Preface

Probably no single experimental organism has contributed as much to our understanding of the structure, function, and mechanism of transmission of eukaryotic chromosomes as has the fruit fly, *Drosophila melanogaster*. Think of a (cyto)genetics principle and chances are it was discovered (or substantially elaborated) using the fly. Indeed, *Drosophila* experimental biology is so powerful precisely because its foundations are built around the "chromosome problem." For the very same reason, cytogenetics methods can benefit practically any area of *Drosophila* study.

Drosophila Cytogenetics Protocols is a laboratory manual aimed primarily at "entry-level" drosophilists, such as graduate students and postdoctoral researchers who may be unfamiliar with fruit fly chromosomes or their methods of study. This is reflected in the detailed step-by-step protocols, the substantial background material, and extensive references to primary literature. The chapters emphasize specimen preparation, from dissection to mounting (this book might have been called *Fly Smooshing*), and both polytene and mitotic/meiotic chromosomes are covered in depth. Techniques for image acquisition are provided where necessary, but, in general, it is assumed that the user will have a working knowledge of his or her particular microscope, or at least access to someone who does. Seasoned drosophilists will likely also find new and interesting material here.

Drosophila Cytogenetics Protocols began as a notion in Dundee, Scotland. There, and later in Cambridge, England, I had the pleasure of working with, and learning from, many talented cytogenetics practitioners, members of David Glover's cell cycle genetics laboratory. Practical cytogenetics is as much an art as a science, and the revolving-door world of laboratory personnel can result in a loss of critical know-how when a researcher moves on, sometimes leaving new arrivals to flounder. Putting together a collection of detailed cytogenetics protocols therefore seemed a worthwhile endeavor, and five of my former Dundee/Cambridge colleagues kindly wrote chapters for this book. Many other skilled drosophilists also saw value in this project and *Drosophila Cytogenetics Protocols* has benefited enormously from their contributions as well. I thank all the authors for their fine chapters, suggestions about content, and endless patience. I am grateful to David Glover for having given me the opportunity to be part of his vibrant lab, John Walker, the series editor, for his encouragement and advice on book matters, Wendy Kopf, production editor, for sterling stewardship, and members of my family for their interest and support.

Daryl S. Henderson

2

Spermatogenesis

Analysis of Meiosis and Morphogenesis

Helen White-Cooper

1. Introduction

1.1. Cytogenetic Analysis of the Drosophila *Testis*

In this chapter, I hope to convince the reader that *Drosophila* spermatogenesis is an ideal system for the cytogeneticist. Spermatogenesis is relatively simple, dispensable for adult viability, and amenable to genetic, cell biological, and biochemical approaches. The stages of spermatogenesis are well defined, the cells are large, easily accessible, and easily identified. Because spermatogenesis initiates with a stem cell division, there is continuous production through the life of the fly. Therefore, a normal adult testis presents all of the stages of spermatogenesis as a spatio-temporal array (*see* **Fig. 1A**). Excellent and comprehensive reviews of the genetics of spermatogenesis and ultrastructure of wild-type spermatogenesis are available ***(1**,**2)***. In this introduction, I will review only the essential features needed to interpret the results of experiments performed according to the protocols given here.

Spermatogenesis follows a multistep differentiation program involving dramatic changes in cell cycle dynamics, gene expression, and morphogenesis. The transformation of a 15-μm-diameter round spermatid into a 1.8-mm-long mature motile sperm is a truly remarkable act of cellular remodeling. This includes changes in mitochondrial morphology that occur nowhere else in the fly; mitochondria aggregate, fuse, and wrap to give a characteristic Nebenkern mitochondrial derivative at the onion stage. The centriole is transformed into a flagellar basal body embedded in the nuclear envelope. Flagellal elongation is accompanied by elongation of the two mitochondrial derivatives. Nuclear shaping and chromatin condensation transforms a round nucleus into a bear-claw-shaped

From: *Methods in Molecular Biology, vol. 247:* Drosophila *Cytogenetics Protocols*
Edited by: D. S. Henderson © Humana Press Inc., Totowa, NJ

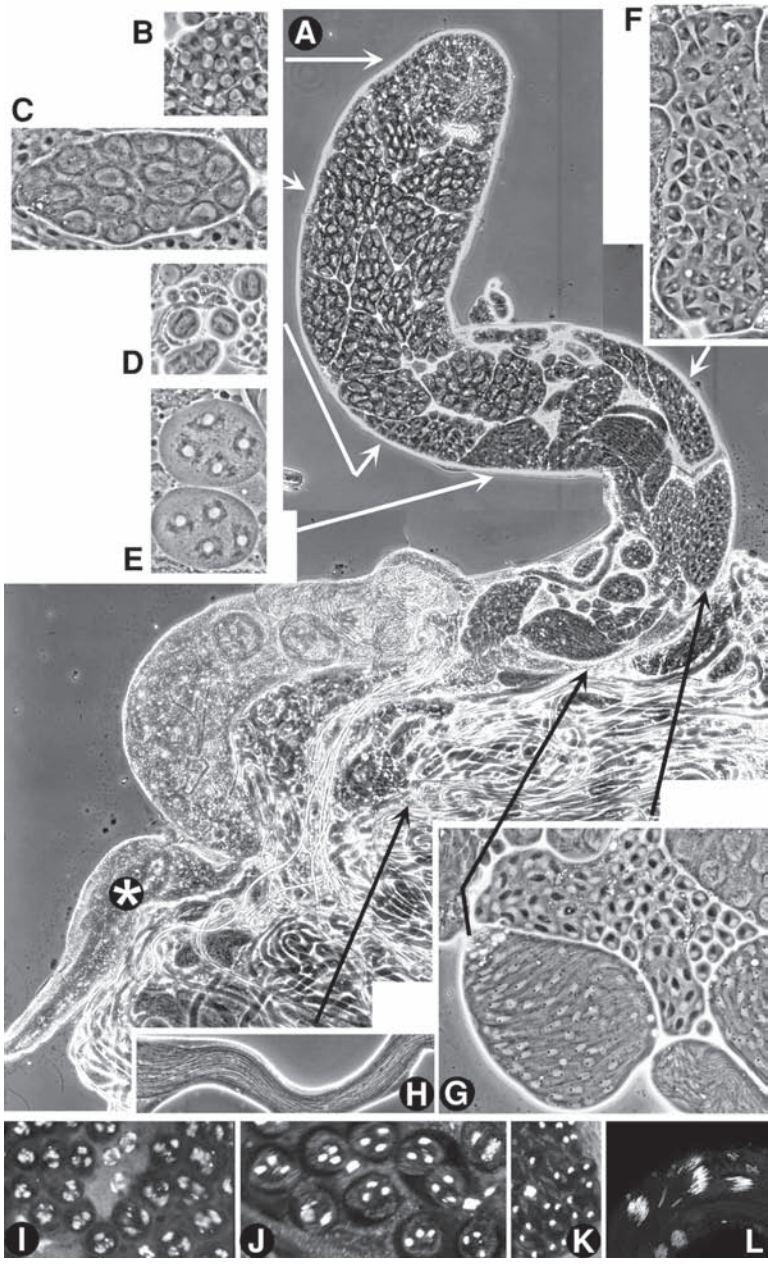


Fig. 1. Phase-contrast microscopy of wild-type testes. The stages of spermatogenesis are easily visible with phase-contrast optics in gently squashed preparations. **(A)** A whole wild-type testis, cut near the distal end, with most of the elongating spermatids spilled out through the cut. There is a temporal progression of cell types from very

highly compact structure. Some processes and gene products are shared with other tissues and developmental stages; others are spermatogenesis-specific. Male meiosis, for example, is much more similar to mitosis than to female meiosis. Many mitotic gene products are used for male meiosis and, therefore the application of a few simple techniques for studying testes can reveal much about the mechanics of cell division. For example, we have learned about the maintenance of sister chromatid cohesion through analysis of *mei-S332* and *ord*. Analysis of weak *ord* mutants suggests Ord is required for proper centromeric cohesion after arm cohesion is released at the metaphase I–anaphase I transition. Ord activity appears to promote centromeric cohesion during meiosis II. Mei-S332 protein is localized to the centromeric region in meiosis; its destruction at the metaphase II–anaphase II transition allows sister chromatid separation. A balance between the activity of Mei-S332 and Ord is required for proper regulation of meiotic cohesion ***(3–7)***. Analysis of *asp* alleles indicates a role for Asp protein in the normal meiotic and mitotic spindle structure. Immunolocalization of Asp in spermatocytes revealed that it is required for the bundling of microtubules at spindle poles, but it is not an integral centrosome component implicated in microtubule nucleation ***(8,9)***. There has been a long-standing debate over the role of asters in determining the position of the cleavage furrow of cytokinesis. Recent evidence from *Drosophila* spermatogenesis has shown that asters are not required for cytokinesis, because *asterless* mutants undergo cytokinesis ***(10)***. Instead, it appears that the cleavage furrow depends on the central spindle ***(9)***. Analysis of cell cycle regulatory genes has revealed that differentiation can continue in the absence of cell cycle progression. Mutation of the meiosis-specific Cdc2-activator *twine* blocks progression through the meiotic divisions; however, the cells continue with aspects of spermatid differentiation, including axoneme elongation and nuclear shaping ***(11–14)***.

A notable feature of the recent completion of the genome sequencing and continuation of the expressed sequence tag (EST) sequencing projects has been

---

early stages at the apical end **(top)** to nearly mature sperm at the distal end **(bottom)**, leading into the seminal vesicle (*). **(B–H)** Higher magnification of specific stages of spermatogenesis; **(B)** polar spermatocytes. **(C)** mature primary spermatocytes; **(D)** part of a cyst in metaphase–anaphase I; **(E)** part of a secondary spermatocyte cyst (meiotic interphase); **(F)** a telophase II cyst; **(G)** onion-stage spermatids (right) and comet stage of early spermatid elongation (left); **(H)** part of a cyst late in elongation before individualization. **(I–L)** Hoechst 33342 labeling of DNA in wild-type live squashes. **(I)** Primary spermatocytes with decondensed chromosomes visible as three discrete regions in each nucleus; **(J)** prophase of meiosis I, partially condensed chromosomes are visible; **(K)** leaf blade stage, DNA is compact within the nucleus, faint staining of the mitochondrial DNA is seen in the Nebenkern; **(L)** tightly clustered and fully shaped nuclei in nearly mature bundles of elongated spermatids, before individualization.

the identification of a large set of testis-specific transcripts. Andrews et al. ***(15)*** sequenced 3141 testis ESTs, representing 1560 contigs, of which 47% were not represented in the 80,000 ESTs sequenced by the Berkley Drosophila Genome Project (BDGP) from other tissues. Sixteen percent had not even been predicted as genes on the first annotation of the genome sequence, and only 11% corresponded to known named genes. This study highlights how little we know and how much more there is to learn about the genes required to carry out this most remarkable cellular process of spermatogenesis. Fortunately, a large set of new male sterile mutants, the tool we need to take a genetic approach to understanding spermatogenesis, is now available. A large-scale mutagenesis screen was undertaken in the Charles Zuker lab and yielded over 12,000 new viable mutagenized chromosomes. These were tested for male and female sterility by Barbara Wakimoto and Dan Lindsley, yielding approx 2000 male sterile lines (cited in **ref. *16***). This collection supplements our existing battery of male sterile mutants (e.g., those generated by Castrillon et al. ***[17]***). They are available to the whole community and have formed the basis of a new wave of excitement and analysis of cytogenetics in *Drosophila* spermatogenesis ***(18–20)***.

