Video Article

Using Fluorescence *In Situ* Hybridization (FISH) to Monitor the State of Arm Cohesion in Prometaphase and Metaphase I *Drosophila* Oocytes

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Abstract

In humans, chromosome segregation errors in oocytes are responsible for the majority of miscarriages and birth defects. Moreover, as women age, their risk of conceiving an aneuploid fetus increases dramatically and this phenomenon is known as the maternal age effect. One requirement for accurate chromosome segregation during the meiotic divisions is maintenance of sister chromatid cohesion during the extended prophase period that oocytes experience. Cytological evidence in both humans and model organisms suggests that meiotic cohesion deteriorates during the aging process. In addition, segregation errors in human oocytes are most prevalent during meiosis I, consistent with premature loss of arm cohesion. The use of model organisms is critical for unraveling the mechanisms that underlie age-dependent loss of cohesion. *Drosophila melanogaster* offers several advantages for studying the regulation of meiotic cohesion in oocytes. However, until recently, only genetic tests were available to assay for loss of arm cohesion in oocytes of different genotypes or under different experimental conditions. Here, a detailed protocol is provided for using fluorescence *in situ* hybridization (FISH) to directly visualize defects in arm cohesion in prometaphase I and metaphase I arrested *Drosophila* oocytes. By generating a FISH probe that hybridizes to the distal arm of the *X* chromosome and collecting confocal Z stacks, a researcher can visualize the number of individual FISH signals in three dimensions and determine whether sister chromatid arms are separated. The procedure outlined makes it possible to quantify arm cohesion defects in hundreds of *Drosophila* oocytes. As such, this method provides an important tool for investigating the mechanisms that contribute to cohesion maintenance as well as the factors that lead to its demise during the aging process.

Video Link

The video component of this article can be found at https://www.jove.com/video/56802/

Introduction

Proper segregation of chromosomes during mitosis and meiosis requires that sister chromatid cohesion be established, maintained, and released in a coordinated fashion[1,2]. Cohesion is established during S phase and is mediated by the cohesin complex, which forms physical linkages that hold the sister chromatids together. In meiosis, cohesion distal to a crossover also functions to hold recombinant homologs together and this physical association helps ensure proper orientation of the bivalent on the metaphase I spindle (**Figure 1**)[3,4,5]. Release of arm cohesion at anaphase I allows the homologs to segregate to opposite spindle poles. However, if arm cohesion is lost prematurely, recombinant homologs will lose their physical connection and segregate randomly, which can result in aneuploid gametes (**Figure 1**).

In human oocytes, errors in chromosome segregation are the leading cause of miscarriages and birth defects, such as Down Syndrome[6], and their incidence increases exponentially with maternal age[7]. Sister chromatid cohesion is established in fetal oocytes and meiotic recombination is completed before birth. Oocytes then arrest in mid-prophase I until ovulation and during this arrest, the continued physical association of recombinant homologs relies on sister chromatid cohesion. Therefore, accurate segregation during meiosis and normal pregnancy outcomes require that cohesion remain intact for up to five decades.

Premature loss of cohesion during the prolonged meiotic arrest of human oocytes has been proposed to contribute to the maternal age effect and multiple lines of evidence support this hypothesis[8,9]. However, given the challenges of studying meiotic cohesion in human oocytes, much of our understanding of this phenomenon relies on the use of model organisms[5,10,11,12,13,14,15].

Drosophila melanogaster oocytes offer numerous advantages for the study of meiotic cohesion and chromosome segregation. A simple genetic assay allows one to recover progeny from aneuploid gametes and measure the fidelity of *X*-chromosome segregation on a large scale[11,16,17]. Moreover, one may also determine whether chromosome segregation errors arise because recombinant homologs missegregate during meiosis I, a phenotype that is consistent with premature loss of arm cohesion[11,18,19]. Direct observation of the state of meiotic cohesion in *Drosophila* oocytes is also possible using fluorescence *in situ* hybridization (FISH). Although fluorescent oligonucleotides that hybridize to repetitive satellite sequences have been used for over a decade to monitor pericentromeric cohesion in mature *Drosophila* oocytes[4,20], analysis of arm cohesion has been much more challenging. Visualization of the state of arm cohesion requires a probe that spans a large region of single copy sequences

and is bright enough to result in visible signals for individual sister chromatids when arm cohesion is absent. In addition, the oocyte fixation conditions and size of the labeled DNAs must facilitate penetration[21] into the large mature *Drosophila* oocyte (200 µm wide by 500 µm long). Recently, an arm probe was successfully utilized to visualize *Drosophila* oocyte chromatids during anaphase I, but the authors stated that they could not detect a signal in prometaphase or metaphase I arrested oocytes[22]. Here we provide a detailed protocol for the generation of *X*-chromosome arm FISH probes and oocyte preparation conditions that have allowed us to assay for premature loss of sister chromatid cohesion in prometaphase I and metaphase I oocytes. These techniques, which have enabled us to identify gene products that are required for the maintenance of meiotic cohesion, will allow others to assay for sister chromatid cohesion defects in mature *Drosophila* oocytes of different genotypes.

Protocol

1. Preparations

1. **Prepare solutions for fluorescence *in situ* hybridization (FISH). Prepare all solutions using ultrapure water.**
    1. Prepare 5x Modified Robb's buffer: 275 mM potassium acetate, 200 mM sodium acetate, 500 mM sucrose, 50 mM glucose, 6 mM magnesium chloride, 5 mM calcium chloride, 500 mM HEPES pH = 7.4. Bring the pH to 7.4 using 10 N NaOH. Filter sterilize and store at -20 °C. Thaw and dilute to 1x as needed and store 1x aliquots at -20 °C.
    2. Prepare 10x Phosphate buffered saline (PBS) using 1.3 M NaCl, 70 mM Na_2HPO_4, 30 mM NaH_2PO_4. Bring pH to 7.0 using 10 N NaOH. Sterilize by autoclaving or filter sterilization.

