Chapter 1

Studying Recombination in Mouse Oocytes

Xianfei Sun and Paula E. Cohen

Abstract

Meiosis is the specialized cell division in sexually reproducing organisms in which haploid gametes are produced. Meiotic prophase I is the defining stage of meiosis, when pairing and synapsis occur between homologous chromosomes, concurrent with reciprocal recombination (or crossing over) events that arise between them. Any disruption of these events during prophase I can lead to improper segregation of homologous chromosomes which can cause severe birth defects in the resulting progeny, and this occurs with alarming frequency in human oocytes. Thus, while the pathways that regulate these events in prophase I are highly conserved in both males and females, the stringency with which these events are monitored and/or controlled appears to be dramatically lower in females. These observations underscore the need to examine and compare meiotic mechanisms across the sexes. However, the study of female meiosis is impeded by the early start of meiosis during fetal development and the very limited amount of ovarian tissue available for meiotic analyses. Here we describe three different techniques which are useful for meiotic prophase I analysis in mouse/human oocytes, ranging from early prophase I events through until the resolution of crossing over at the first and second meiotic divisions.

Key words: Meiosis, Meiotic prophase I, Chromosome spreading, Chiasmata preparation, Spindle preparation, Oocyte

1. Introduction

Meiosis is a special type of cell division in which diploid precursor cells give rise to haploid daughter cells through one round of DNA replication followed by two rounds of cell division. The meiotic division is an integral part of the reproduction in sexually reproducing organisms and haploid gametes are generated from this process.

Meiosis is divided into two stages, based on the two rounds of cellular division: meiosis I and meiosis II. Each stage can be further divided into prophase, metaphase, anaphase, and telophase. Among all of these substages, prophase I is the most remarkable since this stage encompasses the defining and unique features of meiosis.

Hayden A. Homer (ed.), *Mammalian Oocyte Regulation: Methods and Protocols*, Methods in Molecular Biology, vol. 957,
DOI 10.1007/978-1-62703-191-2_1,

These events include pairing of homologous chromosomes through the proteinaceous scaffold structure known as synaptonemal complex (SC), recombination between chromosome pairs and formation of chiasmata between homologs at sites of crossing over. The importance of these events is underscored by the observation that nondisjunction errors occurring in first meiotic division lead to about 50% of all spontaneous miscarriages in human (1, 2).

According to the structure and status of synaptonemal complex (SC), prophase I is further divided into five substages: leptonema (adjective: leptotene), zygonema (adjective: zygotene), pachynema (adjective: pachytene), diplonema (adjective: diplotene), and diakinesis (see Fig. 1). The replicated chromosomes condense and search for their homologous partners in leptonema. The lateral element of SC formed along the axes of homolog chromosomes at this stage. The lateral element is also called axial element prior to synapsis (3). During zygonema, the central element of the SC element forms and "zip" chromosome pairs together. By pachynema, the chromosome pairs are fully synapsed. The SC starts to degrade after this, a process that results in the separation of chromosome pairs in diplonema. By the time the cell has progressed to diakinesis, the chromosomes remain attached only at sites of chiasmata where crossing over, or reciprocal recombination, has occurred.

Although meiosis is an essential process common to all sexually reproducing organisms, it shows tremendous variation between eukaryotic species in terms of its timing, regulation, and success rate. In mammals, this variability is also observed between sexes, and such sexual dimorphism leads to significant differences in the aneuploidy rates observed in male and female gametes. In humans, oocyte aneuploidy rates are as high as 25%, compared to the rates of around 2% reported for human sperm (1, 4), and the majority of these errors (90%), at least in females, are the result of errors in maternal meiosis I (5, 6). Trisomy, as one type of aneuploidy, involves an extra chromosome copy within the nuclei of affected offspring. While the parental origin of the additional chromosome varies in different human trisomic conditions, maternal errors account for more than 60% of these abnormalities. Once again, nondisjunction events in maternal meiosis I are the major cause of these trisomies (1). Trisomy 21, also known as Down syndrome, is one of the most common aneuploid abnormalities in human newborns, and maternal errors account for 88% of all Down syndrome cases, >70% of them occurring during meiosis I (1, 7). These errors correlate well with the heterogeneity of prophase I events observed in human fetal oocytes (8).

The sexual dimorphism observed in mammalian meiosis is also illustrated in transgenic mice bearing mutations in genes essential for meiotic progression. *Sycp3* encodes a protein essential for SC formation and homolog pairing (9). In females homozygous for an

