coverslip to the desired area and seal the edges with rubber cement. Let the probe hybridize for at least 72 h at 37°C in a humid chamber.
12. Pre-warm 0.05 × SSC at 45°C for 5 min.

13. Peal rubber cement off, carefully remove the coverslip, and wash the slide in $0.05 \times$ SSC at 45°C .
14. Apply $20 \mu\text{L}$ of a DAPI solution in Vectashield on a $22 \times 50 \text{ mm}$ coverslip.
15. Add the coverslip on top of the slide and remove excess mounting medium.
16. Analyze the slide using a fluorescence microscope.

3.10. Embedding of the Ovary in Paraffin

1. Cut a quarter of an ovary and fix it in 2 mL of 4% formaldehyde in PBS for 4 h .
2. Wash the sample three times with PBS for 5 min .
3. Wash one more time in $1 \times$ SSC for 5 min .
4. Dehydrate the specimen in successive washes of 30 min in 70% , 80% , and 90% ethanol in water.
5. Wash the sample three times in ethanol for 10 min each.
6. Submerge the ovary piece in a solution containing ethanol and pentane for 20 min .
7. Submerge the sample for 20 min in pentane.
8. Submerge the specimen in a solution containing pentane and paraplast in equal parts at 65°C for 30 min .
9. Finally, submerge the sample in paraplast and let it sit at 65°C overnight.
10. Fish the specimen out and place it into an appropriate mold containing fresh paraplast.
11. Let the wax block harden at room temperature. Unmold the block and proceed to obtain preparations with $5\text{--}10 \mu\text{m}$ thick sections.
12. Store the slides at room temperature.

3.11. FISH on Paraffin Sections

The protocol described here is based on a previously published one with some variations (14). In this protocol, all the washing and fixations are performed in Coplin jars, unless specifically mentioned.

1. First mark the position of the sample on the back of the slide with a diamond pen.
2. Wash the slide two times in xylene at 60°C for 5 min .
3. Wash the slide in 2-propanol for 5 min .
4. Rehydrate the slide with successive washing in 100% , 80% , and 55% ethanol in water for 5 min in each solution.
5. Wash the slide in distilled water for 5 min .
6. Apply $100 \mu\text{L}$ of 1 M NaSCN for 3 h in a humid chamber at 65°C .
7. Wash the slide in distilled water for 5 min .

8. Wash the slide in $1 \times$ SSC for 5 min.
9. Wash the slide in distilled water for 5 more min.
10. Apply pepsin solution at 37°C until most of the cytoplasm of the cells has been digested and cell nuclei are exposed (*see Note 14*).
11. Stop enzymatic digestion by washing the slide in PBS for 5 min.
12. Fix the preparation in 1% formaldehyde in PBS for 10 min.
13. Wash the slide in 0.05% glycine in PBS for 5 min.
14. Apply 4 μL of the probe to the tissue, put a 1 cm-diameter round coverslip on top and seal it with rubber cement.
15. Co-denature the slide and the probe on a hot plate at 75°C for 5 min.
16. Let hybridization occur for at least 72 h at 37°C in a humid chamber.
17. Peal rubber cement off, carefully remove the coverslip, and wash the slide in $0.05 \times$ SSC at 45°C for 5 min to remove nonspecifically bound and unbound probe.
18. Apply 20 μL of a DAPI solution in Vectashield on a 22×50 mm coverslip and put it on top of the slide.
19. Remove excess mounting medium and analyze the slide using a fluorescence microscope.

4. Notes



1. For our purpose, 50 mL of 2-methylbutane, also known as isopentane, can be stored at -80°C to have it always ready to use.
2. Insect mounting pins are used in this technique because they are thinner than most of the needles commonly found in a lab, thus allowing puncturing of the ovary without substantially damaging the sample. This way, a suspension containing a high concentration of oocytes is obtained free of big clumps.
3. Fixative solution must be prepared just before being used and it cannot be stored or recycled once used. To have better results, cold methanol (stored at 4°C) can be used to prepare the methanol:acetic acid fixative solution.
4. Both 50% and 70% formamide in $2 \times$ SSC can be stored for a long period of time at 4°C . Moreover, these solutions can be reused up to ten times if desired.