2. **Prepare poly-L-Lysine coverslips one day before needed.**
    1. Pipette 70% ethanol containing 1% hydrochloric acid onto the surface of an 18 mm x 18 mm #1.5 coverslip and incubate 5 min. Use vacuum aspiration to remove liquid. Repeat with sterile ultrapure water. Cover surface of coverslip with 0.1 mg/mL poly-L-lysine and incubate for 10 min.
    2. Aspirate to remove liquid and air dry for 10 min. Store coverslips poly-L-lysine side up in a plastic container with a tight-fitting lid to avoid dust.

3. Prepare pulled Pasteur pipettes to remove liquid solutions without removing the oocytes. Over a Bunsen burner flame, heat the middle of a long glass Pasteur pipette while gently pulling on each end until the glass melts and pulls apart.

2. Generation of Arm Probe for FISH

NOTE: All centrifuge steps are performed at ~16,000 - 21,000 x g (maximum speed on most table top microcentrifuges). Brief centrifuge spins indicate spinning for 5 - 15 s. Vortex indicates vortexing for ~15 s at max speed unless otherwise noted.

NOTE: BACs for arm probes can be obtained from BAC PAC Resources. Two *X* chromosome euchromatic arm probes have been used successfully with this method. One arm probe was composed of six BAC clones corresponding to cytological bands 6E-7B (BACR17C09, BACR06J12, BACR35J16, BACR20K01, BACR35A18, BACR26L11). The other arm probe consisted of six BAC clones corresponding to cytological bands 2F-3C (BACR13K19, BACR21G11, BACR09H13, BACR30B01, BACR34O03, BACR03D13). BACs to other *Drosophila* chromosome regions may be browsed at: http://www.fruitfly.org/sequence/X1-11-assembly.html. Two pericentric probes that recognize the 359 bp satellite repeat of the *X* chromosome have been used successfully with this method. A 22 nucleotide probe has been used extensively and works well (5'-Cy3-AGGGATCGTTAGCACTCGTAAT)[19,23]. A 28 nucleotide probe was recently tested and also worked well (5'-Cy3-GGGATCGTTAGCACTGGTAATTAGCTGC)[24]. HPLC purified oligonucleotides 5' labeled with a specific fluorophore were ordered from a commercial source (*e.g.*, Integrated DNA Technologies).

1. Prep BAC clone DNA following kit instructions as specified in the **Table of Materials**. Resuspend the DNA pellet obtained from 100 mL of culture in 200 µL TE; the final DNA concentration should range between 5 - 40 ng/µL depending on the BAC clone.
2. **Perform DNA amplification for each BAC using the amplification kit, as specified in the table of materials, and the following protocol. Process BAC DNA for each clone individually. Use enzymes and buffers supplied with the kit in steps 2.2.1 through 2.2.3.**
    1. Random fragmentation of BAC DNA: For each BAC, use TE to prepare 10 µL of a 1 ng/µL DNA solution in a 200 µL PCR tube. Add 1 µL of 10X Fragmentation Buffer. Place the tube in a PCR machine at 95 °C for exactly 4 min. Immediately cool the sample in ice water and centrifuge briefly. The incubation is time sensitive and any deviation may alter results.
    2. Library preparation
    NOTE: This method uses random primers to generate a library of amplifiable fragments.
        1. To the tube containing the DNA add the following: 2 µL of 1x Library Preparation Buffer, 1 µL of Library Stabilization Solution. Mix thoroughly by vortexing, centrifuge briefly, and place the tube in the PCR machine at 95 °C for 2 min. Immediately cool the sample in ice water and centrifuge briefly.
        2. Add 1 µL of Library Preparation Enzyme to the PCR tube, mix and centrifuge briefly. Place PCR tube in the PCR machine and incubate as follows: 16 °C for 20 min, 24 °C for 20 min, 37 °C for 20 min, 75 °C for 5 min, then hold at 4 °C.
        3. Remove samples from the PCR machine and centrifuge briefly. Samples may be amplified immediately or stored at -20 °C for up to three days.
    3. Amplification of BAC DNA Library
        1. Add the following reagents to the entire 15 µL random fragment reaction: 7.5 µL 10x Amplification Master Mix, 47.5 µL Nuclease Free Water, 5 µL WGA DNA Polymerase. Mix thoroughly by vortexing and centrifuge briefly.
        2. Place PCR tube in the PCR machine and incubate as follows: Initial Denaturation 95 °C for 3 min, 14 cycles of denaturation at 94 °C for 15 s, followed by annealing/extension at 65 °C for 5 min, then hold at 4 °C.

3. After cycling is complete, assay the DNA concentration. The concentration of DNA should be at least 800 ng/µL. Keep samples at 4 °C or store at -20 °C until ready for digestion. Optionally, assess DNA fragment size by running 400 ng of DNA on a 2% agarose gel. Fragments should range from 200 bp to 3 kb (**Figure 2**).

3. **Restriction enzyme digestion of amplified DNA**
    1. Place 20 µg of amplified DNA from the previous section in a 1.5 mL microfuge tube. Add 20 units of AluI, HaeIII, MseI, MspI, RsaI, 25 units of BfuCI, 0.5 µL 100x BSA, 5 µL 10x restriction enzyme digestion buffer, and adjust volume to 50 µL by adding the appropriate amount of ultrapure H_2O.
    2. Incubate the digest overnight at 37 °C in an incubator to prevent condensation on the lid. Ethanol precipitate the digested DNA. Add to the digest: 1 µL glycogen, 1/10 volume 3M sodium acetate, 2.5 volumes 100% molecular grade ethanol. Incubate at -80 °C for 1 h or overnight.
    3. Centrifuge for 30 min at 4 °C. Wash DNA pellet with 1 volume of room temp 70% ethanol and spin for 5 min at 4 °C. Remove the supernatant and let the DNA pellet air dry or gently dry with a steady stream of air.
    4. Resuspend the DNA pellet in 36 µL sterile ultrapure H_2O and use 1 µL of DNA to assay the DNA concentration, which should be approximately 285 ng/µL. Store the DNA at -20 °C.
    5. Optionally assess DNA fragment size running 400 ng of DNA on a 2% agarose gel. Most fragments should range from 100 - 200 bp, with a fainter signal up to 500 bp (**Figure 2**).