1.2. Key Phases of Cellular Differentiation During Spermatogenesis

The testis sheath is a closed tube made up of muscle and pigment cells, separated from the lumen by a basement membrane. At the apical tip (the closed end of the tube), the basement membrane is thickened adjacent to a group of approx 20 somatic cells called the hub (*see* **Fig. 2A**). About eight germ-line stem cells are found around the hub, each associated with two cyst progenitor somatic stem cells. Division of the germ-line stem cell, accompanied by division of the two cyst progenitor cells, results in a spermatogonium encapsulated by two cyst cells. These two cyst cells will never divide again, but they will grow and remain intimately associated with the germ-line cells. Division of the spermatogonium generates two spermatogonia, and three subsequent spermatogonial mitotic divisions (*see* **Fig. 3A**) followed by premeiotic S-phase results in a cyst of 16 primary spermatocytes still surrounded by two cyst cells. Cells within each cyst remain interconnected by cytoplasmic bridges called ring canals, derived from the cleavage furrow after incomplete cytokinesis. A specialized membrane-rich region of cytoplasm, the fusome, extends through these bridges to connect all of the cells in the cyst ***(21)***.

The primary spermatocyte period is primarily one of cell growth (approx 25-fold increase in cell volume **ref. *2***), with certain morphological changes indicating the early, middle, and mature primary spermatocyte. Notable is the transition to the polar spermatocyte stage (*see* **Fig. 1B**), where the phase dark mitochondria are aggregated to one side of the cell and the phase light nucleus

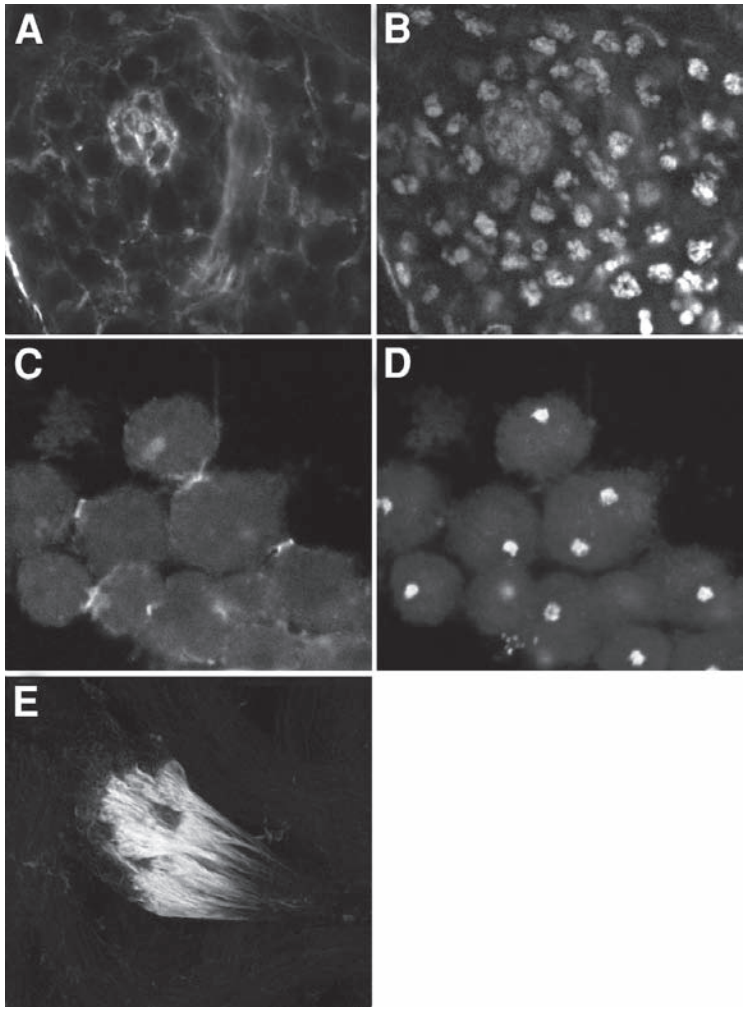


Fig. 2. FITC Phalloidin shows the hub, meiotic cleavage furrows and investment cones. Formaldehyde-fixed testis squash preparations stained with FITC phalloidin (**A,C,E**), which labels F-actin, and counterstained with propidium iodide to reveal the DNA (**B,D**). **(A,B)** The apical tip of a testis. The somatic hub structure appears as a small rosette where there is a more extensive array of F-actin. The large cells around this rosette are the germ-line stem cells. **(C,D)** Several cells at telophase of meiosis I. The F-actin is concentrated in the contractile rings of the cleavage furrows separating sister cells. **(E)** Investment cones are formed around the nuclei of elongated spermatids; they are then displaced from the nuclei as they progress along the cyst, investing each spermatid with its own plasma membrane and extruding the minor mitochondrial derivative and excess cytoplasm into a cytoplasmic waste bag. This set of investment cones has progressed part way along the spermatid tails.

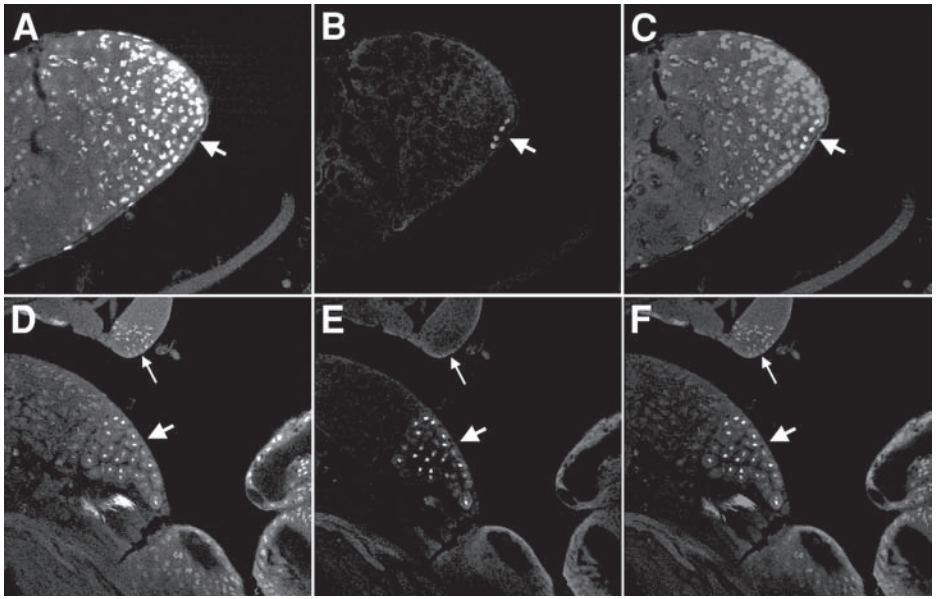


Fig. 3. Phosphorylation of histone H3 identifies mitotic and meiotic chromosomes. Methanol/acetone fixed testes stained with an antibody against phosphorylated histone H3 (**B,E** green [yellow] in **C** and **F**) and counterstained with propidium iodide (**A,D**, red in **C** and **F**). **(A–C)** A cyst of four spermatogonia (arrow) near the apical tip of the testis undergoing mitosis. Note the bright staining of spermatogonial cell DNA and the relatively weak staining of the DNA of the maturing primary spermatocytes to the left of the figure. **(D–F)** A cyst of cells completing meiosis I (large arrow) have phosphorylated histone H3. Early in elongation (small arrow), the nuclei are all clustered to one side of the cyst and histone H3 is no longer phosphorylated. (*See* color plate 1 in the insert following p. 242.)

resides in the other side. Later, the asymmetry is lost as the cells become apolar spermatocytes. Throughout the primary spermatocyte stage, the decondensed chromosomes are in three nuclear domains, corresponding to the two major autosome and the sex chromosome bivalents (*see* **Fig. 1I**). Primary spermatocytes have a very prominent phase dark nucleolus, which is associated with the sex chromosome bivalent. Bulk transcription shuts down as primary spermatocytes mature ***(22,23)***, so transcripts for genes required late in spermatogenesis need to have accumulated in the cells by this stage. They are then stored in RNP particles in the cytoplasm until translation ***(24)*** (*see* **Fig. 4B**).

As mature primary spermatocytes (*see* **Fig. 1C**) enter the meiotic divisions the nucleus becomes rounder and the chromosomes condense and move away from the nuclear envelope (*see* **Fig. 1J**). Mitochondria aligned on the meiotic spindle make this structure readily visible in phase-contrast preparations (*see*

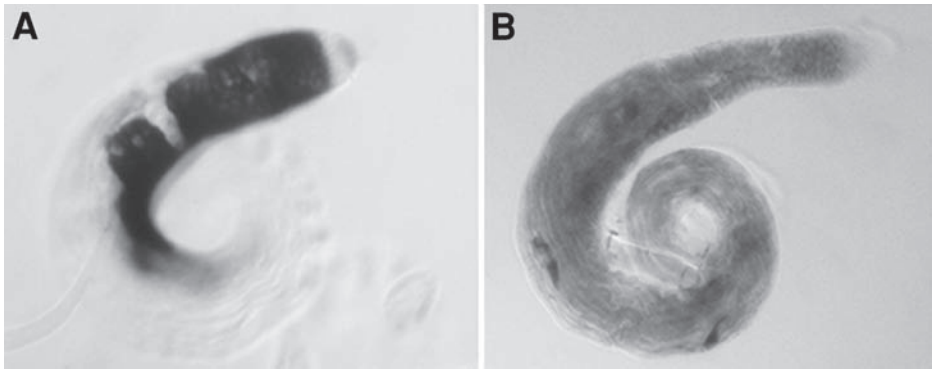
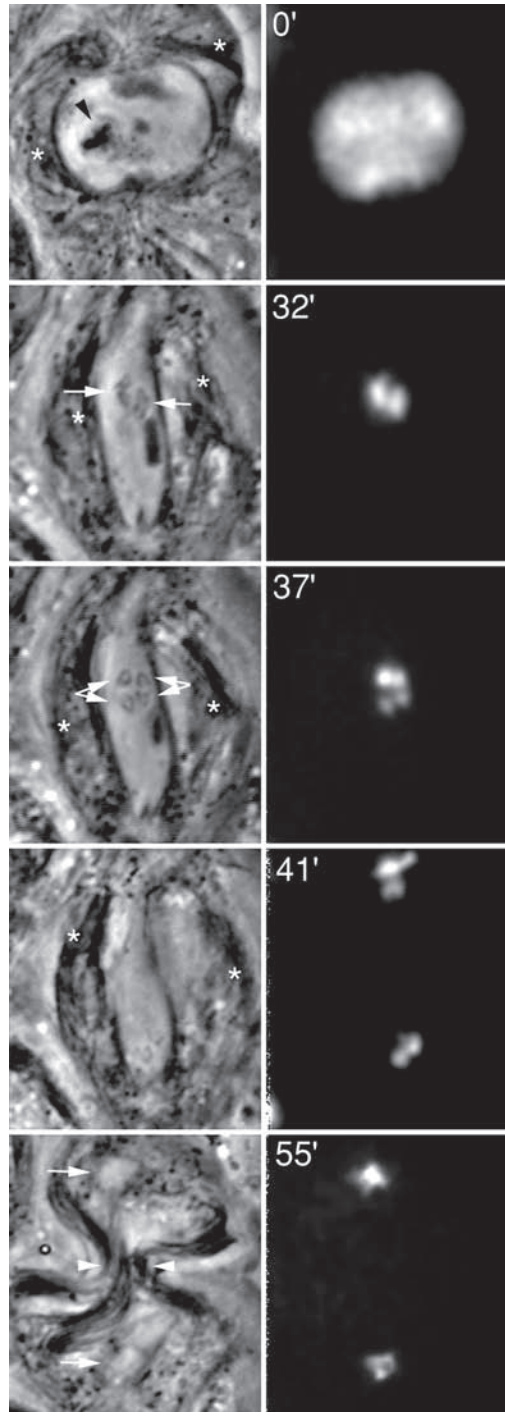