5. Ethanol dilutions should be stored at -20°C and they can be reused up to ten times if desired.
6. Best morphological results are obtained using acid-free formaldehyde. This preparation of formaldehyde contains calcium carbonate which prevents the formation of formic acid, which can harm the sample during fixation. Moreover, formaldehyde fixative solution must be prepared fresh each time and kept at 4°C for every experiment in order to guarantee a proper fixation of the specimen.
7. If the abortion is performed by methods which involve fragmenting the fetus, the ovaries could be collected by washing the sample in clean PBS in a transparent container with light from below. The ovaries can be identified by their ovoid shape. After finding the ovaries, the protocol should continue as described in **Section 3.1**.
8. The best developmental indicator we have found in order to compare different cases is the foot length (8). However, several other parameters, like gestation age, longest biparietal diameter, head circumference, or femur length can be collected in order to evaluate the developmental stage of each fetus.
9. This is a gentle way to obtain a cell suspension containing oocytes. Other methods can be performed to disaggregate the ovary (3, 6), but in our hands, this is the fastest, cleanest and most reliable method to obtain a cell suspension from the ovary almost completely free of cell clumps.
10. Experience will tell you how concentrated you want the cell suspension to be. As a starting point, you can try adding 1 mL of PBS to the cell pellet.
11. Slide denaturing temperature and time may vary depending on each slide. Over-denaturation of the slide should be avoided in order to preserve proper nuclear morphology which is crucial to identify meiotic stage of each oocyte [for an example of how oocytes should look after FISH see (8)]. Thus, in the protocol are cited the ranges of temperatures and times that have previously worked in our hands, but one could first do an experiment trying different times and temperatures to determine the parameters that are enough to denature the nuclear DNA without damaging nuclear morphology.
12. An easy way to remove the coverslip without damaging the specimen is to immerse the slide in a Coplin jar containing $2 \times \text{SSC}$. When the slide is removed from the jar the coverslip should fall by gravity.
13. Let the slide dry until no liquid can be seen, trying not to over-dry it because this may result in an increase of background after immunostaining. The time required depends

on the temperature and humidity of the lab, but to have an approximate idea of how much time it takes, in our lab the slides were almost completely dried in 30–60 min.

14. Pepsin digestion should be controlled by examining slides approximately every 10–15 min under a phase-contrast microscope in order to prevent over-digestion, which may damage the DNA and make FISH impossible.

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Chapter 25

Using RNA FISH to Study Gene Expression During Mammalian Meiosis

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Abstract

During mouse meiosis, gene expression and homologous synapsis are intimately linked. Chromosomes that fail to synapse at the zygotene–pachytene transition become transcriptionally silenced by a process called Meiotic Silencing of Unsynapsed Chromatin (MSUC), and this silencing (or defects in it) may in turn cause germ cell losses and infertility. Gene transcription at the chromosomal level can be readily observed using RNA fluorescence in-situ hybridisation (FISH), and this approach allows one to directly compare expression at a specific locus with the synaptic status of the chromosome domain on which it resides. Here we describe a protocol for carrying out RNA FISH on male meiotic cells, together with detail on the important controls and common problems associated with this technique.

Key words: Fluorescence in-situ hybridisation, meiotic sex chromosome inactivation, meiotic silencing of unsynapsed chromatin.

1. Introduction

RNA fluorescence in-situ hybridisation (FISH) is a powerful technique for visualising transcription of a specific gene at the cellular level. Essentially, the technique entails applying a labelled probe to permeabilised, fixed nuclei, followed by stringency washing and subsequent visualisation of the probe-transcript hybrid. In most instances, probes are designed to recognise transcripts before they are spliced, which allows them to be observed at their point of synthesis. The presence or absence of an RNA FISH signal, most commonly observed as a fluorescent “dot” on the chromosome, provides a visual readout of whether a gene is transcribing or not,

and this allows one to correlate gene transcription with specific features of chromosome behaviour, including subnuclear localisation or chromosome conformation.