4. **3' tailing reaction**
    1. Denature the digested DNA at 100 °C for 1 min in a heat block. Immediately chill in ice water. To the 35 µL of DNA, add the following: 50 µL 400 mM sodium cacodylate, 1 µL 10 mM DTT, and vortex well. Add 4 µL 25 mM $CoCl_2$, 2.5 µL 2 mM 5-(3-aminoallyl)-dUTP from ARES labeling kit as specified in the table of materials, 5 µL 1mM dTTP, 18 µL 5x TdT buffer, and 1 µL terminal deoxynucleotidyl transferase (TdT) 400 U/µL. Vortex and centrifuge briefly.
    CAUTION: $CoCl_2$ is toxic, wear appropriate protection.
    2. Incubate for 2 h at 37 °C in an incubator to prevent condensation. Add 1 µL of 250 mM EDTA to stop the reaction and vortex briefly to mix. Ethanol precipitate the tailed DNA as described above (steps 2.3.2 - 2.3.4). Resuspend the dried DNA pellet in 10 µL sterile ultrapure H_2O. Store the DNA at -20 °C until ready to continue.

5. **Conduct fluorescent dye conjugation using the DNA labeling kit as specified in the Materials Table.**
NOTE: Once dye is added keep samples in the dark.
    1. Denature the tailed DNA at 95 °C for 5 min in a heat block. Immediately cool DNA in ice water and centrifuge briefly. Prepare labeling buffer according to the kit instructions and add 6 µL to each DNA sample. Resuspend each tube of dye in 4 µL of DMSO from the kit. Vortex and centrifuge briefly.
    2. Use one tube of dye for each labeling reaction. Add the dye solution to the DNA, vortex, and centrifuge briefly. Incubate the labeling reaction at room temperature for 2 h in the dark. Add 3 µL of 3M hydroxylamine to stop the reaction followed by 77 µL of the nuclease-free H_2O from the kit. Vortex and centrifuge briefly.

6. Remove non-conjugated dye using the PCR purification kit as specified in the table of materials. Follow manufacturer's instructions. Elute DNA from the column two times using 40 µL of elution buffer each time.
7. Ethanol precipitate the labeled DNA as described previously (steps 2.3.2 - 2.3.4). Resuspend the dried DNA pellet in 10 µL elution buffer.
8. **Prepare Probe Mixture**
    1. For labeled DNA, determine the concentration of the fluorochrome dye in pmol/µL. The fluorophore concentration may range from 30 - 100 pmol/µL, depending on the BAC. The DNA concentration may range from 300 - 800 ng/µL.
    NOTE: The microarray setting works well on a microvolume spectrophotometer, such as that specified in the table of materials. In the microarray window, select DNA-50, and specify the fluorochrome.
    2. Create a master mix by combining equimolar amounts (pmol fluor) of each of the BAC probes. Vortex 30 s before combining. To avoid freeze/thaw cycles, prepare aliquots of the master mix, apply paraffin film to the tubes to prevent evaporation, and store in the dark at -20 °C.
    NOTE: A mastermix containing 250 pmol of each of the 6 individual BAC probes in a total volume of 30 - 50 µL works well. An aliquot containing 25 pmol (fluor) for each BAC will be sufficient for two 40 µL hybridization reactions, with a final probe concentration of 0.31 pmol fluor/µL for each BAC.

3. Dissection and Fixation of Oocytes

1. Collect 30 adult virgin females of the appropriate genotype. To enrich for late stage oocytes, hold females without males in a vial with fly food and dry yeast for 3 - 5 days before dissection[25]. The day before dissection, transfer the females without gas to a fresh vial with yeast.
2. **Perform the dissection.**
NOTE: See **Figure 3** for tools needed. Solutions are removed using a pulled Pasteur pipette unless otherwise noted. When transferring ovaries always use a pipette tip coated with a 10% BSA solution.
    1. In a shallow dissecting dish containing Modified Robb's buffer, use forceps to remove the ovaries from a single female. Use forceps or a tungsten needle to gently splay each ovary, allowing the solution to contact inner ovarioles. Transfer the pair of splayed ovaries to a 1.5 mL microfuge tube containing 1 mL Modified Robb's buffer.
    2. Repeat step 3.2.1 to accumulate ovaries from 20 - 25 females in the 1.5 mL microfuge tube. Finish dissections of the 20 - 25 females within 10 min.

3. **Perform fixation as follows.**
    1. With a pulled Pasteur pipette, remove the liquid in the microfuge tube, use a P1000 to quickly add 500 µL of 37 °C fix solution to the ovaries, and then immediately add 500 µL of room temperature heptane to the ovaries.

2. Shake microfuge tube to mix and place on a nutator for 3 min. Remove the microfuge tube from the nutator and place in a tube rack for 1 min to allow the oocytes to settle to the bottom. The total fixation time is 4 min.
   Caution: Fix solution and heptane are toxic, wear appropriate protection.
3. Remove the fix solution and rinse the ovaries 3 times in 500 µL of PBS containing 0.5% BSA and 0.1% Triton X-100 (PBSBTx)

4. Repeat for remaining genotypes.

4. Removal of Chorions and Vitelline Membranes

NOTE: See **Figure 3** for tools needed.

1. **Separate late stage oocytes**
   1. Add 1 mL PBSBTx to a shallow dissecting dish. Use a P200 with a BSA coated tip to transfer fixed ovaries (in ~150 µL) into the shallow dish. Pipette ovaries up and down with the BSA coated pipette tip to dislodge the mature oocytes from the less mature oocytes.
   2. When late stage oocytes are sufficiently separated, transfer all the tissue to a 500 µL microfuge tube. Remove excess liquid with a pulled Pasteur pipette, leaving about 150 - 200 µL in the tube. Repeat section 4.1 for remaining genotypes.