Fig. 4. *In situ* to wild type with cyclin B, Mst87F. **(A)** In situ hybridization to *cyclin B* transcript reveals a two-phase expression pattern. *cyclin B* message is expressed in the mitotic cells at the apical tip of the testis, but is absent from cells undergoing premeiotic S-phase. *cyclin B* message then reappears in early primary spermatocytes, persists until meiosis, and is degraded before the cysts progress to onion stage. **(B)** *Mst87F* encodes a protein important for the structure of the sperm heads. The transcript first accumulates in primary spermatocytes and persists through to very late stage of spermatogenesis, when it is translated.

Figs. 1D and **5**). An aster can be seen on one side of the nucleus, and this separates as the centrosomes migrate to opposite poles to set up the bipolar spindle. During meiotic telophase the cleavage furrow separating the sisters pinches the spindle to generate a "bow tie" effect (*see* **Fig. 1F**, [telophase II], **Fig. 5** [bottom left panel], and **2C**) and, finally, give a secondary spermatocyte cyst consisting of 32 cells connected by ring canals, encapsulated by 2 cyst cells. Meiosis II follows after a very short interphase (*see* **Fig. 1E**). Morphologically, meiosis II is very similar to meiosis I, although there is clearly half as much DNA, only 1 centriole per centrosome, the cells are smaller, and the final product is a cyst of 64 interconnected spermatids, still surrounded by the 2 cyst cells.

After the second meiotic division, all of the mitochondria aggregate and fuse to form two giant mitochondria. These are interleaved by the onion stage to make a Nebenkern, which, by transmission electron microscopy, resembles an onion slice ***(25)*** and, by phase contrast, is a dark sphere adjacent to the phase light nucleus (*see* **Figs. 1G,J**). The centriole inserts into the nuclear membrane and axoneme elongation initiates ***(2)***. A phase dark dot, the pseudonucleolus or protein body, appears inside the otherwise featureless phase light nucleus. During elongation, the mitochondria unfurl from each other and two distinct phase dark mitochondrial derivatives can be seen elongating alongside


0'
32'
37'
41'
55'

the flagellar axoneme at the comet stage (*see* **Fig. 1H**). All of the spermatids in a cyst develop in synchrony; during elongation, their heads become more closely aligned. This is already visible at the comet stage where the nuclei are found at one side of the cyst, and the tails extending toward the other side (*see* **Fig. 3D**). Intercellular bridges remain at the distal end of the elongating cysts ***(26)***. During elongation, the two cyst cells behave somewhat differently, each encompassing one end of the cyst. The head end cyst cell contacts the terminal epithelium near the base of the testis, so that cysts elongate with their heads anchored and the tails pushing up the length of the testis. During elongation, the nuclei become invisible with phase contrast as they compact and are transformed from a sphere into a needle shape (*see* **Fig. 1L**).

Finally, the fully elongated spermatids individualize and coil. Individualization initiates with an actin-based structure, the investment cone, at the head end of each spermatid ***(27)***. This progresses along the length of each tail (*see* **Fig. 2E**), stripping off all excess cytoplasm and the minor mitochondrial derivative into a cytoplasmic waste bag (*see* **Fig. 6C**) that is eventually shed into the lumen of the testis. Individual sperm are coiled into the seminal vesicle ready for transfer to the female during copulation.

2. Materials

2.1. Phase-Contrast Microscopy

1. Testis buffer: 183 m*M* KCl, 47 m*M* NaCl, 10 m*M* Tris-HCl, pH 6.8 **or** TB1: 15 m*M* potassium phosphate (equimolar dibasic and monobasic), pH 6.7, 80 m*M* KCl, 16 m*M* NaCl, 5 m*M* $MgCl_2$, 1% polyethylene glycol (PEG) 6000.

Fig. 5. (*opposite page*) Meiosis I progression in wild-type spermatocytes observed by time-lapse phase-contrast and fluorescence microscopy. Meiosis was followed from early prometaphase to the end of meiosis I at a rate of 20 frames/min using a combination of phase contrast (left panels) and fluorescence (right panels) microscopy. The chromosomes were labeled with a His2-GFP fusion protein (right panels). Five significant time-points are shown in this figure, reproduced from Rebollo and Gonzalez ***(41)***: 0' corresponds to late prophase, the two centrosomes have migrated to opposite poles and organized large asters. The meiotic spindle is forming and is highlighted with phase dark mitochondria (asterisks). A nonchromosomal phase dark nuclear structure, which remains throughout meiosis, can be seen (arrowhead). At 32', the spermatocyte contains a fully formed elongated spindle. Two bivalents are stabilized at the metaphase plate in this focal plane (arrows). At 37', anaphase has just started. Two pairs of homologous chromosomes can be seen segregating from each other (double arrows). At 41', the chromosomes have reached the poles and are decondensing. Chromosome decondensation is first apparent midway through anaphase. At 55', the two daughter nuclei have formed (arrows) and the cleavage furrow begins to pinch the central spindle (white arrowheads).

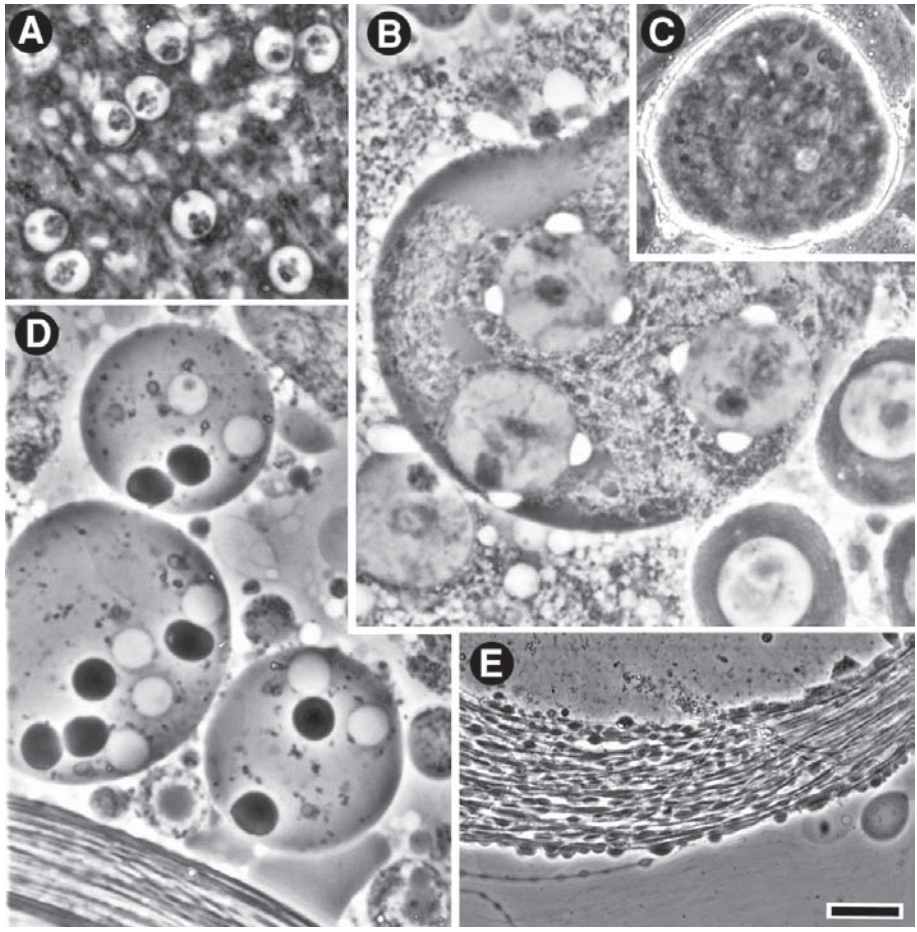


Fig. 6. Other cell types and typical artefacts that should be ignored. **(A)** Cells from the accessory gland can be confused with early primary spermatocytes. However, primary spermatocytes are in cysts, the nuclei of accessory gland cells are more uniform, and there is never a polarity to the accessory gland cytoplasm. **(B)** Oversquashed primary spermatocytes can develop vacuoles adjacent to the nucleus. These can be confused with mutant onion stages; however, the large spermatocyte nuclei are usually still apparent. **(C)** The cytoplasmic waste bag extruded upon individualization can sometimes be confused with a very disorganized early elongation cyst. **(D)** Cells can fuse under pressure from the cover slip. Fused normal onion stage cells can be confused for cytokinesis mutants. True cytokinesis mutants show two or four phase light nuclei adjacent to only one (large) Nebenkern. Here, the nuclei and Nebenkern are similar in size and equal in number. **(E)** Occasionally, when elongated cysts are disrupted just before individualization the sperm tails have a blebby rather than smooth appearance. For this to be classified as the mutant phenotype, it has to be consistently rather than rarely seen. (Scale bar is 10 μm and applies to all panels, except panel **C**.)

2. Dissecting plate; for example, a 10-cm-diameter plastic Petri dish with the sides removed.
3. Sharp forceps (the sharper the better).
4. Tungsten mounted needles (sharp!).
5. Microscope slides and cover slips, 22×22 mm^2.
6. Phase-contrast compound microscope with camera.
7. Kimwipes.
8. Hoechst 33342 (bis-benzimide; e.g., Sigma-Aldrich) (1 mg/mL stock). Store in the dark at 4°C. Dilute in dissection buffer to 2–5 μg/mL when needed.
9. 45% Acetic acid.
10. Halocarbon oil (Voltalef 10S).