A striking example of how changes in chromosome behaviour influence gene expression is seen during meiotic synapsis. In male mice, the transition from zygotene to pachytene is accompanied by a dramatic increase in transcription throughout the nuclear compartment, with the exception of the X and Y chromosome, which become silenced. This process, called Meiotic Sex Chromosome Inactivation (MSCI), results in the formation of a distinct perinuclear compartment called the sex body (1, 2). Two observations suggest that MSCI occurs because the X and Y chromosomes are largely unsynapsed. Firstly, when either sex chromosome is provided with a synaptic partner during meiosis – in the case of the Y chromosome this can be done by generating XYY males – that chromosome synapses and escapes MSCI (3). Furthermore when autosomes fail to synapse, they are also silenced (4, 5). Therefore, a mechanism exists in mammals that senses unsynapsed chromosomes early in pachytene and silences them. This mechanism is called Meiotic Silencing of Unsynapsed Chromatin (MSUC) (6), and it is now clear that MSCI is MSUC affecting the X and Y chromosomes.

In this chapter, we describe the use of RNA FISH to study gene transcription during meiosis, using MSCI/MSUC as a representative example. We also discuss how RNA FISH can be combined with antibody staining and DNA FISH to study the relationship between chromosome synapsis and gene transcription.

2. Materials

2.1. Probe Preparation –

BACs

1. Chloramphenicol stock: 50 mg/mL.
2. LB agar.
3. LB broth.
4. P1 solution: 15 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/mL RNase A.
5. P2 solution: 0.2 M NaOH, 1% SDS – made fresh.
6. P3 solution: 3 M potassium acetate, pH 5.5.
7. Isopropanol ice-cold.
8. Ethanol: 70% and 100%.
9. TE pH 8.0: 10 mM Tris-HCl, 1 mM disodium EDTA.
10. RNase A: 10 mg/mL

11. Phenol.
12. Phenol/chloroform/isoamyl alcohol 25:24:1.
13. Chloroform: isoamyl alcohol 24:1.
14. Sodium acetate: 3 M, pH 5.2.
15. BioNick™ DNA Labelling System (Invitrogen; 18247-015).
16. DIG-Nick Translation Mix (Roche; 11745816910).
17. Salmon sperm DNA: 10 mg/mL.
18. Mouse Cot-1 DNA: 1 µg/µL (Invitrogen; 18440-016).
19. Deionised formamide.

2.2. Probe Preparation –

Long-Range PCR

Products

1. Template: 100 ng/µL genomic DNA or 50 ng/µL BAC DNA.
2. PCR primers: 5 pmol/µL.
3. KOD XL polymerase kit (Novagen; 71087-3).
4. Qiagen gel purification kit.

2.3. RNA FISH

1. Mouse testis material – fresh or snap frozen (stored –80°C).
2. RPMI medium + L-glutamine, ice cold.
3. CSK buffer: 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES. Adjust pH to 6.8 with NaOH. Filter sterilise and keep at –20°C in 25 mL aliquots.
4. CSK buffer supplements: 0.5% Triton X-100, 1 mM EGTA, 2 mM vanadyl ribonucleoside (NEB; S1402S). Add fresh to defrosted CSK buffer, mix thoroughly at room temperature, then cool on ice.
5. 4% PFA (paraformaldehyde) pH 7.0–7.4 – made fresh, filter sterilised, ice cold.
6. Ethanol series: 70%, 80%, 95%, 100%, ice cold.
7. Dulbecco's PBS, ice cold.
8. 2 × Hybridisation buffer: 4 × SSC, 50% dextran sulphate, 2 mg/mL BSA, 2 mM vanadyl ribonucleoside.
9. Stringency wash solution A: 50% formamide, 1 × SSC pH 7.2–7.4.
10. Stringency wash solution B: 2 × SSC pH 7.2–7.4.
11. Stringency wash solution C: 4 × SSC, 0.1% Tween-20 pH 7.2–7.4.
12. Blocking Buffer: 4 × SSC, 4 mg/mL BSA, 0.1% Tween-20.
13. Detection Buffer: 4 × SSC, 1 mg/mL BSA, 0.1% Tween-20.
14. Streptavidin, Alexa Fluor® 555 conjugate (Invitrogen S21381). Dilute 1:100 in detection buffer and spin 16,000*g*, 15 min immediately before use.