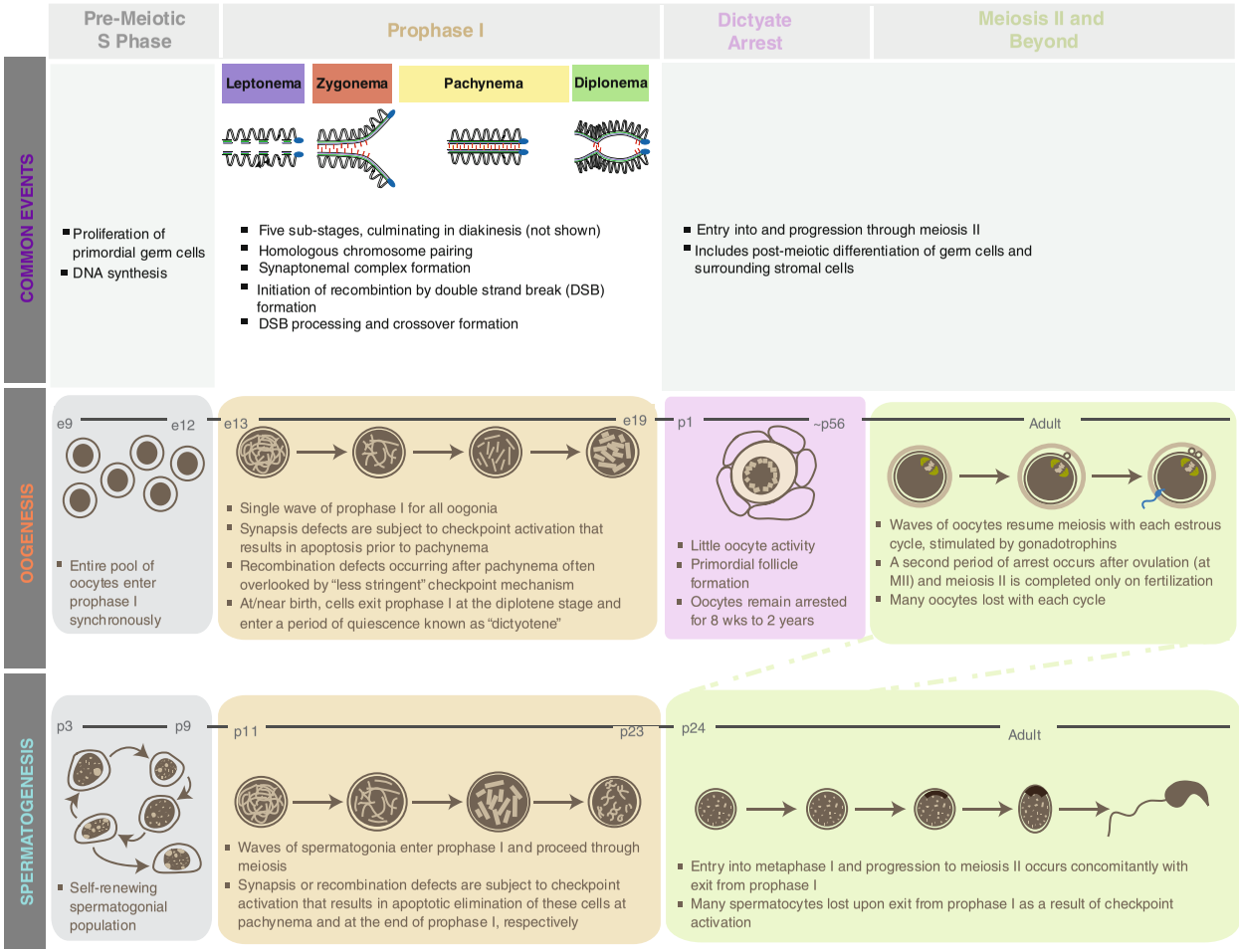


Fig. 1. Gametogenesis and meiosis in mice. The *top panel* shows the sequential events during pre-meiotic S phase and meiosis common in both sexes. The *middle and bottom panels* illustrate the gametogenesis in female and male mouse separately, demonstrating obvious sexual dimorphism not only in their different meiotic products but also in timing and checkpoint control during meiosis.

Sycp3 deletion, synapsis and chiasmata formation are reduced, leading to a high aneuploidy rate in oocytes and a subfertility phenotype (10, 11). By contrast, *Sycp3*–/– males are totally sterile. The spermatocytes in *Sycp3* knockout males show seriously disrupted synapsis and undergo apoptosis at a cytologically defined zygotene stage (9, 12).

A major difference in male and female meiosis, and one of the principal roadblocks in understanding female meiotic errors, comes from the fact that female meiosis starts during fetal development, whereas males initiate meiosis during early postnatal life (in the mouse). Mouse oocytes initiate prophase I entry soon after the oogonia populate the fetal ovary at embryonic day 10.5–12.5 (13). The oocytes progress into leptonema from embryonic day 13.5 (14). At around the time of birth, the oocytes start to enter diakinesis and arrest at a prolonged quiescent state known as dictyate (13). By the fifth day after birth, all oocytes are arrested in dictyate, remaining dormant until puberty when only a few will be selected to resume meiosis at each estrous cycle. Even for these selected oocytes, they will not complete meiosis but get arrested again in metaphase II unless they can be fertilized by sperm after ovulation. By contrast, male germ cells enter meiosis after birth. At puberty, a group of male germ cells are recruited to enter meiosis and produce mature sperm. Meiotic entry then proceeds in waves throughout the lifetime of the animal (see Fig. 1).

The temporal differences mentioned above illuminate the inherent difficulty in studying female meiosis. In mouse, while a small piece of testicular tissue from an adult male could provide enough meiotic and post-meiotic germ cells for most experimental purposes, and would certainly generate hundreds of "chromosome spread" preparations for prophase I analysis, the meiotic material obtained from an individual female is very limited. Thus the efficient processing of the scant tissue samples is extremely important for female study.

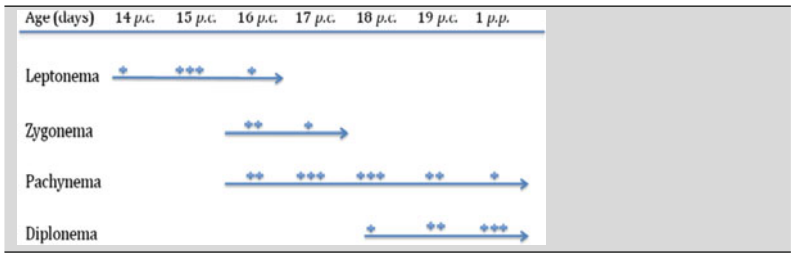
The following protocols are designed to provide three different methods that can be used in the female meiotic studies with restricted amounts of experimental material. Subheading 3.1 will introduce a method for preparing chromosome spreads from late stage mouse fetal ovaries and labeling key meiotic markers of prophase I with fluorescent antibodies, often termed indirect immunofluorescence. In Subheading 3.2, a technique will be described for staining diakinesis stage mouse oocytes and detecting chiasmata in vitro. Often, the abnormality in meiotic recombination during prophase I may not show up until later, which could display as a disrupted spindle structure in metaphase I and/or a lower rate for the first polar body extrusion. Subheading 3.3 will demonstrate how to make spindle preparations. Collectively, and in conjunction with fertility assessment studies, these techniques allow for a comprehensive analysis of prophase I events from beginning to end in mouse/human oocytes.