2.2. Immunostaining

2.2.1. Whole Mount Immunohistochemistry

1. Testis buffer or TB1 (*see* **item 1, Subheading 2.1.**). For monoclonal antibodies directed against phosphorylated epitopes, use TBPi buffer: 10 m*M* Tris-HCl, pH 6.8, 180 m*M* KCl, 50 m*M* NaF, 1 m*M* Na_3VO_4, 10 m*M* Na β-glycerophosphate.
2. 4% Formaldehyde (from 40% stock) in phosphate-buffered saline (PBS), or make up fresh 4% paraformaldehyde in PBS or HEPES buffer: 100 m*M* HEPES, pH 6.9, 2 m*M* $MgSO_4$, 1 m*M* EGTA. HEPES buffer may be stored at –20°C, but check pH before (re)use.
3. PBS+0.1% Triton-X100 (PBSTx).
4. Fetal calf serum (FCS). Sterile, but out of date will do; it is used as a blocking agent.
5. 24-Well tissue culture plates and tissue culture inserts with 8-μm mesh (Falcon no. 3097).
6. Primary antibody.
7. Secondary antibody, biotin-conjugated (Vector Laboratories).
8. ExtrAvidin-HRP conjugate (Sigma-Aldrich), or Vectastain ABC reagents (Vector Laboratories).
9. 3,3'-Diaminobenzidine (DAB; available in tablet form from Sigma). DAB is a potent carcinogen. Wear gloves and use caution.
10. Hydrogen peroxide.
11. Microscope slides and cover slips.
12. Mounting medium: 85% Glycerol. Add 2.5% *n*-propyl-gallate if counterstaining with Hoechst.
13. Hoechst 33258 (e.g., Sigma-Aldrich) (optional; *see* **Subheading 3.2.1.**).

2.2.2. Formaldehyde Fixation for Confocal Microscopy

1. Testis buffer or TB1 (*see* **item 1**, **Subheading 2.1.**).
2. Liquid nitrogen, scalpel, safety glasses.
3. 100% Ethanol (chilled on dry ice).
4. 4% Formaldehyde in PBS.
5. PBST: PBS + 0.1% Triton X-100 or 0.1% Tween-20.

6. PBST-DOC: PBS + 0.3% Triton X-100 + 0.3% sodium deoxycholate (from 10% stock).
7. FCS (sterile, but does not have to be in date), or bovine serum albumin (BSA).
8. Poly-L-lysine-coated slides.
9. Siliconized 22 × 22-mm^2 cover slips.
10. Evostick impact adhesive or rubber cement.
11. Clear nail polish.
12. Humid chamber (e.g., sandwich box with wet tissue paper).
13. Primary antibody.
14. Fluorescently conjugated secondary antibody (Jackson ImmunoResearch Laboratories or Vector Laboratories).
15. Propidium iodide or DAPI (4,6-diamidino-2-phenylindole) if staining for DNA.
16. RNAse A, 10 mg/mL stock (store at –20°C). Required if using propidium iodide.

2.2.3. Methanol/Acetone Fixation for Confocal Microscopy

As per **Subheading 2.2.2.**, except replace **item 3** (ethanol) with methanol cooled on dry ice, **item 4** (4% formaldehyde) with acetone cooled on dry ice, and **items 5** and **6** (PBST and PBS-DOC) with PBS + 1% Triton X-100 + 0.5% acetic acid (PBS-T-AA).

2.3. X-Gal Staining

1. 1% Glutaraldehyde in PBS.
2. 20% 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide (DMF). Store at –20°C.
3. X-gal buffer: 150 m*M* NaCl, 7.2 m*M* Na_2HPO_4, 2.8 m*M* NaH_2PO_4, 1 m*M* $MgCl_2$. Keep as 5X stock.
4. Staining solution: X-gal buffer containing 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$. Store 50 m*M* stock solutions in the dark.
5. 85% Glycerol.
6. Microscope slides and cover slips for mounting.

2.4. RNA In Situ *Hybridization*

2.4.1. Probe

1. Plasmid clone of cDNA with suitable RNA polymerase promoter sites (e.g., pBluescript, Stratagene).
2. 10X DIG RNA labeling mix (Roche): 10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 6.5 m*M* UTP, 3.5 m*M* DIG-UTP in Tris-HCl, pH 7.5.
3. RNA polymerase and buffer (for an antisense transcript, the one that transcribes from the 3' end of the gene) (New England Biolabs [NEB] or Roche).
4. RNAse-free water.
5. 2X Carbonate buffer: 60 m*M* Na_2CO_3, 40 m*M* $NaHCO_3$, pH 10.2.
6. 2X Neutralization buffer: 200 m*M* sodium acetate, 1% (v/v) acetic acid.

2.4.2. Hybridization

1. Fix: 4% Paraformaldehyde in 100 m*M* HEPES, pH 6.9, 2 m*M* $MgSO_4$, 1 m*M* EGTA. May be stored at –20°C. Check pH before (re)use.
2. PBS + 0.1% Tween-20 (PBST).
3. Proteinase K (Roche). Stock is 19 mg/mL. Store at –20°C.
4. 2 mg/mL Glycine in PBST. Glycine stock is 200 mg/mL (store at room temperature).
5. Hybridization buffer (HB): 50% Formamide, 5X SSC, 100 µg/mL denatured sonicated salmon sperm DNA, 50 µg/mL heparin, 0.1% Tween-20, adjust to pH 4.5 with 2 *M* citric acid (approx 100 m*M* final concentration). Store at –20°C. Preheat for 65°C washes. 20X SSC is 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0.
6. RNA probe (*see* **Subheading 3.4.1.**).
7. High pH buffer (HP): 100 m*M* NaCl, 100 m*M* Tris-HCl, pH 9.5, 50 m*M* $MgCl_2$, 0.1% Tween-20. Make up fresh, and add $MgCl_2$ last to prevent precipitation.
8. Nitroblue tetrazolium (NBT): 18.75 mg/mL in 70% DMF stock (Roche).
9. X-Phosphate (5-bromo-4-chloro-3-indolyl-phosphate [BCIP]): stock solution is 50 mg/mL in DMF (Roche).
10. Alkaline phosphatase conjugated antidigoxygenin antibody (Roche), preadsorbed against embryos (*see* **Note 1**).
11. Gary's Magic Mountant (GMM): 1.6 g/mL Canada balsam (powder) in methyl salicylate. If only liquid Canada balsam is available, mix 4 : 1 with methyl salicylate.
12. 24-Well tissue culture plates and tissue culture inserts with 8-µm mesh (Falcon no. 3097).
13. Glass staining blocks.
14. Microscope slides and cover slips.

3. Methods

3.1. Phase Contrast Microscopy of Live Testes

This is the first technique to apply when asking the question "Why are my mutant males sterile?" Because the key stages of spermatogenesis have a very distinctive appearance in wild-type testes (*see* **Fig. 1**), it is relatively easy to look at a squash and preliminarily classify the mutant based on the morphologies observed. **Table 1** gives a key to the types of defects you may observe; these give clues as to what cytological process may be affected and, therefore, will direct your future experiments. This list is by no means exhaustive; many characterized phenotypes are not listed here. Additionally, as more mutations are characterized the number of distinct phenotypes seen will increase substantially. **Figure 7** shows examples of some mutant phenotypes seen in phase-contrast squash preparations. As with analysis of mutant phenotypes in other stages of the life cycle (e.g., embryogenesis), it is critical to identify the earliest stage at which faults are detectable. Sometimes, mutant males will show more than one defect; the challenge then becomes to

Table 1
Some Well-Characterized Testes Phenotypes

Observation	Avenues to follow	Example	Ref.
There are motile sperm in the seminal vesicle	Fertilization/paternal effect	*sneaky*	***28***
	Mating or courtship behavior	*fru*	***29***
	Nondisjunction in meiosis	*asp* (weak)	***8***
Mature sperm bundles are present, no other defect seen	Individualization	*Chc4*	***27***
	Axoneme structure	*wrl*	***30***
Classic male sterile phenotype	Nuclear shaping. Mitochondrial morphogenesis	*moz*	
Unequal nuclear and Nebenkern size at onion stage	Chromosome nondisjunction	*asp*	***8***
Too few cells at onion stage	Meiotic division failed?	*twine*	***11***
16 cells, 16 large nuclei, 16 large Nebenkern	No nuclear or cell division, nuclei are 4N	*mgr*	***31***
16 cells, 64 normal nuclei, 16 large Nebenkern	Nuclear division occurred, but cytokinesis failed	*fwd*	***32***
Nebenkern looks abnormal at onion stage and early elongation	Mitochondrial aggregation	*nmd*	***1***
	Mitochondrial fusion	*fzo*	***33***
	Mitochondrial unfurling		
Primary spermatocytes look normal, no later stages seen	Meiotic arrest	*mia*	***34***
Spermatogonial cysts contain more than 16 cells	Failure to activate spermatocyte differentiation program	*bam*	***35***
Many small cells present, not all in cysts	Stem cell overproliferation	*egfr* *raf*	***36,37***
No germ-line cells in testis	Maternal effect, pole cell formation failed	*tud*	***38***
	Stem cell maintenance		

identify whether one is primary and the other secondary, or whether the gene in question acts directly in both affected processes. For example, spermatids in males mutant for proteins required for nuclear shaping fail to individualize, because correct shaping of the nucleus is required for the normal assembly of the investment cone ***(27)***. Mutations affecting spindle structure may show defects in both chromosome segregation (uneven nuclear size at onion stage) and cytokinesis (Nebenkern larger than wild type and fewer in number) because of the relationship between spindle structure, especially the central spindle, and the cleavage furrow.

Cell division is an extremely dynamic process; however, the transient nature of standard squash preparations observed by phase contrast yields only snapshot images of meiosis. In many cases, it is desirable to observe cells undergoing the divisions using time-lapse microscopy ***(40,41)***. By observing living cells, we can characterize in much more detail the exact nature of any meiotic defect. For example, a four-wheel-drive phenotype, where karyokinesis is unaffected but cytokinesis fails (e.g., **Fig. 7D**), could be the result of one of two fundamentally different defects. There may be a failure in the contractile ring, such that there is no contraction; alternatively, the intercellular bridge that should remain after incomplete cytokinesis may not be stabilized ***(42)***. Observations of living cells can rapidly distinguish between these possibilities ***(32)***. For analysis of live specimens undergoing meiosis *see* **Subheading 3.1.3.** and Chapter 3.