15. Biotinylated anti-streptavidin (Vector; BA0500). Dilute 1:100 in detection buffer and spin 16,000*g*, 15 min immediately before use.
17. Vectashield mounting medium with DAPI (Vector; H-1200).
18. PBT: Dulbecco's PBS, 0.1% Tween-20, 1.5 mg/mL BSA.
19. Coplin jars, sterilised by baking. Boil slides in water for 10 min in microwave to clean.
20. Ice-cold platform.
21. Humid chamber.
22. 80°C hot block.
23. 37°C incubator.
24. 42°C water bath.
25. Shaker.
26. Tip-Top vulcanising solution (Rema; 5059056) – optional.

2.4. DNA FISH

1. Dulbecco's PBS.
2. Pepsin solution: 500 µg/mL in 10 mM HCl.
3. Denaturation solution: 70% deionised formamide: 30% 2 × SSC.
4. Ethanol series: 70%, 80%, 95%, 100%, ice cold.
5. 2 × Hybridisation buffer: 4 × SSC, 50% dextran sulphate, 2 mg/mL BSA, 2 mM vanadyl ribonucleoside.
6. Stringency wash solution A: 50% formamide, 1 × SSC.
7. Stringency wash solution B: 2 × SSC.
8. Stringency wash solution C: 4 × SSC, 0.1% Tween-20.
9. Blocking Buffer: 4 × SSC, 4 mg/mL BSA, 0.1% Tween-20.
10. Detection Buffer: 4 × SSC, 1 mg/mL BSA, 0.1% Tween-20.
11. FITC-conjugated anti-digoxigenin (Roche; 11207741910). Dilute 1:10 in detection buffer and spin 16,000*g*, 15 min immediately before use.
12. Vectashield mounting medium with DAPI (Vector; H-1200).
13. Coplin jars, slides, and coverslips.
14. Humid chamber.
15. 80°C hot block.
16. 37°C incubator.
17. 75–80°C water bath.
18. 42°C water bath.
19. Shaker.
20. Tip-Top vulcanising solution (Rema; 5059056) – optional.

3. Methods

Although riboprobes were used in classical RNA FISH studies, most modern applications use DNA probes, which are equally effective and much easier and cheaper to prepare. Unlike riboprobes, DNA probes must obviously be denatured before use. We routinely use BAC DNA (100–200 kb) or long-range PCR product (~12 kb) probes. In our experience, BAC probes are more sensitive than PCR products but they are less specific, as their large size raises the possibility of hybridisation to more than one transcript within a given chromosome region. In either case, the probe should include intronic sequence in order to ensure hybridisation to primary (pre-spliced) transcripts. Even when these criteria are met, the success of RNA FISH varies from gene to gene. Choosing genes with large primary transcripts will theoretically provide greater sensitivity, but even in these cases an RNA FISH signal may not be visible, either because the expression level of the gene is low or the rate of processing of the primary transcripts is high.

A major concern when performing RNA FISH is RNA degradation. This is circumvented by maintaining cells and RNA FISH solutions on ice and in the presence of the RNase inhibitor vanadyl ribonucleoside. For the same reason, when combining RNA FISH with antibody staining and/or DNA FISH, the RNA FISH is performed first. We always perform DNA FISH after RNA FISH, because this provides added confidence that the signal observed following RNA FISH corresponds to the transcript of interest and is not spurious (7). DNA FISH involves heating cells to high temperatures and this can result in loss of RNA FISH signals, so we advise capturing RNA FISH images prior to carrying out DNA FISH.