2. Materials

2.1. Ovarian Chromosome Spreads

1. Mice: Mouse embryos are used. To set up timed mating, female mice (2 months to 6 months old) are bred to males (2 months to 1 year old). Start checking vaginal plug the next day. Noon on the day of plug is 0.5 day post coitum (dpc) (see Table 1).
2. Dissecting equipment and dissecting tools: dissecting scope, microdissecting scissors (11-1165 and 11-1025, Biomedical Research Instruments, Inc., Silver Spring, MD 20914), fine forceps (watchmaker No. 5), surgery scalpel (B-P blade handle No. 5 and compatible scalpel blade No. 11), disposable 1 ml syringe and needle (30G1/2, 305106, Becton Dickinson & Co., Franklin lakes, NJ 07417).
3. Culture plates: Sterile 100 mm and 60 mm culture.
4. 15 ml Conical centrifuge tubes.
5. Two-well Concavity slides (71878-08), Electron Microscopy Sciences (EMS), 1560 Industry Road (Hatfield, PA 19440).
6. Six-well microscope slides (63423-08, EMS) (see Note 1).
7. Microscope coverslips (24 mm × 60 mm).
8. Humid chamber: Put wet towel papers on both sides of a square plastic bioassay plate (431272, Corning Incorporated), One Riverfront Plaza (Corning, NY 14831), leaving the middle section dry for slides.
9. Coplin staining jars (70315, EMS).

Table 1
Percentage of oocytes in different prophase I stages from the 14th day of fetal life to the first day after birth[a] (adapted from Evan et al., 1982 (14))



p.c. post coitum, *p.p.* post partum
[a]The number of "+" indicates the relative percentage of each population in a particular developmental stage

10. 1× PBS: 8 g NaCl, 0.2 g KCl, 0.92 g Na_2HPO_4, 0.2 g KH_2PO_4. Adjust volume to 1 L with ddH_2O. Adjust pH to 7.4 with NaOH.
11. 10× Sodium borate buffer (pH 8.2, 1 M): Dissolve 61.83 g boric acid in 500 ml ddH_2O. Adjust pH to 8.2 with solid NaOH pellets. Bring final volume to 1 L with ddH_2O. Use filter to sterilize.
12. Hypotonic extraction buffer (HEB): 100 µl of 500 mM EDTA (final concentration 5 mM), 300 µl of 1 M Tris–HCl (pH 8.5) (final concentration 30 mM), 10 µl of 500 mM dithiothreitol (DTT) (final concentration 0.5 mM), 50 µl of 100 mM phenylmethylsulfonylfluoride (PMSF) (final concentration 0.5 mM), 0.051 g trisodium citrate (final concentration 20 mM), and 0.172 g sucrose (final concentration 50 mM).

    Combine all ingredients and bring the final volume to 10 ml (see Note 3). The pH should be between 8.2 and 8.4. If not, adjust pH with sodium borate buffer. Stocks of DTT and PMSF should be stored at −20°C in small aliquots.
13. Hypotonic sucrose solution (100 mM): Dissolve 0.3423 g sucrose in 10 ml ddH_2O. Adjust pH to 8.2 (see Note 2).
14. 1% Paraformaldehyde (PFA): Add 0.5 g of PFA (19200, EMS) to 50 ml of pre-warmed ddH_2O (at approximately 65°C). Add one drop of 1 N NaOH and shake to dissolve. Adjust pH to 9.2 with sodium borate buffer. Add 75 µl Triton X-100 and mix well but gently (see Note 2).
15. Antibody dilution buffer (ADB): 10 ml Normal goat serum (NGS), 3 g bovine serum albumin (A9647, Sigma), 50 µl Triton X-100, and 90 ml 1× PBS. Combine the ingredients above and mix well. The solution can be stored at 4°C up to 1 week (see Note 3).
16. 0.4% Photoflo/PBS: 400 µl Photoflo in 100 ml of PBS.
17. 0.1% Triton/PBS: 100 µl Triton X-100 in 100 ml of PBS.
18. 10% ADB/PBS: 10 ml ADB in 90 ml of PBS.
19. 0.4% Photoflo/H_2O: 400 µl Photoflo in 100 ml of ddH_2O.
20. Primary antibodies: Many antibodies are compatible with prophase I chromosome spreads. These include:
    (a) Mouse or rabbit SYCP3 (available from many labs or commercial resources (8, 15–18)).
    (b) Goat SYCP3 (SC-20845, Santa Cruz).
    (c) Mouse Monoclonal MLH1 (550838, BD Biosciences).
    (d) Mouse or rabbit γH2AX (05-636 or 07-164, Upstate).
    (e) Rabbit RAD51 (PC 130, Calbiochem).
    (f) Rabbit SYCP1 (ab 15090, Abcam (17)).

21. Secondary antibodies:
    (a) Goat anti-mouse Alexa Fluor 488 (A11017, Invitrogen; 1:1,000 dilution).
    (b) Goat anti-rabbit Alexa Fluor 488 (A11070, Invitrogen; 1:1,000 dilution).
    (c) Goat anti-mouse Alexa Fluor 555 (A21425, Invitrogen; 1:1,000 dilution).
    (d) Goat anti-rabbit Alexa Fluor 555 (A21430, Invitrogen; 1:1,000 dilution).
22. Antifade with DAPI: Mix 5 ml of 1× PBS, 50 µl of 1 mg/ml DAPI stock (1 µg/ml final), 1.2 g 1,4-Diazabicyclo[2.2.2] octane (DABCO) (24 mg/ml final), and 45 ml glycerol (90% final). Dissolve well. Store at –20°C in small (~1 ml) aliquots in brown 1.5 ml tubes to protect from light. Alternatively, use commercial antifade mounting medium, such as Prolong Gold antifade reagent with DAPI (P36935, Invitrogen).
23. Parafilm (13-374-10, Fisher Scientific).