2. **Prepare for rolling**
   1. Pre-wet a deep well dish with 200 µL of PBSBTx. Cover and set aside. Obtain 3 frosted glass slides and set slide #3 aside. Gently rub the frosted glass regions of slides #1 and #2 together. Rinse them in deionized water to remove any glass shards and dry with a disposable wipe.
   2. Coat the frosted regions of slides #1 and #2 with PBSBTx by adding 50 µL of PBSBTx to one slide and rubbing this region with the other slide. Remove liquid with a disposable wipe.
   3. Place the slides under a dissecting microscope in the configuration shown in **Figure 4A**. Keep the frosted regions of slides #1 and #2 in contact, with slide #3 supporting slide #2.

3. **Roll oocytes; ensure that the direction of rolling is always in a straight line and never a circular motion.**
   1. Prewet a P200 pipette tip in PBSBTx and disperse the oocytes in the microfuge tube by pipetting up and down. Transfer 50 µL of liquid containing oocytes to the center of the frosted glass part of slide #1. Lift slide #2 to do this.
   2. Slowly lower slide #2 until the surface tension of the liquid creates a seal between the two frosted glass regions. There should be enough liquid to cover the frosted area but none should be seeping out. If liquid is overflowing, use a pulled Pasteur pipette to remove excess liquid.
   3. Hold the bottom slide (#1) in place with one hand and use the other hand to move the top slide (#2) back and forth in a horizontal direction, keeping slide #2 level and supported on slide #3. Perform under a microscope for easy visualization of oocyte movements and progress.
      NOTE: This movement will generate friction and cause the oocytes to "roll" and lose their chorions. Minimal pressure should be used and movements should be short and quick.
   4. After a few movements in the horizontal direction, slightly change the angle of movement (**Figure 4B**). In multiple increments, gradually increase this angle to 90° until movement of the top slide (#2) is perpendicular to the starting direction (**Figure 4B**). Note that empty chorions will be visible in the liquid and oocytes lacking chorions will appear longer and thinner.
   5. Repeat steps 4.3.3 - 4.3.4 about 7 - 10 times until the solution becomes slightly cloudy. Stop rolling when the majority of oocytes (75 - 85%) appear to have lost their vitelline membranes.
      NOTE: A distinctive pointed end is often visible on oocytes lacking vitelline membranes. Vitelline membranes are more difficult to remove than chorions. Light pressure may be applied to the top slide (slide #2) while rolling to achieve removal of vitelline membranes. Trying to remove vitelline membranes from all the oocytes often results in destruction of other oocytes.
   6. Gently lift the top slide (#2), dragging one of its corners to the center of the frosted region of the bottom slide (#1) so that rolled oocytes accumulate in the center of the frosted region. Rinse oocytes from both slides with PBSBTx into the deep well dish containing PBSBTx.
   7. Clean slides #1 and #2 with ultrapure water, dry with a disposable wipe, and reset. Repeat steps 4.3.1 - 4.3.6 until all the oocytes of the same genotype have been rolled. This usually requires 3 - 4 rounds of rolling per genotype.

4. **Remove debris after rolling**
   1. Add 1 mL PBSBTx to a 15 mL conical tube. Swirl the liquid to coat the sides of the tube.
   2. Using a PBSBTx coated P1000 pipette tip, transfer the rolled oocytes from the deep well dish to the conical tube containing 1 mL of PBSBTx. Add an additional 2 mL of PBSBTx to the conical tube containing the oocytes.
   3. Let the oocytes begin to settle to the bottom, then remove the top 2 mL of solution containing debris (chorions, vitellines, *etc.*) with a P1000 and discard. If needed, hold the conical tube against a dark background to see the opaque oocytes as they sink.
   4. Add an additional 2 mL of PBSBTx to the oocytes, and repeat step 4.4.3. Repeat 4.4.3. for a total of 3 rounds of debris removal.
   5. Using a PBSBTx coated P1000 pipette tip, transfer oocytes back to the original 500 µL microfuge tube. 20 - 25 females should yield approximately 50 µL of rolled mature oocytes.

5. Repeat section 4 for the remaining genotypes using fresh frosted slides, a clean deep well dish, and a new conical tube for each genotype.
6. If storage is necessary, transfer oocytes to 1x PBS with 0.1% TritionX-100 and store overnight at 4 °C. Long-term storage is not recommended because formaldehyde crosslinking can be reversed by non-ionic detergents.

5. FISH

NOTE: All washes are performed on a nutator at room temperature unless otherwise noted. Oocytes that have been rolled take longer to settle to the bottom of the microfuge tube, especially in solutions that contain formamide. It is important to be patient when changing solutions so that

oocytes are not lost in the process. This may require waiting 5 - 15 min to let oocytes settle after rinses and washes. Also note that oocytes in formamide are less opaque.

1. **Extraction and RNAse treatment**
    1. Rinse oocytes with 500 µL of PBS containing 1% Triton X-100 (PBSTx). Remove the liquid and add 500 µL Extraction Buffer (PBSTx containing RNAse). Incubate on nutator at room temperature for 2 h.

2. **Pre-hybridization washes**
    1. Rinse oocytes 3 times with 500 µL 2x Saline Sodium Citrate containing Tween 20 (SSCT). Preheat an aliquot (~500 µL/genotype) of 2x SSCT + 50% formamide to 37 °C.
    Caution: formamide is toxic, wear appropriate protection.
    2. Wash oocytes 3 times for 10 min each in 500 µL 2x SSCT. Wash oocytes for 10 min in 500 µL 2x SSCT + 20% formamide. Wash oocytes for 10 min in 500 µL 2x SSCT + 40% formamide. Wash oocytes for 10 min in 500 µL 2x SSCT + 50% formamide.
    3. Remove liquid and add 500 µL of 37 °C 2x SSCT + 50% formamide from step 5.2.1 to sample and incubate for 2 h at 37 °C with rotation.
    NOTE: A hybridization oven equipped with a rotator works well for this. A foam microfuge tube "float" attached to the rotator can be used to secure the tubes.