1. Dissect testes from a newly eclosed male in fresh testis buffer (0–1 d old) (*see* **Note 2**).
2. Place a drop of testis buffer on a clean microscope slide, using the surface tension of the buffer transfer the testes to this drop.
3. Open up the testes (and seminal vesicles if looking for motile sperm) by cutting them open with the tungsten needles or by ripping with the forceps (*see* **Note 3**).
4. Place a clean cover slip over the testes; this will gently squash the cells. Squashing can be increased to give better phase contrast by wicking buffer out using a Kimwipe. This can be done while observing the cells under the phase-contrast microscope (*see* **Note 4**).

3.1.1. Analyzing Nuclear Morphology

Nuclear morphology can be examined in live squashes by staining the DNA with the vital dye Hoechst 33342. This is included in the testes buffer at 2–5 μg/mL during dissection.

1. Proceed as described in **Subheading 3.1.**, but allow the testes to sit in the buffer for 5 min after dissection and before adding the cover slip (*see* **Fig. 1I–L**). Alternatively, chromatin can be observed using a stock carrying a His2-GFP transgene ***(41)*** (*see* **Fig. 5**).

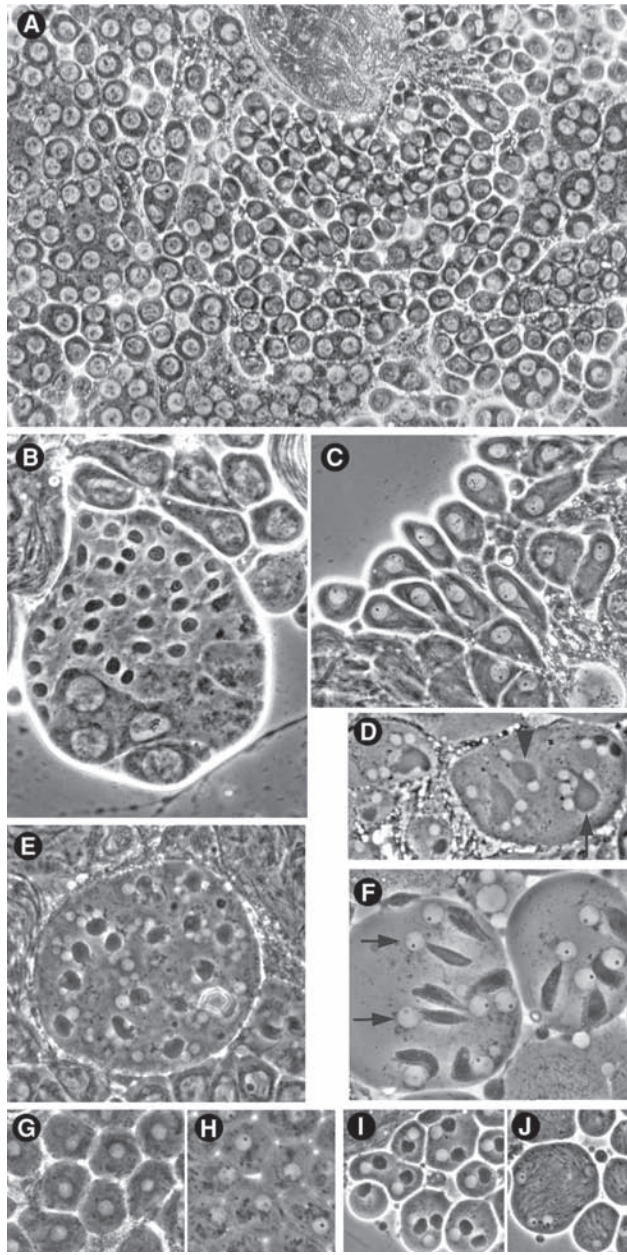


Fig. 7. Some examples of mutant phenotypes. **(A)** Meiotic arrest (e.g., *aly*, *can*, *mia*, *sa*): Testes mutant for meiotic arrest genes accumulate morphologically normal primary spermatocytes. No meiotic or postmeiotic cells are present. **(B)** Intracyst asynchrony (e.g., *polo*): Some alleles of cell cycle mutants can result in asynchrony within cysts. Here, half of the cyst has progressed to onion stage, four cells are attempting

3.1.2. Analyzing Meiotic Chromosome Morphology

Meiotic chromosome morphology can be assessed in acetic acid squashes. Because acid destroys much of the cellular structure, the morphology of the squashed testis will be compromised; however, condensed meiotic chromosomes and sperm heads will be visible ***(14)***.

1. Dissect testes as described in **Subheading 3.1., step 1**, then transfer to 45% acetic acid on the slide, and allow to swell for 15 s before cutting.
2. Proceed to **step 3** of **Subheading 3.1.**

3.1.3. Time-Lapse Microscopy

1. Dissect out the testes and place into a large drop of halocarbon oil on a cover slip. Remove as much nontestis material as possible from the preparation.
2. Cut the testes open and spill the contents out by pulling the sheath around the cover slip.
3. Pick up the cover slip with a slide and observe with phase-contrast optics for up to 1 h ***(32)***. The addition of extra cover slips (or pieces of cover slip) as supports alongside the testes prevents oversquashing and increases the viability of the cells to 2 h (*see* **Note 5**).

Fig. 7. (*continued*) meiosis, and four are still primary spermatocytes. **(C)** No meiotic divisions (e.g., *twine*, *boule*, *mgr*, β*2-tubulin*): Failure to form a meiotic spindle results in failure of both chromosome segregation and cell division. Onion stage cysts can be seen containing 16 rather than 64 cells, each having a large (4N) nucleus and a large (usually misshapen) Nebenkern. **(D)** Cytokinesis failure (e.g., *fwd*, *shank*): Normal chromosome segregation followed by failure of cytokinesis at both meiotic divisions results in an onion stage cell (arrow) containing four nuclei associated with just one very large mitochondrial derivative. If only one cytokinesis fails, two nuclei are associated with one large mitochondrial derivative (arrowhead). **(F)** Chromosome nondisjunction (e.g., *asp*, compound chromosomes): Chromosome nondisjunction results in one daughter nucleus having more DNA than its sister nucleus. Because the nuclear size at onion stage is directly proportional to DNA content, this is manifest as variable nuclear diameters in early spermatids (compare arrowed nuclei). **(E)** Cytokinesis failure and chromosome nondisjunction: Mutants that affect both cytokinesis and chromosome segregation can result in postmeiotic cysts containing 64 variable-sized nuclei associated with 16 large Nebenkern. **(G,H)** No mitochondrial derivative (e.g., *nmd*): Failure of mitochondrial aggregation and fusion results in a cloud of mitochondria in onion-stage cells **(G)**. At the leaf blade stage **(H)**, they are scattered, mostly to one side of the nucleus, but a normal Nebenkern is never formed. **(I,J)** Mitochondrial fusion (e.g., *fzo*): At onion stage, mitochondria aggregate but fail to fuse. Wrapping of pairs of mitochondria forms a Nebenkern, which at this stage (**I**) looks somewhat lumpy. By the comet stage **(J)**, many individual mitochondrial pairs are seen elongating from each nucleus. Panel **E** was provided by Cayetano Gonzalez (EMBO), and panels **G–J** were provided by Karen Hales (Davidson College).

Because in each preparation only one field can be imaged, it is wasteful to dissect more than one male per slide. Care should be taken in choosing the image field; cells near the edge of the displaced testis contents may be easier to see, but will die sooner than those that remain surrounded by other testis material in slightly more physiological conditions. Cytokinesis seems to be more sensitive to perturbation than chromosome segregation. If the aim of the experiment is to observe cytokinesis in a mutant, it is best to select cells in anaphase at the start of the recording session. Cells can easily fuse during preparation of the slide; if two cells in meiotic prophase are artificially fused, both spindle morphology and kinetochore attachment to microtubules may be disrupted. The effect will be an aberrant meiosis, and often failed cytokinesis, which could be misinterpreted as representing the true mutant phenotype.

3.2. Immunostaining

Phase-contrast observation of mutant testes can only give a broad indication of the cytological defect in any particular mutant. Immunostaining mutant testes can give information on both cell-type distributions and subcellular structures. At the subcellular level, for example, staining of the meiotic spindle with anti-tubulin antibodies and a DNA dye will reveal much about the cytological mechanism underlying a chromosome nondisjunction phenotype. Antibodies or markers can also reveal which cell types are present in the testes. This is especially important in situations where relatively few, or very small, cells are seen in phase contrast. Are these germ-line or somatic cells? Are they stem cells or spermatogonia? Is the hub present and normal? Many of these cell type-specific markers are LacZ enhancer traps, so staining can be done either with an anti-LacZ antibody (**Subheading 3.2.**) or with a β-galactosidase activity assay (*see* **Subheading 3.3.**). A selection of useful antibodies and markers for probing subcellular structure and cellular identity in the testis is given in **Tables 3** and **4**. The detailed analysis of mutant phenotypes is, of course, not the only reason for wanting to immunostain testes. Having generated an antibody to a protein expressed in testes, one will always want to know its cellular and subcellular distribution patterns.

The choice of the following protocols depends on the exact question being addressed in the experiment. If information is needed on the pattern of protein localization at a global scale, then clearly whole-mount immunohistochemistry is required. This has the advantage of maintaining the temporal pattern of cysts in the testes; however, the resolution at the subcellular level is relatively low. This can be somewhat helped by counterstaining the preparation with Hoechst 33258. Immunofluorescence of squashed preparations has greater resolution of subcellular structures, but at the cost of loss of the temporal

Table 3
Choosing Antibodies and Reagents for Labeling

Antibody/probe	Structure revealed	Suggested conc.	Supplier (ref.)
DAPI	DNA	1 µg/mL	Molecular Probes
Propidium iodide (*see* **Figs. 2** and **3**)	DNA	1 µg/mL	Sigma
Hoechst 33342 (*see* **Fig. 1**)	DNA (vital dye)	2–5 µg/mL	Sigma ***(34)***
Phospho-Histone H3 (*see* **Fig. 3**)	Mitotic/meiotic chromosomes	1:200	Upstate Biotechnology ***(36)***
Phalloidin (*see* **Fig. 2**)	F-actin Hub Fusome Cleavage furrow Investment cone Muscle of sheath	5 µg/mL	Sigma/Molecular Probes ***(27)***
Tubulin (YL1/2)	Microtubules	1:20 (tissue culture supernatant)	Serolab ***(14)***
Fasiclin III	Hub	1:50	***(43)***
Phospho-tyrosine	Ring canals	1:100	Sigma/Upstate Biotechnology ***(26)***
Spectrin mAb1b1 (adducin)	Fusome	1:50	***(44,45)***
Bam-C	Spermatogonia	1:1500	***(46)***
Anillin	Nuclei and ring canals	1:300	***(26)***
Gamma-tubulin	Centrosomes	1:400	***(47)***
MPM2	DNA spindles and centrosomes		Upstate Biotechnology ***(14)***
β-galactosidase	LacZ reporter patterns		Promega ***(18)***

Table 4
Enhancer Traps That Reveal Specific Cell Types and GFP Reporters for Substructures

Reporter line	Cell type/structure	Ref.
M34a	Germ-line stem cells and gonialblasts	***36***
LacZ600, eyes absent	Hub and cyst cells	***37,48***
Vein	Cyst cells	***36***
M5-4	Hub, germ-line stem cells and gonialblasts	***49***
Don Juan GFP	Sperm tails	***50***
His2-GFP	Meiotic chromosomes	***41***

sequence of cysts. Two different fixation protocols for immunofluorescence of squashed preparations are given here. The choice of which to use is partly personal preference and partly antibody/antigen-specific. As a general rule, if trying a new antibody, do both protocols. I find that the methanol/acetone method (**Subheading 3.2.3.**) gives better fixation of meiotic spindles. However, phalloidin staining of F-actin is significantly better with formaldehyde fixation (**Subheading 3.2.2.**).