3.1. BAC DNA Purification

1. Streak BAC-containing bacteria onto LB plates containing relevant antibiotic (usually 12.5 µg/mL chloramphenicol). Grow overnight 37°C.
2. Inoculate a single colony into 10 mL LB medium containing relevant antibiotic (usually 12.5 µg/mL chloramphenicol). Grow overnight at 37°C.
3. Add 1.5 mL culture into each of two Eppendorf tubes. Centrifuge at 16,000*g*, room temperature, for 30 s to pellet bacteria.
4. Discard supernatant. Repeat step 3 twice, such that a final culture volume of 4.5 mL has been pelleted in each tube.
5. Discard supernatant. Resuspend pellet in 300 µL of P1 solution by vortexing.

6. Add 300 μ L of P2 solution. Gently shake to mix. Incubate at room temperature for 5 min.
7. Add 300 μ L of P3 solution. Gently shake to mix. Incubate on ice for 5 min.
8. Centrifuge at 16,000*g*, room temperature, for 10 min.
9. Gently remove 800 μ L of supernatant from white precipitate and place in fresh Eppendorf tube.
10. Add 0.6 vol (480 μ L) ice-cold isopropanol. Invert to mix. Immediately centrifuge at 16,000*g*, room temperature, for 15 min.
11. Discard supernatant. Wash BAC DNA pellet in 500 μ L 70% ethanol by inverting. Centrifuge at 16,000*g*, room temperature, for 5 min.
12. Aspirate 70% ethanol, air dry pellet and resuspend in 30 μ L TE pH 8.0.
13. Combine two tubes of DNA for each clone, giving 60 μ L in total for each original clone.
14. Add 1 μ L RNase A and incubate overnight 37°C (*see Note 1*).
15. Supplement BAC DNA solution to 400 μ L TE pH 8.0.
16. Remove RNase A by extraction in equal vol of phenol, phenol:chloroform:isoamyl alcohol and finally chloroform:isoamyl alcohol, once each. In each case, mix the solutions by inverting, centrifuge at 16,000*g*, room temperature, for 5 min, and transfer the upper phase to a new Eppendorf tube.
17. Estimate final volume of BAC DNA. Precipitate using 0.6 vol ice-cold isopropanol. Immediately centrifuge at 16,000*g*, room temperature, for 15 min.
18. Discard supernatant. Wash BAC DNA pellet in 500 μ L 70% ethanol by inverting. Centrifuge at 16,000*g*, room temperature, for 5 min.
19. Aspirate 70% ethanol, air dry pellet and resuspend in 30 μ L TE pH 8.0.
20. Estimate BAC DNA concentration by spectrophotometry.

3.2. Long-Range PCR Product Preparation

1. Design PCR primers using the Primer3 algorithm (8) with parameters: T_m [min = 68°C, opt = 70°C, max = 71°C], primer length [min = 25 bp, opt = 28 bp, max = 31 bp].
2. Set up 50 μ L long-range PCR exactly according to manufacturer's instructions, using either 100 ng genomic DNA or 50 ng BAC DNA as template (*see Note 2*).
3. Carry out PCR using following conditions: 1 cycle of 95°C for 1 min, 30 cycles of 94°C for 30 s and 68°C for 10 min.

4. Run 4 µL of long-range PCR product on an agarose gel to visualise product.
5. Combine long-range PCR products and gel purify product using the Qiagen PCR Gel Extraction kit exactly according to manufacturer's instructions.
6. Estimate PCR product concentration by spectrophotometry.

3.3. Probe Labelling

1. Label 1 µg of BAC DNA or purified long-range PCR product using the BioNick™ DNA Labelling System for biotin labelling or the DIG-Nick Translation Mix for digoxigenin labelling exactly according to manufacturer's instructions (*see Note 3*). The reaction should be stopped when the probe length reaches 50–200 bp (*see Note 4* and Fig. 25.1).