3. **Denaturation and hybridization**
    1. For each tube of oocytes, use 40 µL of 1x hybridization buffer containing 2.5 ng/µL centromeric probe and 0.31 pmol fluor/µL of each BAC probe. Prepare a solution of probe in hybridization buffer sufficient for all the genotypes. Vortex probe mix 30 s before adding.
    2. Using a BSA-coated P200 pipette tip, transfer oocytes to a 200 µL PCR tube. Keep samples at 37 °C and let oocytes settle to the bottom, then use a pulled Pasteur pipette to remove as much liquid as possible.
    3. Add 40 µL of hybridization solution containing probe (prepared in section 2) to the oocytes. Place the PCR tube containing oocytes in the PCR machine and incubate as follows: 37 °C for 5 min, 92 °C for 3 min, then 37 °C overnight. After probe is added to the oocytes, keep them in the dark as much as possible.

4. **Post-hybridization washes**
    1. Preheat the 2x SSCT + 50% formamide to 37 °C and keep it in the hybridization incubator. With a BSA coated P200 pipette tip, add 100 µL of 37 °C 2x SSCT + 50% formamide to the PCR tube with the oocytes and transfer the oocytes to a new 500 µL microfuge tube.
    2. Add an additional 400 µL of 37 °C 2x SSCT + 50% formamide to the same tube and let the oocytes settle at 37 °C in the incubator.
    NOTE: Settling often takes longer at this step. It is not unusual to take 15 min or longer. A lack of patience will result in significant loss of oocytes.
    3. Wash the oocytes 3 times for 20 min each in 500 µL 37 °C 2x SSCT + 50% formamide at 37 °C with rotation. Keep oocytes at 37 °C while they settle.
    4. Wash the oocytes 3 times for 10 min each in 500 µL 37 °C 2x SSCT + 50% formamide at 37 °C with rotation. Keep oocytes at 37 °C while they settle.
    5. At room temperature, wash the oocytes for 10 min in 500 µL 2x SSCT + 40% formamide on a nutator. Wash the oocytes for 10 min in 500 µL 2x SSCT + 20% formamide. Wash the oocytes for 10 min in 500 µL 2x SSCT.

5. **Stain with DAPI**
CAUTION: DAPI is toxic, wear appropriate protection.
    1. Wash oocytes for 20 min in 500 µL DAPI solution (1 µg/ml) in 2x SSCT on the nutator. Rinse the oocytes 3 times in 500 µL 2x SSCT. Wash oocytes 2 times for 10 min each in 500 µL 2x SSCT on the nutator. Samples can be stored for up to 4 h at room temperature in the dark until they are ready to mount on coverslips.

6. **Mounting**
NOTE: See **Figure 3** for tools needed.
    1. Place a poly-L-lysine coated coverslip under the dissecting microscope. Remove liquid from the tube of oocytes, adjusting the total volume to about 150 µL. With a BSA coated P200 pipette tip, pipette up and down to disperse oocytes throughout the solution, and then transfer 40 µL of the oocytes/solution to a poly-L-lysine coated coverslip.
    2. Remove some of the liquid from the coverslip using a pulled Pasteur pipette until the oocytes stick to the coverslip but are still surrounded by liquid. With forceps, hold down the cover slip on one side and use a tungsten needle to gently dissociate clumps of oocytes, moving them to achieve a single layer of non-overlapping oocytes.
    3. Remove the remainder of the liquid around the oocytes with a pulled Pasteur pipette. Take a clean glass slide and use compressed gas to blow off any dust. Add 22 µL of mounting media (*e.g.*, Prolong-GOLD) to the middle of the slide. Slowly lower the slide towards the coverslip, with the mounting media facing the sample, until the media touches the sample. Surface tension will cause the coverslip to adhere to the slide.
    4. Place slides in a plastic container with a tight-fitting lid that contains a layer of fresh dessicant (*e.g.*, drierite). Let slides dry for 3 - 5 days in the dark before imaging. Drying time may vary depending on the humidity of the room.

6. Imaging

1. Upon removing dry slides from the box of dessicant, clean them to remove any dust particles. Seal coverslips with nail polish. Sealed slides may be stored indefinitely at -20 °C.
2. Acquire laser scanning confocal images using a high numerical aperture 40X oil objective with 6X digital zoom.
NOTE: Ideally, system software will allow one to find and mark the X-Y location of meiotic chromosomes for multiple oocytes while viewing them using epifluorescence. This capability saves time during an imaging session. Rapid bleaching with a high numerical aperture 60X objective made its use unsuitable with the chosen confocal system, but it may work for other systems.

3. Capture a Z series for each oocyte, setting the top and bottom of the series using the DAPI signal.
   NOTE: Gain, offset, and laser power will need to be determined empirically using a subset of oocytes. Once suitable parameters are found, only minor adjustments should need to be made.
   If altered acquisition settings are required, use the previously collected image stack to estimate the necessary changes. To avoid bleaching, refrain from pre-imaging the arm probe before capturing the Z series. With a step size of 0.25 µm, Z series typically range from 25 to 30 steps depending on the orientation and placement of the chromosomes. A smaller step size may improve one's ability to assess whether two FISH spots are separated in the axial dimension, but there is a trade-off with the time required to capture smaller steps and loss of signal intensity due to bleaching. A 40X objective with 6X digital zoom and a 1,024 x 512 image field generates images with a pixel size of 0.05 µm.

7. Image analysis and scoring for cohesion defects

1. Deconvolve the Z series to optimize the signal to noise ratio for each oocyte[19].
   NOTE: A software package such as the one specified in the **Materials Table** allows one to deconvolve using integrative restoration, as well as crop and contrast-enhance images. Importantly, a software package that allows one to view images and score for cohesion defects in three dimensions is imperative.
2. For each oocyte, tabulate the number of arm and centromeric foci that colocalize with the DAPI signal. Loss of cohesion results in three or four distinct and separated signals for a specific probe. It is not uncommon to observe two foci that are connected by a thin thread. By the most stringent criteria, these foci would not be considered "separated."