3.2.1. Whole Mount Immunohistochemistry

1. Dissect the testes in the appropriate buffer and transfer to an Eppendorf tube (*see* **Notes 2** and **6**).
2. Fix the testes in 4% formaldehyde in PBS, for 20–30 min at room temperature.
3. Rinse once, transfer to tissue culture inserts in a 24-well tissue culture plate, then wash three times in PBSTx, 20 min each wash (*see* **Note 7**).
4. Block 30 min in PBSTx + 5% FCS (*see* **Note 8**).
5. Incubate in 1° antibody diluted in PBSTx + 5% FCS overnight at 4°C, or at room temperature for 2 h (*see* **Note 9**).
6. Rinse once, then wash three times in PBSTx, 20 min each.
7. Incubate in biotinylated 2° antibody diluted 1 : 2000 in PBSTx + 5% FCS for 1 h at room temperature.
8. Rinse once, then wash three times in PBSTx, 20 min each. (If using Vectastain ABC kit, *see* **Note 10**.)
9. Incubate for 30 min at room temperature with ExtrAvidin-HRP conjugate diluted 1 : 1000 in PBSTx.
10. Rinse once, then wash three times in PBSTx, 20 min each.
11. Stain with 0.7 mg/mL DAB, 0.001% H_2O_2 in PBS (*see* **Note 11**).
12. Stop the reaction by rinsing once, then washing several times (10 min each) in PBS.
13. Counterstain DNA with 1 µg/mL Hoechst 33258 in PBS for 15 min if required.
14. Mount on slides in mounting medium and observe with Nomarski optics (*see* **Note 12**) (*see* **Fig. 8**).

3.2.2. Formaldehyde Fixation for Confocal Microscopy

1. Dissect testes from young adults in testis buffer, four or five pairs per slide (*see* **Note 2**).
2. Transfer the testes to a drop of testis buffer on a poly-L-lysine-treated slide and cut open to spill the contents (*see* **Notes 3** and **13**).
3. Squash gently under a 22×22-mm^2 siliconized cover slip.
4. Freeze immediately in liquid nitrogen (wear safety glasses) and pop off the cover slip using a scalpel.
5. Immediately place in chilled (on dry ice) 100% ethanol, 10 min.
6. Place the slide flat and flood with 0.5–1 mL of 4% formaldehyde solution; incubate for 7 min.
7. Tip off the formaldehyde onto paper towels (wear gloves). If staining with phalloidin, *see* **Note 14**.
8. Permeabilize the testes by incubating twice in PBST-DOC, 15 min each.
9. Store in PBST until all the slides have been prepared.
10. Make a well on the slide with Evostick (rubber cement), block for a minimum of 30 min in PBST + 3% BSA or PBST + 5% FCS (*see* **Note 15**).
11. Incubate with primary antibody diluted in PBST + BSA (or PBST + FCS) for a minimum of 2 h at room temperature, or overnight at 4°C in a humid chamber (*see* **Notes 9** and **16**).
12. Remove the evostick. Wash 4 times, 15 min each, in PBST.
13. Incubate with secondary antibody 1 : 500–1 : 1000 (in fresh Evostick wells) for at least 1 h at room temperature, or overnight at 4°C, in a humid chamber (*see* **Note 16**).
14. Remove the Evostick. Wash four times in PBST, 15 min each.
15. Optional: Incubate 10 min in 1 µg/mL DAPI in PBST.
16. Wash once in PBS for 10 min.
17. Mount under a siliconized cover slip in 85% glycerol + 2.5% *n*-propyl gallate (containing 1 µg/mL propidium iodide if required). Seal with nail polish.
18. Image with conventional epifluorescence or confocal laser scanning microscopy.

3.2.3. Methanol/Acetone Fixation for Confocal Microscopy

1. Dissect testes from young adults in testis buffer or TB1, four or five pairs per slide (*see* **Note 2**).
2. Transfer to a drop of testis buffer on a poly-L-lysine-treated slide and cut open to spill the contents (*see* **Note 3**).
3. Squash gently under a 22×22-mm^2 siliconized cover slip.
4. Freeze immediately in liquid nitrogen (wear safety glasses) and pop off cover slip using a scalpel.
5. Transfer to methanol, chilled on dry ice, and incubate for 5 min.
6. Transfer to acetone, chilled on dry ice, and incubate for 5 min.
7. Incubate in PBS-T-AA for 10 min at room temperature.
8. Store in PBST until all the slides are prepared.
9. Continue from **step 10** of **Subheading 3.3.2.**

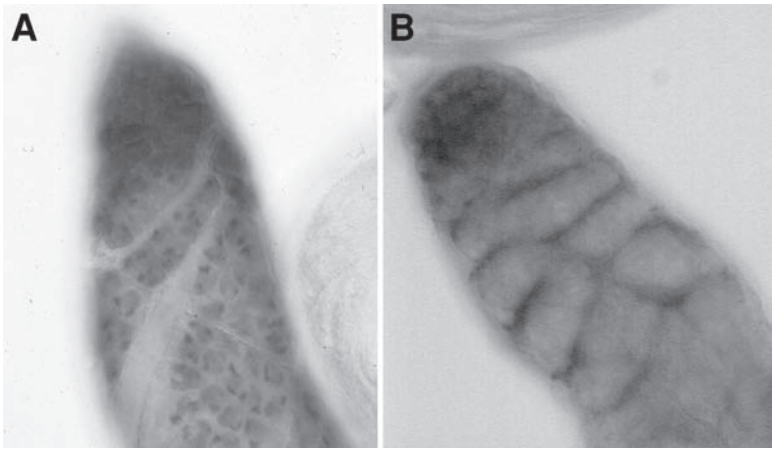


Fig. 8. Immunohistochemistry of Aly and dpERK protein localization. Immunohistochemical staining retains the spatial relationship between different stages of differentiation in the testes, and so allows temporal changes in the cellular or subcellular distribution of an antigen to be assessed. **(A)** The tip of a testis stained with an antibody against Aly protein. Aly is not expressed in the mitotic domain (out of focus in this image). When Aly is first detected, it is both cytoplasmic and nuclear in the germline cells. As the cysts mature, the localization resolves to be concentrated on chromatin in primary spermatocytes. Aly protein is not found in cyst cells; hence, the gaps seen between the areas of staining. **(B)** A monoclonal antibody specific to the activated (diphosphorylated) form of ERK/MAP kinase stains only the cyst cells near the apical tip of the testis. This staining is particularly sensitive to buffer conditions and only works when testes are dissected in TBPi buffer.

3.3. X-Gal Staining

1. Dissect testes in testis buffer or TB1 (*see* **Note 2**). Transfer to a 24-well tissue culture dish or glass staining block (*see* **Note 17**).
2. Fix in 1% glutaraldehyde (wear gloves) in PBS for 15 min (*see* **Note 18**).
3. Rinse three times in X-gal buffer. Leave in this buffer for at least 30 min.
4. Prepare 5 mL of staining solution and warm to 37°C.
5. Add 50 µL of 20% X-gal. Vortex. Keep at 37°C.
6. Incubate tissue in staining solution plus X-Gal at 37°C for 1 h to overnight. Monitor the reaction and leave until color develops.
7. Wash in X-gal buffer, counterstain (if desired) by incubating in 1 µg/mL Hoechst 33258 for 15 min, and mount in 85% glycerol.

3.4. RNA In Situ *Hybridization*

The pattern of expression of your gene can give a clue as to its function. Most testis transcripts are made in primary spermatocytes and stored until use.

A transcript that persists along the testis is more likely to encode a protein needed late in differentiation. Early degradation of a transcript suggests an earlier function (*see* **Fig 4**).

3.4.1. Preparation of Probe

Essentially follow the labeling protocol given with the Roche RNA labeling mix, outlined as follows.

1. Linearize plasmid with a suitable restriction enzyme (usually one that cuts in the polylinker at the 5' end of the gene).
2. Set up a 20–µL reaction using 1 µg of linear template and the relevant RNA polymerase. A typical reaction contains 2 µL of 10X RNA labeling mix, 2 µL of 10X RNA polymerase buffer, 1 µg of template DNA in 14 µL of RNAse-free water, and 2 µL of RNA polymerase.
3. Allow the transcription reaction to continue for 2 h at 37°C, then stop the reaction by adding 2 µL of 0.5 *M* EDTA.
4. Hydrolyze the probe by diluting with distilled water to 100 µL and adding 100 µL of 2X carbonate buffer; incubate at 60°C. Probe hydrolysis times vary according to transcript length. The aim is to generate short RNA fragments (approx 100 bp) that will penetrate the tissue more easily. I allow 15 min per 500 bp (i.e., incubate a 2-kb probe for 1 h).
5. Neutralize by adding 200 µL of 2X neutralization buffer.
6. Precipitate the RNA product by adding 3 vol of ethanol and incubating at –20°C for at least 30 min.
7. Spin in a microcentrifuge 15 min, wash with 70% ethanol, dry the pellet, and resuspend in 200 µL of RNAse-free water. Store the probe at –70°C. For hybridization, use 0.5–1 µL per 100 µL of hybridization buffer.

3.4.2. Hybridization

1. Dissect testes from young adults (0–1 d old) in testis buffer and transfer to a 1.5 mL tube (*see* **Notes 2**, **6**, and **19**).
2. Fix in 4% paraformaldehyde in HEPES buffer for 20–60 min; agitation is not required—simply leave the tube on its side.
3. Wash three times in PBST, 5 min each.
4. Incubate in 50 µg/mL of proteinase K in PBST for 5–7 min (*see* **Note 20**).
5. Remove the proteinase K solution and stop the digestion by incubating the testes in 2 mg/mL glycine in PBST for 2 min.
6. Wash twice in PBST, 5 min each.
7. Refix in 4% paraformaldehyde in HEPES buffer for 20 min.
8. Wash three times in PBST, 10 min each.
9. Incubate in 1 : 1 PBST : HB for 10 min.
10. Wash in HB for 10 min.
11. Transfer testes into tissue culture inserts in a 24-well tissue culture plate (*see* **Note 7**).