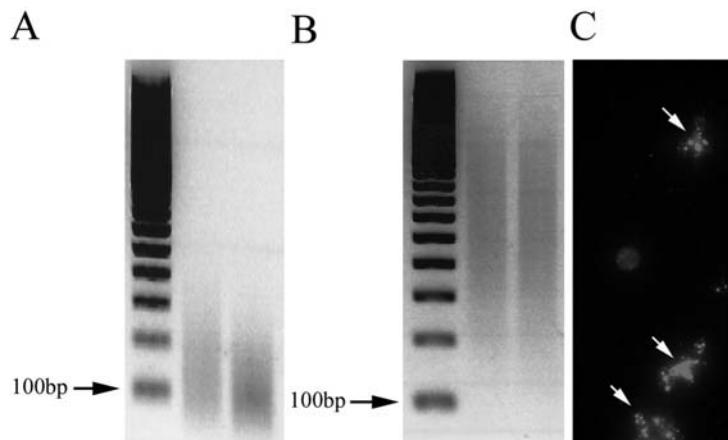


Fig. 25.1. Probe labelling for RNA FISH. (A) Successful probe labelling – lane 1:100 bp ladder, lanes 2 and 3: two different probes post-labelling – both migrate between approximately 50 and 200 bp. (B) Unsuccessful probe labelling – lane 1:100 bp ladder, lanes 2 and 3: two different probes post-labelling – both migrate between 200 bp and 2 kb. (C) Probes that are insufficiently labelled will form aggregates on the slide (arrows).

2. Combine one tenth of the reaction volume in a screw-capped 1.5 mL tube together with 3 µL mouse Cot1 DNA and 1 µL salmon sperm DNA.
3. Precipitate by adding 2.5 vol 100% ethanol. Centrifuge at 16,000*g*, at 4°C, for 10 min.
4. Discard supernatant. Wash pellet in 100 µL 70% ethanol by inverting. Centrifuge at 16,000*g*, at 4°C, for 5 min.
5. Aspirate 70% ethanol, air dry pellet and resuspend in 10 µL formamide.
6. Store at –20°C.

3.4. RNA FISH Plus
Antibody Staining

1. Place clean slides on an ice-cold, horizontal platform.
2. Take freshly harvested or frozen (stored at -80°C) testis material (*see Note 5*) and make a cell suspension in ice-cold RPMI + L-glutamine in a Petri dish using sterile scalpel blades. Mix up and down with plastic Pasteur pipette and tilt to allow tubule remnants to sink.
3. Place two drops of suspension on each slide (*see Note 6*).
4. Permeabilise the cells by adding ice-cold CSK buffer + supplements, dropwise, until the slide is flooded but not over-flowing. Leave for 10 min (*see Note 7*).
5. Drain CSK from slide either by aspirating gently from one end of the slide using a plastic Pasteur pipette, or by placing a paper towel at one end of the slide and then gently tilting the cold platform.
6. Fix the cells by flooding the slide with ice-cold 4% PFA. Leave for 10 min.
7. In the meantime, denature probes at 80°C for 10 min. Following denaturation, add 10 µL 2 × hybridisation buffer, vortex and pre-hybridise probes at 37°C for 30 min (*see Note 8*).
8. Remove the slide from the cold platform, pour off the PFA, and rinse in ice-cold PBS. Transfer slides into ice-cold 70% ethanol.
9. Dehydrate the slide through an ice-cold ethanol series, beginning with a second wash in 70% followed by 80%, 95%, and 100%, for 3 min each.
10. Air-dry until no traces of ethanol are left on slide.
11. Add pre-hybridised probe directly onto centre of slide, avoiding air bubbles, add coverslip. Seal coverslips with Tip-Top vulcanising solution. If not sealing, place slides in a humid chamber. Incubate overnight 37°C.
12. The next day, pre-heat stringency wash solutions A and B at 42°C.
13. If sealant was used on slides, remove it at this stage. Transfer slides into a Coplin jar, add stringency wash solution A and shake at room temperature, 5 min.
14. Carefully remove coverslips. Wash two more times in stringency wash solution A at room temperature, 5 min, with shaking.
15. Wash three times in stringency wash solution B at room temperature, 5 min, with shaking.
16. Add stringency wash solution C (kept at room temperature) and keep slides in this solution until ready for next step.
17. Remove slides from stringency wash solution C, drain briefly, and place horizontally in a humid chamber. Add 100 µL Blocking Buffer, apply coverslip and incubate 37°C for 30 min.