2.2. Chiasmata Prep

1. Sterile 60 mm culture dish.
2. Watch glasses.
3. Collection Medium: 45 ml Waymouth's medium with 1% penicillin–streptomycin solution, 5 ml fetal bovine serum. Add 2.5 mg/ml sodium pyruvate before use. Medium without sodium pyruvate can be stored at 4°C in the dark for up to 1 month.
4. KSOM medium (MR-020P-5F, Millipore).
5. Light mineral oil (ES-005-C, Millipore).
6. Hand-pulled micropipettes and suction mouth piece: use a fine flame and pull 100 µL capillary glass pipettes (53432-921, VWR). Snip the glass pipette in the middle. Micropipettes with small openings (about 0.3 mm in diameter) should be obtained in this way. Then attached the micropipettes to the mouth piece (A5177-5EA, Sigma).
7. Microscope slide preparation: Use china marker pen to mark the area on the underside of a microscope slide.
8. Hypotonic solution: Dissolve 1 g sodium citrate in 100 ml ddH_2O (1% final).
9. Carnoy's fixative: Mix three parts methanol and one part glacial acetic acid. Make fresh immediately prior to use.
10. Giemsa stain (GS500, Sigma-Aldrich).
11. Histological mounting medium (Permount SP15-100, Fisher Scientific).
12. Coplin staining jars (70315, EMS).
13. Microscope coverslip (24 mm × 60 mm).

2.3. Spindle Prep

1. Sterile 60 mm culture dish.
2. Watch glasses.
3. Lysine coated slide preparation: Make a stock solution of 2 mg/ml Poly-d-lysine hydrobromide (P1024, Sigma-Aldrich). Boil microscope slides in soapy water, rinse well, wash in 95% ethanol, and then dry either in the air or with a lint-free cloth. Immerse the bottom half of the slides in poly-d-lysine solution for 15 min at room temperature and rinse well in ddH_2O. Let the slides dry in the air. Then use rubber cement to make separate wells on the slides to hold medium drops.
4. Slide mailer (HS15986, Fisher Scientific).
5. Hand-pulled micropipettes and suction mouth pieces: Please refer to Subheading 2.2, item 6 for details.
6. Collection medium: Please refer to Subheading 2.2, item 3 for details.
7. KSOM medium (MR-020P-5F, Millipore).
8. Light mineral oil (ES-005-C, Millipore).
9. 5× Stabilization buffer (SB) stock: Add 7.55 g of PIPES, 0.25 g of $MgCl_2$, and 0.235 g of EGTA to 50 ml ddH_2O. Can be stored at 4°C for 1 month (see Note 4).
10. Spindle Fixative: Dissolve 0.2 g of PFA to 5 ml of ddH_2O (add one drop of 1 N NaOH and heat to 65°C to dissolve). Add 2 ml of 5× SB stock, 50 µl 20% Triton X-100 to the dissolved PFA. Bring the total volume to 10 ml with ddH_2O. Make fresh prior to use.
11. 0.1% NGS wash: 50 µl NGS with 50 ml 1× PBS. Can be stored at 4°C for 1 month.
12. 10% NGS wash: Add 50 µl of Triton X-100 and 5 ml of NGS to 45 ml of 1× PBS. Can be stored at 4°C for 1 month. Add 0.01 g sodium azide if long time storage is desired.
13. Ringer solution: Add 0.45 g of NaCl, 0.021 g KCl, and 0.0125 g $CaCl_2$ to 50 ml of ddH_2O. Sterilize with syringe filter.
14. Fibrinogen solution: Add 400 µl Ringer's solution to 0.005 g fibrinogen. Incubate at 37°C for 10 min to dissolve.
15. Thrombin: Reconstitute 250 U of lyophilized thrombin with 1 ml ddH_2O. Add 1.5 ml 1× PBS for a final volume of 2.5 ml. Store in 20 µl aliquots at –20°C.
16. Primary antibody: Monoclonal mouse anti-β-tubulin (T4026, Sigma-Aldrich).
17. Secondary antibody: Goat anti-mouse AlexFluor 488 (A11017, Invitrogen).
18. 2% Triton/PBS: 200 µl Triton-X-100 in 10 ml PBS.

19. 0.4% Photoflo/ddH_2O: 40 µl Photoflo in 10 ml ddH_2O.
20. Antifade with DAPI: Please refer to Subheading 2.1, item 22 for details.
21. Microscope coverslips.

3. Methods

3.1. Chromosome Spreads from Mouse Fetal Ovaries for Prophase I Analysis

Since meiosis begins during fetal development in females, ovaries obtained from fetuses at the appropriate developmental stage will be used. Timed matings need to be set up ahead of time. Noon on the day of a copulation plug is counted as embryonic day (E) 0.5 (see Note 5). The timing of meiotic progression may vary among different mouse strains. Generally, leptotene- and zygotene-stage cells can be found at E15.5-16.5. Pachytene- and diplotene-stage cells predominate at E18.5-19.5 (see Table 1).

3.1.1. Chromosome Spreading Procedure

1. Euthanize pregnant female and remove the gravid uterine horns. Place uteri containing embryos into a 100 mm culture dish containing 1× PBS on ice.
2. Dissect ovaries from female mouse embryos. Do one embryo at a time and maintain the rest in 1× PBS on ice. Remove the embryo from uterus, decidual tissue, and yolk sac. Sacrifice the embryo by removing the head and tape the torso down on a paper towel, ventral surface up. A testis should be easily distinguished from an ovary at this stage. Testes are located in the caudal region of the pelvis while ovaries are located near the kidneys (see Fig. 2). To locate the ovaries, first identify the bladder and, protruding out from there, identify each uterine horn traveling outwards and anteriorly from the bladder. Follow the

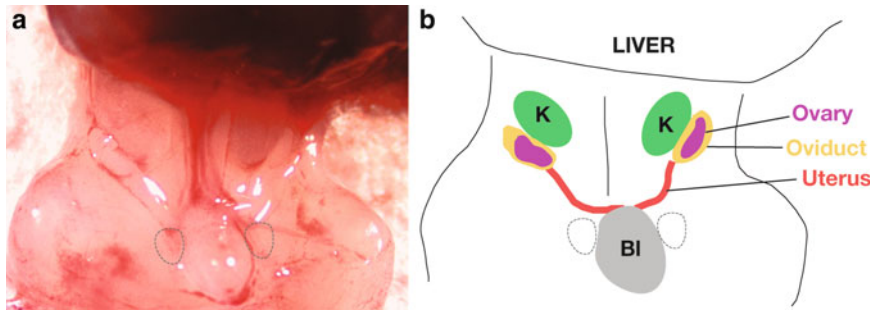


Fig. 2. Illustration of late stage mouse fetal ovaries and testes. (**a**) The abdominal part of a 16.5 dpc female embryo is shown with the abdominal cavity open and liver/intestines flipped to the side. The *dotted circles* indicate the location of the testes in a male embryo at this age. Panel (**b**) represents the cartoon version of (**a**) with the neighboring organs labeled. *K* kidney, *Bl* bladder.

uterine horns up towards the kidney, being careful not to tear each horn, until you locate the larger clear "jelly like" structure at the end of each horn. This is the ovary and oviduct. Pinch off entire structure with forceps or scissors. You can dissect the ovary away from the other tissues in a drop of PBS.