12. Prehybridize in HB at 65°C for at least 1 h (*see* **Notes 21** and **22**).
13. Dilute the RNA probe (from **Subheading 3.4.1.**) in HB, heat denature at 80°C for 10 min, and briefly chill on ice.
14. Hybridize at 65°C overnight.
15. Wash at least six times, 30 min each, in HB at 65°C (*see* **Note 23**).
16. Wash once in 4 : 1 HB : PBST for 15 min at room temperature.
17. Wash once in 3 : 2 HB : PBST for 15 min at room temperature.
18. Wash once in 2 : 3 HB : PBST for 15 min at room temperature.
19. Wash once in 1 : 4 HB : PBST for 15 min at room temperature.
20. Wash twice in PBST, 15 min each.
21. Incubate overnight at 4°C in preadsorbed (*see* **Note 1**) alkaline phosphatase-conjugated antidigoxygenin antibody diluted 1 : 2000 in PBST.
22. Wash four times in PBST, 20 min each.
23. Wash three times in freshly made buffer HP, 5 min each.
24. Make staining solution in HP buffer by adding 4.5 μL of NBT and 3.5 μL of X-phosphate per milliliter.
25. Add the color reaction solution to the testes and leave to develop in the dark. The signal typically takes 10 min to 1 h, although for some transcripts incubation for several hours may be required. The staining needs to look quite dark, and purple not pink, at this stage to get good pictures at high magnification.
26. Stop the reaction by washing three times in PBST, 5 min each.
27. Dehydrate through an ethanol series: 10 min in each of 30%, 50%, 70%, 90%, and 100% (twice) ethanol. Transfer testes into a glass staining block (*see* **Note 24**).
28. Incubate 15 min in 1 : 1 ethanol : methyl salicylate, then in 100% methyl salicylate (*see* **Note 25**).
29. Mount in GMM and observe with Nomarski optics.

4. Notes

1. To preadsorb the antibody, fix embryos using a protocol suitable for immunostaining (*see* Chapter 9). Dilute the antibody 1 : 20 in PBST and incubate with the rehydrated fixed embryos for 2 h. Remove the antibody solution from the embryos and store at 4°C.
2. Newly eclosed males are used for all of these protocols, as they show the best morphology. To dissect testes from flies, place an anesthetized male next to a drop of testis buffer on the dissecting dish. Hold near the top of the abdomen with a pair of fine forceps in your left hand (or your right hand if you are left-handed). Grasp the external genitalia with the other pair of forceps and pull into the drop of buffer. The male genital tract, including testes, should come clear of the carcass. If it does not come clear, you will have to "fish" for the testes in the abdomen. Transfer the genital tract into a fresh drop of testis buffer and dissect the coiled testes and attached seminal vesicle from the rest of the tissues. If scoring for sperm motility, it is important to note that many males have no mature sperm for about 12 h after eclosion. To be sure of the absence of motile sperm from a

mutant, keep males isolated from females for 3 d, then dissect their seminal vesicles.

3. Generally, it is best to cut halfway along the straight portion of the testis. The contents should partially spill out, and this can be encouraged by gently tapping on the slide. The diameter of the drop of buffer should be approx 7 mm to get good preparations under a 22 × 22-mm^2 cover slip. Cysts of spermatocytes or spermatids should stay intact. The seminal vesicle from males that produce normal motile sperm will normally be slightly opaque. When it is opened, the sperm spill out. Motile sperm will show a shimmering effect visible even under the dissecting microscope.
4. Take pictures first, ask questions later. Photography can be done with either a digital camera or using black-and-white film. Squash preparations are only good for approx 20 min. After that, the cells are usually too flat and dead to observe. **Figure 6** shows some typical squash artifacts.
5. Alternatively, preparations can be made for visualization with an inverted microscope by using slides with a hole cut in them, sealing a cover slip over the bottom of the hole with vaseline, and dissecting the male into halocarbon oil in the chamber thus generated. Under these conditions, the cells remain viable for at least 3 h ***(40**,**41)***. (*See* Chapter 3.)
6. To transfer testes, place a small drop of testis buffer in the lid, put the testes into this, add 600 µL of fix to the tube, close the lid, and mix. Testes stick to tweezers if you try to put them directly into fix.
7. Add testes and washes into inserts; remove by lifting up the insert and aspirating solution from the well (*see* **Fig. 9**). Be careful; sometimes the mesh at the bottom detaches. Check for loose testes before aspirating. Do not fix testes in these dishes, as they may stick to the mesh.
8. Blocking does not seem to be essential for whole-mount immunohistochemistry, but it may improve the staining with certain antibodies.
9. The optimal dilution for each antibody needs to be empirically determined. As a first guess try using it two to four times more concentrated than gives an acceptable signal on Western blots. Immunohistochemistry often works with the antibody more dilute than is needed for immunofluorescence. For example, if an antibody works well at 1 : 4000 on Western blots, try 1 : 2000 for whole-mount immunohistochemistry and 1 : 1000 for immunofluorescence on squashed preparations. Antibody incubations can generally be done for a few hours at room temperature or overnight at 4°C. The choice is usually governed by convenience, although some primary antibodies work better overnight at 4°C.
10. The Vectastain ABC kit gives a slightly stronger signal, which decreases the development time, and may be useful for some proteins with relatively low levels of expression. Substitute the following for **step 9** of **Subheading 3.2.1.**: Incubate testes in 0.5X ABC reagent (50 µL solution A, 50 µL solution B, 5 mL PBS mixed 30 min before use) for 30 min. Go to **step 10** of **Subheading 3.2.1.**
11. DAB is a potent carcinogen; wear gloves and inactivate DAB with bleach after use. Tablets from Sigma are 0.7 mg and should be dissolved in 1 mL of PBS.

Lowering the concentration of DAB to 0.35 mg/mL (i.e., one tablet in 2 mL) does not significantly compromise the staining.

12. To mount stained testes in 85% glycerol, first transfer to a glass staining block. Remove the PBS and replace with glycerol. Mix well with a tungsten mounted needle or tweezers. Leave the testes in glycerol for 15–30 min before transferring to a cover slip, cutting off accessory gland and picking up with a clean slide. Imaging stained tissue under Nomarski optics usually requires that the optics be somewhat compromised, because the stain rarely shows up well when the Nomarski is set up to give the most structural information. Photograph using a tungsten balanced color slide film or a daylight correction filter and normal color slide film.
13. Write the genotype on the slide with pencil or a diamond pen; marker dissolves in ethanol! Use at least two slides per genotype, per antibody combination.
14. For phalloidin staining, testes do not need to be permeabilized with DOC. Instead, wash in PBST, then block in PBST-FCS. Dry the required amount of labeled phalloidin (stored at –20°C in methanol) in a Speed Vac just before use, and resuspend in the appropriate volume of PBST. Incubate for 2 h at room temperature, wash three times in PBST for 15 min each, then counterstain and mount. If costaining with an antibody, the phalloidin can be included in the secondary antibody incubation. **Caution:** Phalloidin is extremely hazardous! Wear protective clothing and be aware of the risks and safety procedures before using this compound.
15. To make Evostick wells, dry off the surface of the slide around the testes, taking care not to let the testes dry out. Paint a ring of Evostick or rubber cement around the area containing the testes and leave to dry for about 1 min (*see* **Fig. 10**). This forms a barrier, keeping a good depth of antibody solution above the tissue. Use 100 μL of diluted antibody per slide. Some antibodies penetrate the tissue much better under these conditions. Additionally, the testes remain stuck more firmly to the slide than if they are covered with a cover slip during antibody incubations. Evostick can be removed from the slide with tweezers.
16. If propidium iodide will be used to stain DNA, then RNAse A (0.5–1 mg/mL) must be added to one of the antibody incubations to reduce background fluorescence. I generally add it to whichever antibody incubation is being carried out at room temperature.
17. Do not fix in the tissue culture inserts used for other protocols, because glutaraldehyde makes testes stick to the mesh. Testes can be transferred to the inserts during the washes after fixation, but because the whole protocol is short, little time is saved.
18. Four percent formaldehyde may be substituted for glutaraldehyde. It is less toxic, but the staining intensity will be lower, so it is only suitable for reporters that express well.
19. Prepare about 10 males per probe. If many probes are to be used, process the testes together until the transfer into tissue culture inserts step. Removal of the accessory glands and other bits of genital tract is not essential; they can provide a nice in-sample negative control. If any mutant testes being used are different enough to be easily told apart from wild-type by Nomarski optics, then an excel-

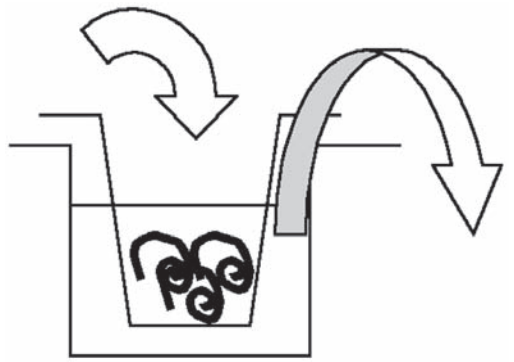


Fig. 9. Diagram of an insert and well of a tissue culture plate containing testes bathed in wash solution (*see* **Note 7**).

lent control is to mix wild type and mutant testes together after fixation. This allows a somewhat more accurate assessment of relative transcript levels, as the treatment of both genotypes will have been identical.

20. New batches of proteinase K should be checked. Overdigestion results in testes that are extra fragile and sticky, with a tendency to clump together.
21. To incubate at 65°C, the tissue culture dish can be left floating in a water bath.
22. Testes may be stored in HB at –20°C for up to 1 wk (in Eppendorf tubes).
23. The numbers of washes given here is a minimum. Increasing the number and total time of washing, especially in HB, can improve the signal-to-noise ratio.
24. To transfer stained testes, use a 200–µL pipet tip with the end cut off. Watch under the dissecting microscope to ensure complete transfer, especially of lightly stained tissue. Transfer of the testes at this stage is essential because methyl salicylate dissolves the tissue culture plastic.
25. Methyl salicylate clears the testes, but also dissolves some of the color product. Therefore, this incubation will make background staining disappear, but care must be taken to prevent the real staining from disappearing too. Canada balsam stabilizes the color, so remove the methyl salicylate and add GMM to the staining block when ready. The color should look quite intense under the dissecting microscope, in order to obtain good higher-magnification pictures. To mount the stained testes, transfer them in GMM onto a 22×22-mm^2 cover slip with a cut-off pipet tip. Methyl salicylate also makes the testes very brittle so that any dissection (e.g., separation of pairs of testes) can be done by prodding or cutting with a tungsten needle.

Acknowledgments

I would like to thank David Neville and Daimark Bennett for proofreading this manuscript. Thanks to Natasha Vereshegina and Liz Benson for testing protocols, Karen Hales for figures of mutants and Cayetano Gonzalez for pictures of mutants and for the time-lapse figure.