18. Remove coverslip, drain Blocking Buffer and add 100 μ L diluted streptavidin AlexaFluor® 555 conjugate (*see Note 9*). Apply coverslip and incubate 37°C for 30 min.
19. Remove coverslip. Wash three times in stringency wash solution C at room temperature, 2 min, with shaking.
20. Add 100 μ L diluted biotinylated anti-streptavidin, diluted 1:100 in Detection Buffer. Apply coverslip and incubate 37°C for 30 min.
21. Remove coverslip. Wash three times in stringency wash solution C at room temperature, 2 min, with shaking.
22. Add 100 μ L diluted streptavidin AlexaFluor® 555 conjugate. Apply coverslip and incubate 37°C for 30 min.
23. Remove coverslip. Wash three times in stringency wash solution C at room temp, 2 min, with shaking.
24. Slides can proceed directly to antibody staining. Otherwise they can be drained, mounted in Vectashield mounting medium with DAPI, observed or stored at -20°C.
25. For antibody staining, add antibody of choice diluted in PBT at concentration, temperature, and time predetermined for each specific antibody (*see Note 10*). An example of RNA FISH for the X-linked gene *Zfx*, combined with antibody staining for the MSCI marker γ H2AX is shown in Fig. 25.2.

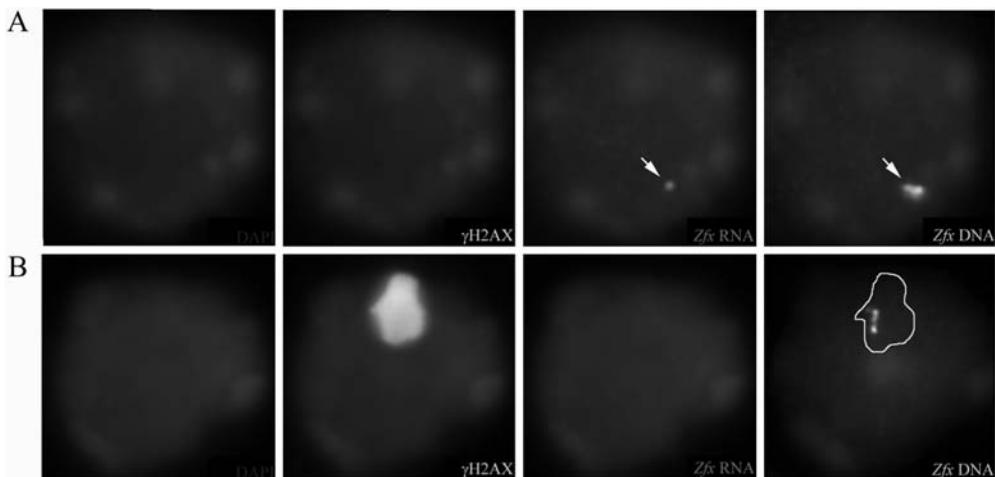


Fig. 25.2. RNA FISH before and during meiosis for the X-linked gene *Zfx*. **(A)** Spermatogonium – during this stage, the X chromosome is active, as shown by the absence of the sex body marker γ H2AX (*second column*). *Zfx* is active, as shown by the *pinpoint signal* (*third column*). This signal colocalises with the *Zfx* locus, as shown by DNA FISH in the *last column*. **(B)** Pachytene. MSCI has taken place, and the γ H2AX-positive sex body is shown (*second column*). *Zfx* is silent as a result of MSCI (*third column*). The *Zfx* locus lies within the sex body, as inferred from the γ H2AX staining, which is shown as an outline in the *last column*.