3. Place ovaries in HEB and incubate on ice for 30 min (see Note 6).
4. Add 40–60 μl hypotonic sucrose solution to the concave well of the concavity slide and transfer two to three pairs of fetal ovaries to the sucrose drop (or 20 μl hypotonic sucrose solution to one pair if embryos are being treated separately). Use one surgery scalpel blade (size #11) and one 30-guage needle to tease the ovaries apart and release cells. Remove the larger pieces of ovaries from the sucrose solution and pipette sucrose solution up and down to resuspend cells.
5. Place 35 μl PFA drops on each well of the six-well slides. Pipette 5 μl of the sucrose solution to each PFA drop until the sucrose solution is exhausted. You should have enough cell slurry to prepare 2 six-well slides from three pairs of fetal ovaries. Again, if each pair of ovaries is being treated separately, apply cell slurry from embryo 1 to well 1 of each of your slides. Then apply the cell slurry from embryo 2 to well 2 of each slide, and so on. In this way, each of the slides will have 6 wells, each containing cells from a distinct animal. The entire slide will be processed together, such that each well will be subjected to the same treatment, and hence each slide will contain a mixture of genotypes ALL receiving the same antibodies.
6. Incubate the six-well slides in a closed humid chamber overnight at 4°C.
7. Open the chamber and let the slides dry completely.
8. Wash slides in 0.4% Photoflo/H_2O for 5 min. Repeat the wash two more times and then air-dry. At this point, the slides can be either used for immunostaining or stored at −80°C (see Note 7).

3.1.2. Indirect Immunofluorescence of Chromosomes from Prophase I Oocytes

1. Blocking for primary antibody: Soak the slides in 0.4% Photoflo/PBS for 10 min, then in 0.1% Triton/PBS for 10 min, followed by another 10 min in 10% ADB/PBS. From this point onwards, never allow slides to dry out.
2. Dilute primary antibodies in ADB (see Note 8). Maintain antibodies on ice until needed.
3. Cut one piece of parafilm and place it in the central dry area of the humid chamber. Use strips of parafilm to lift the slides off the surface of the underlying film and create room for the antibody dilutions. Add 80 μl of antibody dilution on the parafilm.

Put the slides face down to the antibody drop on the parafilm and expel air bubbles out. Seal the humid chamber with saran wrap.

4. Incubate overnight at room temperature.
5. Remove the slides from the humid chamber and repeat the blocking steps: 10 min in 0.4% Photoflo/PBS, 10 min in 0.1% Triton/PBS, and 10 min in 10% ADB/PBS.
6. Dilute secondary antibodies in ADB.
7. With the same method described in step 3 of this section, add 80 μl of secondary antibody dilution on the parafilm in a humid chamber and place the slides face down to the antibody dilution drop (see Note 9).
8. Seal the humid chamber and wrap the box with aluminum foil as well.
9. Incubate in the dark for 2 h at 37°C.
10. Carefully lift the slides off the parafilm. It is often useful to flood the parafilm with 0.4% Photoflo/PBS first. Transfer slides to Coplin jars and wash twice in 0.4% Photoflo/PBS for 5 min and twice in 0.4% Photoflo/H_2O for 5 min.
11. Air-dry the slides thoroughly in the dark and add antifade with DAPI. Then mount with glass coverslip and blot excess liquid (see Note 10).
12. Dry on the bench for at least 4 h before visualizing. The slides can be stored at 4°C in the dark (before or after drying) or immediately visualized using an epifluorescence microscope (see Fig. 3, for example).

3.2. Chiasmata Preparation Using Diakinesis Stage Mouse Oocytes

The following protocol is modified from the method described previously by Tarkowski and others (15, 19–21). A nice scattering of oocyte nuclei and spreading of chromosomes can be obtained using this technique. It can be applied not only to chiasmata analysis on diakinesis-stage oocytes but also to studies for meiosis I disjunction study on metaphase II arrested oocytes.

1. Remove ovaries from unstimulated female mice at 24–28 days of age (see Note 11), remove fat and other tissue, and transfer them into the oocyte collection media.
2. Grasp ovary with blunt forceps in one hand and release the oocytes from the ovaries by puncturing the ovaries with one 30-gauge needle in the other hand.
3. Transfer the oocytes with germinal vesicle (nuclear membrane) intact into 20 μl KSOM drop under light mineral oil using mouth pipette and hand-pulled pipette (see Note 12).
4. Incubate the oocytes in a cell culture incubator (37°C, 5% CO_2) until the oocytes enter metaphase I (see Note 13).