Representative Results

Figure 5 presents images obtained with an arm probe that hybridizes to cytological region 6E-7B on the *X* chromosome. This probe results in a signal that co-localizes with that of DAPI, is easily distinguishable from the background, and has been used successfully to quantify arm cohesion defects in different genotypes[19]. Quantification of cohesion defects was restricted to prometaphase I and metaphase I stages; oocytes prior to nuclear envelope breakdown were excluded from analysis[19]. An intact nuclear envelope is evident when scanning in the DAPI channel because the oocyte chromosomes will be surrounded by a discrete circular area that is darker than the surrounding cytoplasm.

Figure 5B shows maximum intensity projections of individual Z series after deconvolution and contrast enhancement. Quantification of cohesion defects requires determining for each probe the number of FISH signals that overlap with the DAPI signal. For such an analysis, visualization of the merged images in three dimensions is imperative. Only FISH spots that are clearly associated with the DAPI signal in all three dimensions should be included in the analysis.

Intact sister chromatid cohesion is evidenced as two FISH spots for both the arm and pericentric probes (**Figure 5B**, left). Oocytes containing three or four separated FISH spots for either probe are considered to have cohesion defects (**Figure 5B**, right). To be classified as individual spots, two FISH signals must be minimally separated in all three dimensions by a distance corresponding to the diameter of the smallest spot. Thin faint threads connecting two FISH spots are not uncommon. Such signals are not considered completely separated and therefore are not counted as instances of cohesion defects.

With the 6E-7B arm hybridization probe, approximately 15 - 20% of the oocytes imaged completely lacked signal or the signal was too weak to confidently score. In addition, roughly 5% of the oocytes imaged exhibited a large area of diffuse chromosomal signal and these were discarded from the analysis. In contrast, it was rare to encounter oocytes for which the pericentric hybridization signal was missing or too diffuse to score.

The 6E-7B arm probe has been used extensively to quantify arm cohesion defects in prometaphase and metaphase I arrested *Drosophila* oocytes of different genotypes[19]. When the cohesin subunit SMC3 was knocked down during mid-prophase, 16.3% of the mature oocytes analyzed contained greater than two arm spots, indicative of premature loss of arm cohesion. In contrast, we observed separated arm spots in only 5.1% of oocytes that contained normal levels of SMC3[19]. Interestingly, although the frequency of arm cohesion defects was elevated in multiple knockdown genotypes, sister chromatids were rarely separated in their pericentric regions[19].

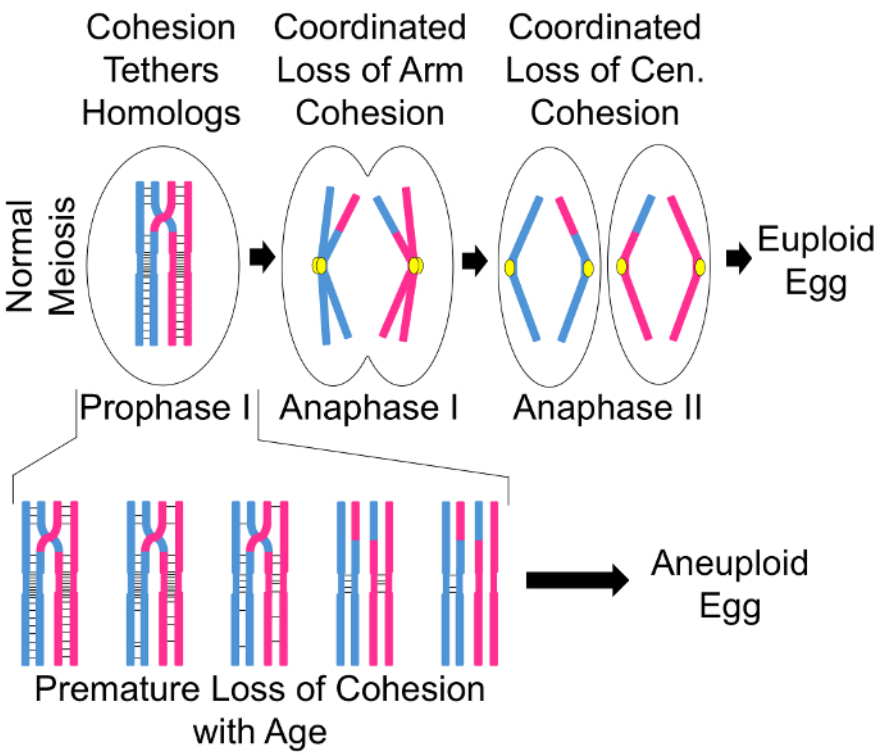

Figure 1: Age-dependent loss of cohesion during the extended prophase I arrest of female meiosis can result in errors in chromosome segregation and aneuploidy in the egg. Cohesion is represented by black bars between the sister chromatids. Arm cohesion functions to hold recombinant homologs together and is required for accurate segregation at anaphase I. If arm cohesion is lost prematurely, chromosome missegregation can occur, resulting in an aneuploid egg. This figure was adapted from reference[19]. Please click here to view a larger version of this figure.