3.5. DNA FISH After RNA FISH

1. Remove coverslips and wash slide three times in PBS at room temperature, 5 min, with shaking.
2. Incubate slides in pepsin solution at room temperature, 10 min.
3. Wash slides three times in PBS, 5 min.
4. In the meantime, denature probes at 80°C for 10 min. Following denaturation, add 10µL 2 × hybridisation buffer, vortex and pre-hybridise probes at 37°C for 30 min (*see Note 8*).
5. Rinse slides in stringency wash solution B.
6. Incubate slides in stringency wash solution B at 75–80°C for 5 min.
7. Denature the slides in denaturation solution at 75–80°C for 3 min (*see Note 11*).
8. Quench slides in ice cold 70% ethanol for 3 min.
9. Carry out slide dehydration, probe hybridisation and stringency washes exactly as described for RNA FISH (*see Section 3.4*, steps 9–17).
10. Remove coverslip, drain blocking buffer and add 100 µL FITC-conjugated anti-digoxigenin diluted 1:10 in detection buffer. Apply coverslip and incubate 37°C for 60 min.
11. Remove coverslip. Wash three times in stringency wash solution C at room temperature, 2 min, with shaking.
12. Drain slides, mount in Vectashield mounting medium with DAPI. Observe immediately or store at –20°C. An example of DNA FISH for the X-linked gene *Zfx* is shown in Fig. 25.2.

4. Notes



1. RNase treatments can be carried out for shorter periods, e.g., 30 min at 37°C.
2. An appropriate number of 50 µL PCRs should be set up in order to generate a final DNA concentration of 2 µg for the probe labelling step. The number needed will depend on the efficiency of the given PCR primer pair and therefore must be determined empirically.
3. For rapid generation of labelled probes, reactions can be carried out at 37°C rather than 15°C. Note that long-range PCR products label much more quickly than BAC DNA due to their smaller size. It is advisable to set up more than one labelling reaction per DNA sample, as some of the reaction will be used up in assessing the labelling efficiency.

4. The duration of probe labelling is the single most important step in FISH and therefore probes should always be run on agarose gels before they are used. A common problem is under-labelling, which can be seen as a smear of high molecular weight on the gel (**Fig. 25.1B**). If probes are too long, they will be unable to pass through the nuclear membrane and will form brightly staining aggregates on the slide (**Fig. 25.1C**). Probes that are under-labelled following the recommended time of incubation can be placed at 37°C for short bursts until the required length is reached. Additional labelling mix can also be added.
5. In our experience, testis material stored at -80°C gives equivalent RNA FISH results to fresh material.
6. Alternatively, cells can be placed onto coverslips. Although coverslips provide the advantage that smaller volumes of solutions are required, we find handling of slides much more straightforward.
7. A number of variations exist for preparing cells for RNA FISH. Some protocols fix before permeabilising, or use alternative methods of permeabilisation, e.g., saponin, freeze-thawing (9). We find that permeabilisation before fixation gives the best results for testis cells.
8. This step is used to allow the Cot1 DNA to hybridise to repetitive sequences in the probe, thus reducing background.
9. This protocol describes amplification of the RNA FISH signal using multiple rounds of antibody incubations, which will increase the sensitivity of the procedure but can also increase background. Several variations on the theme exist. A single round of diluted streptavidin AlexaFluor® 555 conjugate incubation can be performed, or probes can be directly labelled (10), thus removing the need to develop the probe signal and allowing direct visualisation after stringency washing.
10. Although we do not generally do it, antibody staining can be performed prior to RNA FISH in the presence of RNase inhibitors, as described (10). This alternative approach is often favoured because epitopes may be destroyed by the conditions required for RNA FISH. Note that blocking has already been performed in step 19 and so does not need to be repeated prior to antibody staining.
11. In our experience, the time of denaturation can vary from gene to gene – some require short periods, e.g. 3 min, while others can be left for as long as 30 min. Unlike some other protocols, we do not refix cells between RNA FISH and DNA FISH because this cross-links the DNA and reduces the efficiency of DNA FISH.

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