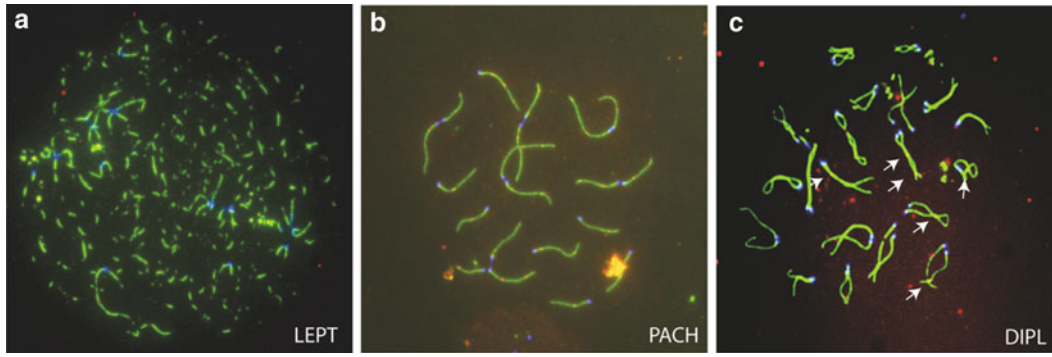


Fig. 3. Examples of female chromosome spreads made from fetal mouse ovaries. Shown are chromosome spreads immunostained for SYCP3 (*green*), centromeres (using CREST autoimmune serum, *green*), and MLH1 (*red*) at leptonema (LEPT), pachynema (PACH), and diplonema (DIPL). The structure of the synaptonemal complex (SC), which is stained with SYCP3 antiserum in this figure, changes during the progression of prophase I. (**a**) The components of SC start to accumulate on the chromosome core during leptonema, shown as fragmented SYCP3 signals. (**b**) In pachynema, the homologous chromosomes are fully synapsed illustrated by associated SYCP3 at full length. MLH1, a key marker of the homologous recombination site, is detected in this stage, shown as *red/orange* foci on the chromosome core. (**c**) Homologous chromosomes start to desynapse during diplonema. MLH1 foci (*yellow dots* indicated by *arrow*) are gradually lost from the crossing over site. SYCP3 is represented by the *green* signal in (**a**–**c**). The centromeres are detected by CREST autoimmune serum (*blue* in all three).

5. Move oocytes to hypotonic solution and incubate for 15 min at room temperature.
6. Use a fine hand-pulled micropipette and transfer a small drop (1–2 μl) of hypotonic solution onto the upper surface of the slides with markers underneath.
7. Transfer a few oocytes from hypotonic solution to the drop on the slides. Remove excess fluid from the drop until the oocytes appear to be attached to the slides (see Note 14).
8. Add one drop of Carnoy's fixative onto the top of the oocytes without dispersing them. Pause briefly to let the fixative disperse and quickly add another two drops of fixative (see Note 15, Fig. 4).
9. Allow the slides to air-dry. The spreading of chromosome will be achieved during this drying process (see Note 16).
10. Stain the slides with Giemsa solution for 3 min. Wash the slides with water for three times, 3 min each.
11. Air-dry completely. Load mounting medium and put on coverslip carefully to avoid air bubbles. When the slide is dried, the oocytes can be visualized under the microscope.

3.3. Oocyte Spindle Preparation

This method is designed to observe the metaphase spindle configuration in oocytes following meiotic resumption. The three-dimensional structure of spindles and the developing oocytes are

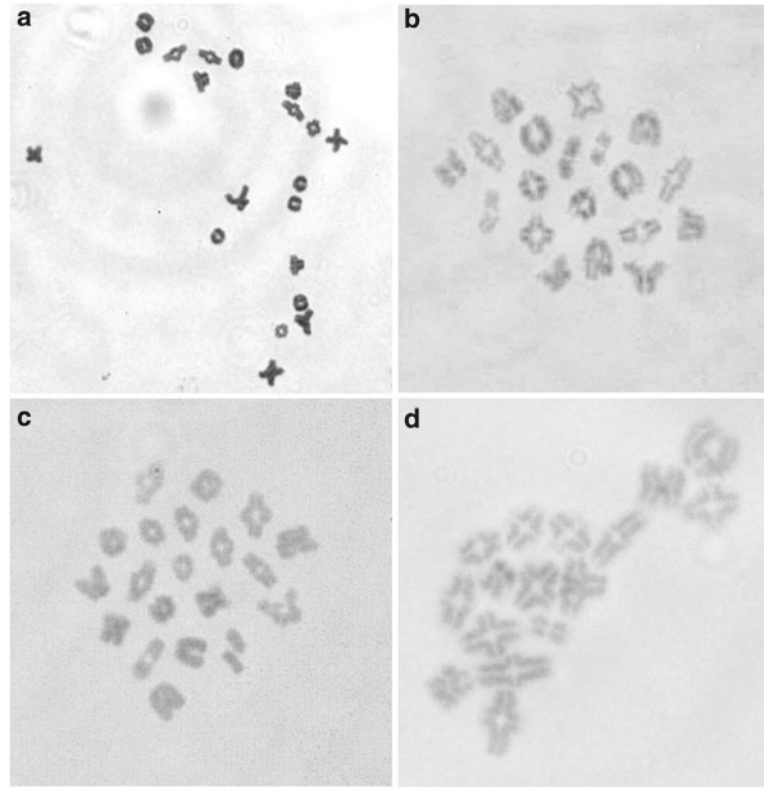


Fig. 4. Giemsa-stained chiasmata preparation of diakinesis mouse oocytes. Shown are giemsa-stained chiasmata preparations of wild type mouse oocytes under different evaporation conditions. The density of the chromosomes will vary widely responding to subtle changes of the local humidity level. The slower the fixative evaporates, the more condense the chromosomes appear. When the evaporation is sped up, the chromosomes tend to be more spread out. (**a**–**d**) Oocytes at diakinesis stage are fixed under slow to higher evaporation conditions.

well preserved using this technique, which makes the temporal analysis of chromosome behavior during this process possible (21) (see Fig. 5).

1. Follow steps 1–4 in Subheading 3.2 to collect oocytes from ovaries of 24–28-day-old mice and culture them in KSOM medium until the oocytes reach metaphase I or metaphase II (see Note 13).
2. Place four to six 2 μl drops of fibrinogen solution within each rubber cement well on the poly-lysine coated slides and cover with oil. Incubate the slides at 37°C for 10 min.
3. Transfer five to ten oocytes to each fibrinogen drop (see Note 17).
4. Add 2 μl thrombin to the fibrinogen drop using fine hand-pulled micropipette (see Note 18).