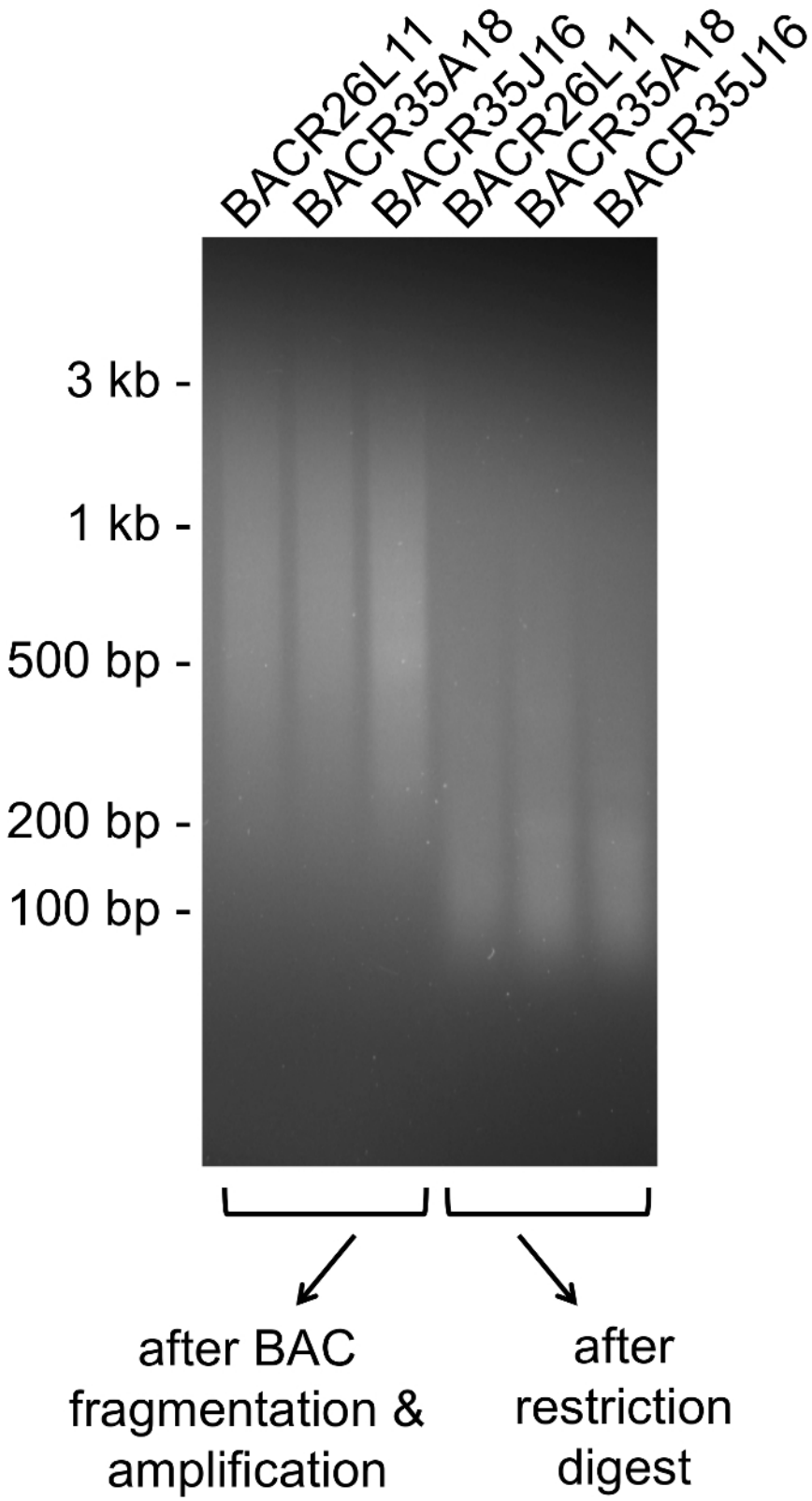

Figure 2: Size of DNA molecules after BAC fragmentation and amplification (left) and after restriction enzyme digestion (right) for three different BACs. 400 ng of amplified DNA products are shown in the first three lanes. The last three lanes show DNA fragments after the overnight restriction digest. The amplified DNA products range from 200 bp up to 3 kb. After the restriction digest, most fragments ranged from 100 - 200 bp, but larger fragments (up to 500 bp) were visible. Please click here to view a larger version of this figure.

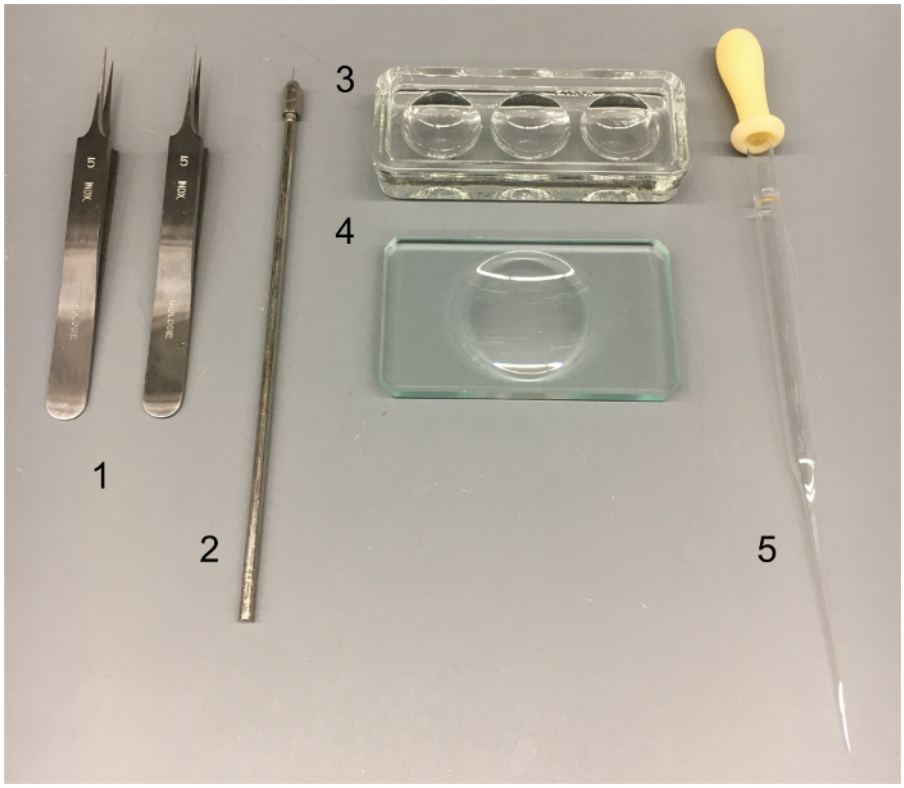

Figure 3: Cytology tools. Tools used in protocol: (1) pair of forceps (#5 Dumont); (2) tungsten needle; (3) deep well dish with cover; (4) shallow glass dissecting dish; (5) pulled Pasteur pipette. Please click here to view a larger version of this figure.