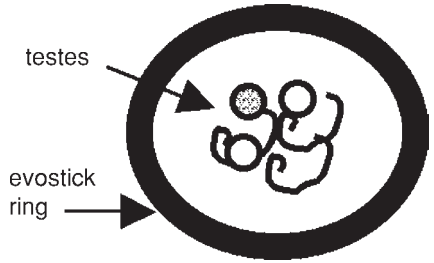


Fig. 10. Diagram of testes incubating in an Evostick well on a microscope slide (*see* **Note 15**).

References

1. Fuller, M. T. (1993) Spermatogenesis, in *The Development of Drosophila* (Bate, M. and Martinez-Arias, A., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 71–147.
2. Lindsley, D. and Tokuyasu, K. T. (1980) Spermatogenesis, in *Genetics and Biology of Drosophila* (Ashburner, M. and Wright, T. R. F., eds.), Academic, New York, pp. 225–294.
3. Kerrebrock, A. W., Miyazaki, W. Y., Birnby, D., and Orr-Weaver, T. L. (1992) The *Drosophila mei-S332* gene promotes sister-chromatid cohesion in meiosis following kinetochore differentiation. *Genetics* **130,** 827–841.
4. Kerrebrock, A. W., Moore, D. P., Wu, J. S., and Orr-Weaver, T. L. (1995) Mei-S332, a *Drosophila* protein required for sister-chromatid cohesion, can localize to meiotic centromere regions. *Cell* **83,** 247–256.
5. Miyazaki, W. Y. and Orr-Weaver, T. L. (1992) Sister chromatid misbehavior in *Drosophila ord* mutants. *Genetics* **132,** 1047–1061.
6. Bickel, S. E., Moore, D. P., Lai, C., and Orr-Weaver, T. L. (1998) Genetic interactions between *mei-S332* and *ord* in the control of sister-chromatid cohesion. *Genetics* **150,** 1467–1476.
7. Bickel, S. E., Wyman, D. W., and Orr-Weaver, T. L. (1997) Mutational analysis of the *Drosophila* sister-chromatid cohesion protein ORD and its role in the maintenance of centromeric cohesion. *Genetics* **146,** 1319–1331.
8. Casal, J., Gonzalez, C., Wandosell, F., Avila, J., and Ripoll, P. (1990) Abnormal meiotic spindles cause a cascade of defects during spermatogenesis in *asp* males of *Drosophila*. *Development* **108,** 251–260.
9. Wakefield, J. G., Bonaccorsi, S., and Gatti, M. (2001) The *Drosophila* protein asp is involved in microtubule organization during spindle formation and cytokinesis. *J. Cell Biol.* **153,** 637–648.
10. Bonaccorsi, S., Giansanti, M. G., and Gatti, M. (1998) Spindle self-organization and cytokinesis during male meiosis in *asterless* mutants of *Drosophila melanogaster*. *J. Cell Biol.* **142,** 751–761.

11. Alphey, L., Jimenez, J., White-Cooper, H., Dawson, I., Nurse, P., and Glover, D.M. (1992) *twine*, a *cdc25* homologue that functions in the male and female germlines of Drosophila. *Cell* **69,** 977–988.
12. Eberhart, C. G., Maines, J. Z. and Wasserman, S. A. (1996) Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature* **381,** 783–785.
13. Eberhart, C. G. and Wasserman, S. A. (1995) The *pelota* locus encodes a protein required for meiotic cell division: an analysis of G2/M arrest in *Drosophila* spermatogenesis. *Development* **121,** 3477–3486.
14. White-Cooper, H., Alphey, L., and Glover, D. M. (1993) The cdc25 homologue twine is required for only some aspects of the entry into meiosis in Drosophila. *J Cell Sci* **106,** 1035–1044.
15. Andrews, J., Bouffard, G. G., Cheadle, C., Lu, J., Becker, K. G., and Oliver, B. (2000) Gene discovery using computational and microarray analysis of transcription in the *Drosophila melanogaster* testis. *Genome Res.* **10,** 2030–2043.
16. Wakimoto, B. T. (2000) Doubling the rewards: testis ESTs for *Drosophila* gene discovery and spermatogenesis expression profile analysis. *Genome Res.* **10,** 1841–1842.
17. Castrillon, D. H., Gönczy, P., Alexander, S., et al. (1993) Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single P element mutagenesis. *Genetics* **135,** 489–505.
18. Maines, J. Z. and Wasserman, S. A. (1999) Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule. *Nature Cell Biol.* **1,** 171–174.
19. Tomkiel, J. E., Wakimoto, B. T., and Briscoe, A. (2001) The *teflon* gene is required for maintenance of autosomal homolog pairing at meiosis I in male *Drosophila melanogaster*. *Genetics* **157,** 273–281.
20. White-Cooper, H., Leroy, D., MacQueen, A., and Fuller, M. T. (2000) Transcription of meiotic cell cycle and terminal differentiation genes depends on a conserved chromatin associated protein, whose nuclear localisation is regulated. *Development* **127,** 5463–5473.
21. McKearin, D. (1997) The *Drosophila* fusome, organelle biogenesis and germ cell differentiation: if you build it. . . . *Bioessays* **19,** 147–152.
22. Gould-Somero, M. and Holland, L. (1974) The timing of RNA synthesis for spermiogenesis in organ cultures of *Drosophila melanogaster* testes. *Wilhelm Roux. Arch.* **174,** 133–148.
23. Olivieri, G. and Olivieri, A. (1965) Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. *Mutat. Res.* **2,** 366–380.
24. Schafer, M., Nayernia, K., Engel, W. and Schafer, U. (1995) Translational control in spermatogenesis. *Dev. Biol.* **172,** 344–352.
25. Tates, A. D. (1971) Cytodifferentiation during spermatogenesis in *Drosophila melanogaster*: an electron microscope study. Thesis, Rijksuniversiteit, Leiden.

26. Hime, G. R., Brill, J. A., and Fuller, M. T. (1996) Assembly of ring canals in the male germ line from structural components of the contractile ring. *J. Cell Sci.* **109,** 2779–2788.
27. Fabrizio, J. J., Hime, G. R., Lemmon, S. K., and Bazinet, C. (1998) Genetic dissection of sperm individualisation in *Drosophila melanogaster*. *Development* **125,** 1833–1843.
28. Fitch, K. R. and Wakimoto, B. T. (1998) The paternal effect gene *ms(3)sneaky* is required for sperm activation and the initiation of embryogenesis in *Drosophila melanogaster*. *Dev. Biol.* **197,** 270–282.
29. Ryner, L. C., Goodwin, S. F., Castrillon, D. H., et al. (1996) Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. *Cell* **87,** 1079–1089.
30. Green, L. L., Wolf, N., McDonald, K. L., and Fuller, M. T. (1990) Two types of genetic interaction implicate the *whirligig* gene of *Drosophila melanogaster* in microtubule organization in the flagellar axoneme. *Genetics* **126,** 961–973.
31. Gonzalez, C., Casal, J., and Ripoll, P. (1988) Functional monopolar spindles caused by mutation in *mgr*, a cell division gene of *Drosophila melanogaster*. *J. Cell Sci.* **89,** 39–47.
32. Brill, J. A., Hime, G. R., Scharer-Schuksz, M., and Fuller, M. T. (2000) A phospholipid kinase regulates actin organisation and intercellular bridge formation during germline cytokinesis. *Development* **127,** 3855–3864.
33. Hales, K. G. and Fuller, M. T. (1997) Developmentally regulated mitochondrial fusion mediated by a conserved novel predicted GTPase. *Cell* **90,** 121–129.
34. Lin, T.-Y., Viswanathan, S., Wood, C., Wilson, P. G., Wolf, N., and Fuller, M. T. (1996) Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in *Drosophila* males. *Development* **122,** 1331–1341.
35. Gönczy, P., Matunis, E., and DiNardo, S. (1997) *bag-of-marbles* and *benign gonial cell neoplasm* act in the germline to restrict proliferation during *Drosophila* spermatogenesis. *Development* **124,** 4361–4371.
36. Kiger, A. A., White-Cooper, H., and Fuller, M. T. (2000) Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature* **407,** 750–754.
37. Tran, J., Brenner, T., and DiNardo, S. (2000) Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. *Nature* **407,** 754–757.
38. Boswell, R. E. and Mahowald, A. P. (1985) *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. *Cell* **43,** 97–104.
39. Kiger, A. A., Gigliotti, S., and Fuller, M. T. (1999) Developmental genetics of the essential Drosophila nucleoporin *nup154*: allelic differences due to an outward-directed promoter in the P-element 3' end. *Genetics* **153,** 799–812.
40. Church, K. and Lin, H.-P. P. (1985) Kinetochore microtubules and chromosome movement during prometaphase in *Drosophila melanogaster* spermatocytes studied in life and with the electron microscope. *Chromosoma* **92,** 273–282.
41. Rebollo, E. and Gonzalez, C. (2000) Visualizing the spindle checkpoint in *Drosophila* spermatocytes. *EMBO Rep.* **1,** 65–70.

42. Giansanti, M. G., Bonaccorsi, S., Williams, B., et al. (1998) Cooperative interactions between the central spindle and the contractile ring during *Drosophila* cytokinesis. *Genes Dev.* **12,** 396–410.
43. Patel, N. H., Snow, P. M., and Goodman, C. S. (1987) Characterization and cloning of Fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. *Cell* **48,** 975–988.
44. Pesacreta, T. C., Byers, T. J., Dubreuil, R., Kiehart, D. P., and Branton, D. (1989) *Drosophila* spectrin: the membrane skeleton during embryogenesis. *J. Cell Biol.* **108,** 1697–1709.
45. Zaccai, M. and Lipshitz, H. D. (1996) Differential distributions of two adducin-like protein isoforms in the *Drosophila* ovary and early embryo. *Zygote* **4,** 159–166.
46. McKearin, D. and Ohlstein, B. (1995) A role for the *Drosophila* bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121,** 2937–2947.
47. Callaini, G., Whitfield, W. G. F., and Riparbelli, M. G. (1997) Centriole and centrosome dynamics during the embryonic cell cycles that follow the formation of the cellular blastoderm in Drosophila. *Exp. Cell Res.* **234,** 183–190.
48. Gönczy, P., Viswanathan, S., and DiNardo, S. (1992) Probing spermatogenesis in *Drosophila* with P-element enhancer detectors. *Development* **114,** 89–98.
49. Gönczy, P., Matunis, E., and DiNardo, S. (1997) *bag-of-marbles* and *benign gonial cell neoplasm* act in the germline to restrict proliferation during *Drosophila* spermatogenesis. *Development* **124,** 4361–4371.
50. Santel, A., Blumer, N., Kampfer, M., and Renkawitz-Pohl, R. (1998) Flagellar mitochondrial association of the male-specific Don Juan protein in *Drosophila* spermatozoa. *J. Cell Sci.* **111,** 3299–3309.