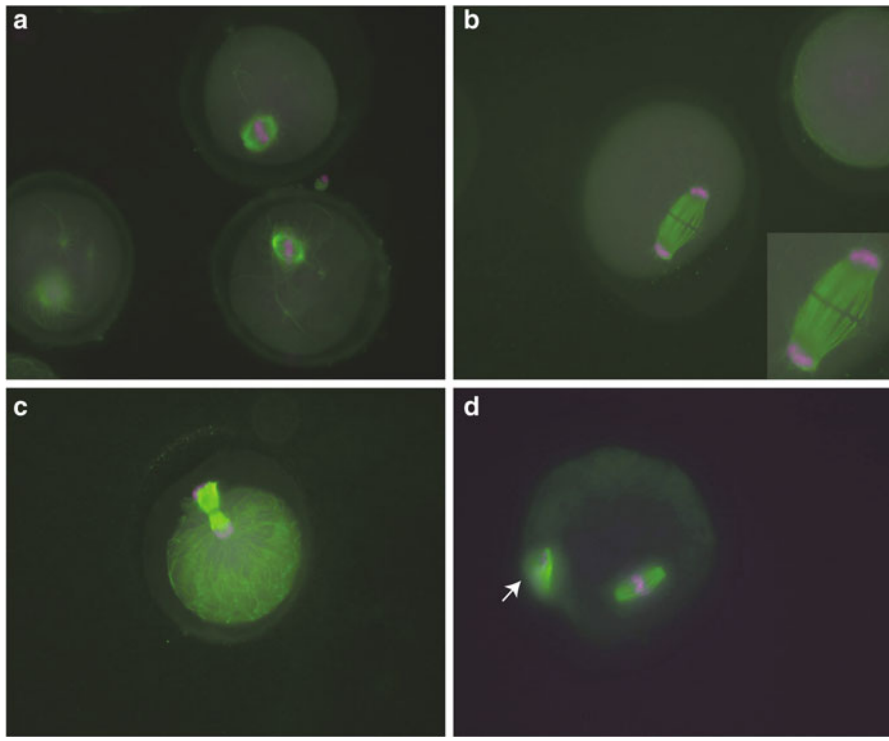


Fig. 5. Confocal images of wild type mouse oocytes from spindle preparation with immunofluorescence staining of β-tubulin. Shown are oocyte spindle preparations immunostained for β-tubulin (*green*) and DNA (*magenta*). The transition from metaphase I to metaphase II can be observed by culturing the mouse oocytes and then fixing them at different time points after the resumption of meiosis. (**a**) 7 h after germinal vesicle break down (GVBD), the oocytes are in early metaphase I with chromosomes align on the spindle equator. (**b**) An oocyte with a characteristic anaphase I configuration with chromosomes on the opposite poles of the spindle is shown. (**c**) The first polar body is being extruded out in telophase I. (**d**) The oocytes get arrested again in metaphase II with the first polar body (*arrow*). Spindle is visualized by β-tubulin staining (*green*); chromosomes are detected by DAPI (*magenta*).

5. Keep the slides at 37°C for 1 min and check the formation of the clot.
6. Carefully drain off oil and rinse the slide well in PBS containing 2% Triton X-100 for 3 min.
7. Put the slide in Spindle Fixative solution for 30 min in a 37°C incubator.
8. Wash in 0.1% NGS for 15 min at 37°C.
9. Transfer the slide to 10% NGS at 37°C for 1 h. Alternatively, the slide can be stored at 4°C in 10% NGS overnight.
10. Dilute mouse anti-β-tubulin antibody (1:800) in 10% NGS (see Note 19). Add the diluted antibody to the slides and incubate at 37°C for 1 h.
11. Wash the slides in 10% NGS for 10 min at 37°C. Repeat the washing two more times.

12. Apply diluted secondary antibody in 10% NGS (1:1,000) to the slides for 1 h at 37°C.
13. Wash the slide in 10% NGS for 10 min at 37°C. Repeat the washing two more times.
14. Briefly immerse the slide in ddH_2O containing 0.4% Photoflo.
15. Carefully remove the rubber cement well around the clots.
16. Mount antifade with DAPI to the slide and put on coverslip. Blot the excess mounting medium and seal with rubber cement.
17. Dry in the dark for at least 2 h at room temperature or overnight at 4°C before analyzing under fluorescence microscope.

4. Notes

1. These slides can be reused by washing them well with soapy water, followed by 70% ethanol and distilled water consecutively. Let the slides dry in the air and store them in a clean box.
2. The HEB, hypotonic sucrose solution, and 1% PFA should be made fresh within 2 h of use and maintain on ice.
3. If anti-goat primary antibody will be used later, replace NGS with serum from the same species in which the secondary antibodies are raised (e.g., donkey or chicken serum).
4. Notice: PIPES will not dissolve until pH reaches 6.1–7.5. Add solid NaOH a few pellets a time until dissolve.
5. Plug date is not always an accurate indicator of gestational age. The actual date may vary ±0.5 day.
6. If mice are of a specific genotype, keep each pair of ovaries separate from those of other embryos in the litter. If tissue is required for genotyping, obtain this from inside the peritoneal cavity of the embryo because the outside surface of each embryo will be contaminated with maternal tissue/fluids.
7. The slides can be stored in a −80°C freezer up to 1 week. Since some epitopes are not very stable, immediate staining of the slides is highly recommended.
8. Antibodies from different vendors or different batches may vary significantly. The dilution of antibodies may require adaptation from time to time.
9. It is ESSENTIAL not to let the slides dry out in this step. So 80 μl is the minimum amount you should use. If in doubt, use more (up to 120 μl or so).

10. It is very important to press the coverslip down firmly at several points and to "wiggle" the coverslip gently across the surface of the slide. This helps to flatten the coverslip on the slide, prevents uneven build-up of antifade, and removes excess antifade.
11. Although one should be able to collect enough oocytes from female mice at this age, stimulation by exogenous hormone can also be used to increase the yield of the eggs harvested. Intraperitoneal injection of two hormones will be needed in this procedure: pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). These two hormones are used to mimic the actions of FSH and LH, respectively. The injection timing is dependent on the light/dark cycle of the mouse room. For example, in a room following a 7 am to 7 pm light cycle, inject 5 IU PMSG to each female in the late afternoon on day 1. After 46–48 h, inject each animal with 5 IU hCG (day 3). Oocytes are supposed to be ovulated 10–13 h after hCG injection (day 4). Note that oocytes obtained by superovulation are often more difficult to handle and more susceptible to damage. To increase the efficiency of the hormonal regimen, always use mice as young as possible (3 weeks of age is optimal).
12. Move the oocytes through two to three 20 μl KSOM drops to dilute the somatic cells transferred with oocytes. Each 20 μl KSOM drop can hold up to 30 oocytes.
13. The cell cycle progression may vary among different mouse strains. For the most commonly used C57B6 strain, it usually takes about 2.5 h for the oocytes to loose their germinal vesicle, which is a sign of meiotic resumption. 8–10 h after break down of germinal vesicle, most oocytes will in metaphase I. For observation of oocytes at metaphase II, overnight culture (16–18 h) can be used.
14. It is critical to have the right amount of fluid with the oocytes. If too much fluid remains, the oocytes will not adhere onto the surface of the slide and easily get washed away later by fixative. Meanwhile, it is also necessary to have enough fluid with the oocytes to prevent drying before fixative is applied.
15. Three drops of freshly prepared fixative are recommended for most conditions. However, the total number of the drops added to the oocytes also depends on the humidity condition of the room where the experiment is performed (see Fig. 4). Thus, the optimal amount of the fixative used for a particular experiment needs to be tested individually.
16. The chromosomes can be observed as light reflecting spots under dissecting microscope during drying but become much less visible when the slide is totally dry. So it will be helpful for

later visualization to remember the location of the oocytes using markers underneath the slides before the fixative on the slide is completely dried.

17. If desired, wash the oocytes through two to three fibrinogen drops. This could help a lot in later staining step since extra somatic cells will increase background staining.
18. Inject thrombin into fibrinogen drop slowly and evenly throughout the drop from different directions. Don't leave the tip of glass pipette with thrombin in the same spot for too long. The tip may get stuck!
19. Depending on the batch and freshness of this antibody, the dilution factor may vary from 200 to 1,000 in our hands.

Acknowledgements

This work was supported by funding from the NICHD to P.E.C. (HD041012) and by a student fellowship from the Cornell Center for Vertebrate Genomics to X.S. We thank members of the Cohen lab for their helpful comments and suggestions during the preparation of this manuscript.

References

1. Hassold T, Hunt P (2001) To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet 2:280–291
2. Hunt PA, Hassold TJ (2008) Human female meiosis: what makes a good egg go bad? Trends Genet 24:86–93
3. Page SL, Hawley RS (2004) The genetics and molecular biology of the synaptonemal complex. Annu Rev Cell Dev Biol 20:525–558
4. Morelli MA, Cohen PE (2005) Not all germ cells are created equal: aspects of sexual dimorphism in mammalian meiosis. Reproduction 130:761–781
5. Hassold T, Hall H, Hunt P (2007) The origin of human aneuploidy: where we have been, where we are going. Hum Mol Genet 16 Spec No. 2: R203–R208
6. Cohen PE, Holloway JK (2010) Predicting gene networks in human oocyte meiosis. Biol Reprod 82:469–472
7. Hulten MA, Patel S, Jonasson J, Iwarsson E (2010) On the origin of the maternal age effect in trisomy 21 Down syndrome: the Oocyte Mosaicism Selection model. Reproduction 139:1–9
8. Lenzi ML, Smith J, Snowden T, Kim M, Fishel R, Poulos BK, Cohen PE (2005) Extreme heterogeneity in the molecular events leading to the establishment of chiasmata during meiosis I in human oocytes. Am J Hum Genet 76: 112–127
9. Yuan L, Liu JG, Zhao J, Brundell E, Daneholt B, Hoog C (2000) The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. Mol Cell 5:73–83
10. Yuan L, Liu JG, Hoja MR, Wilbertz J, Nordqvist K, Hoog C (2002) Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. Science 296:1115–1118
11. Hamer G, Novak I, Kouznetsova A, Hoog C (2008) Disruption of pairing and synapsis of chromosomes causes stage-specific apoptosis of male meiotic cells. Theriogenology 69:333–339
12. Pelttari J, Hoja MR, Yuan L, Liu JG, Brundell E, Moens P, Santucci-Darmanin S, Jessberger R, Barbero JL, Heyting C, Hoog C (2001) A meiotic chromosomal core consisting of cohesin complex proteins recruits DNA recombination

proteins and promotes synapsis in the absence of an axial element in mammalian meiotic cells. Mol Cell Biol 21:5667–5677

13. Borum K (1961) Oogenesis in the mouse. Exp Cell Res 24:495–507
14. Evans CW, Robb DI, Tuckett F, Chanlloner S (1982) Regulation of meiosis in the foetal mouse gonad. J Embryol Exp Morphol 68: 59–67
15. Kan R, Sun X, Kolas NK, Avdievich E, Kneitz B, Edelmann W, Cohen PE (2008) Comparative analysis of meiotic progression in female mice bearing mutations in genes of the DNA mismatch repair pathway. Biol Reprod 78: 462–471
16. Kolas NK, Svetlanov A, Lenzi ML, Macaluso FP, Lipkin SM, Liskay RM, Greally J, Edelmann W, Cohen PE (2005) Localization of MMR proteins on meiotic chromosomes in mice indicates distinct functions during prophase I. J Cell Biol 171:447–458
17. Wojtasz L, Daniel K, Roig I, Bolcun-Filas E, Xu H, Boonsanay V, Eckmann CR, Cooke HJ, Jasin M, Keeney S, McKay MJ, Toth A (2009) Mouse HORMAD1 and HORMAD2, two conserved meiotic chromosomal proteins, are depleted from synapsed chromosome axes with the help of TRIP13 AAA-ATPase. PLoS Genet 5:e1000702
18. Bolcun-Filas E, Bannister LA, Barash A, Schimenti KJ, Hartford SA, Eppig JJ, Handel MA, Shen L, Schimenti JC (2011) A-MYB (MYBL1) transcription factor is a master regulator of male meiosis. Development 138:3319–3330
19. Williams DL, Lafferty DA, Webb SL (1970) An air drying method for the preparation of dictyotene chromosomes from ovaries of Chinese hamsters. Stain Technol 45:133–135
20. Tarkowski AK (1966) An air-drying method for chromosome preparations from mouse eggs. Cytogenetics 5:394–400
21. Woods LM, Hodges CA, Baart E, Baker SM, Liskay RM, Hunt PA (1999) Chromosomal influence on meiotic spindle assembly: abnormal meiosis I in female Mlh1 mutant mice. J Cell Biol 145:1395–1406