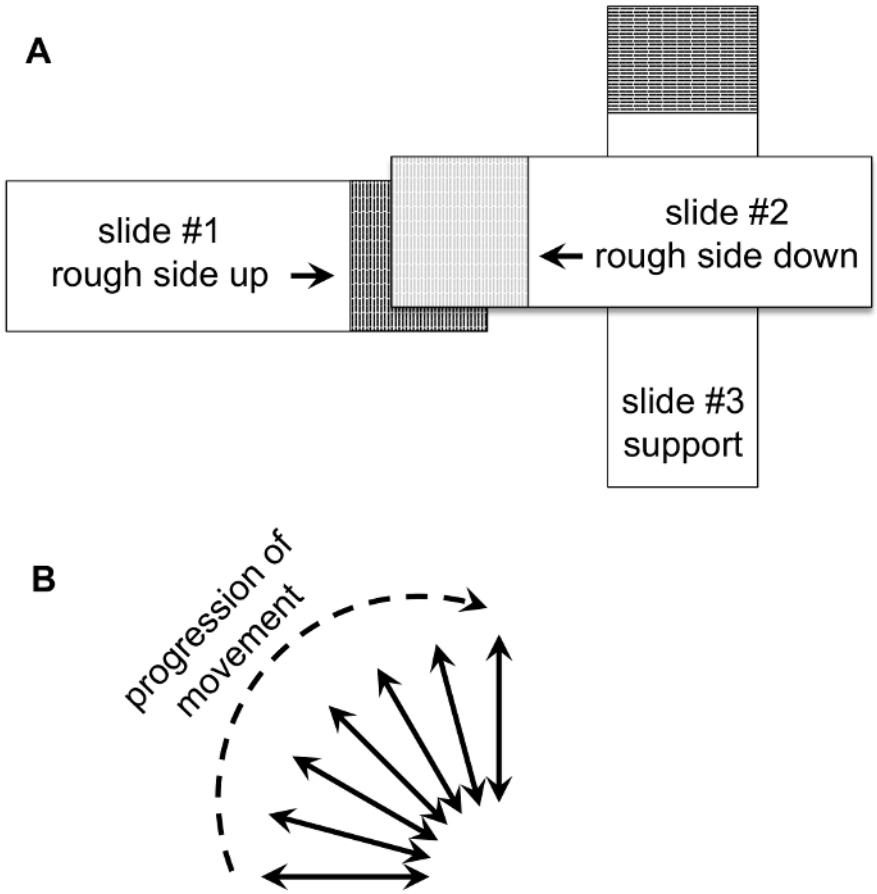

Figure 4. Arrangement and movement of slides for rolling oocytes. (A) Diagram of the slide set up for rolling of oocytes as described in the protocol. The darker area denotes the frosted glass part of the slide. **(B)** Direction of movement vectors for slide #2 during oocyte rolling. Please click here to view a larger version of this figure.

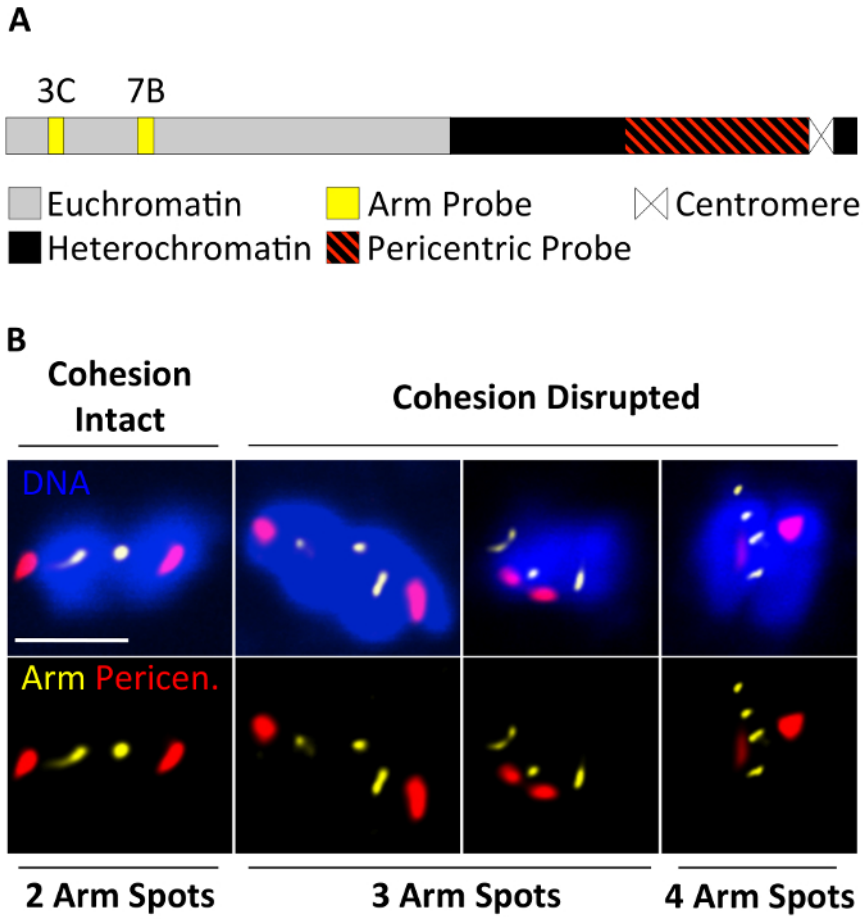

Figure 5. FISH assay results. (A) Schematic depicts the *Drosophila X* chromosome and where the two arm probes (6 BACs each) and the 22 oligonucleotide pericentromeric probe described in the protocol hybridize. For simplicity, the 6E-7B probe is labeled 7B and the 2F-3C probe is labeled 3C. **(B)** Representative images of FISH results (6E-7B probe) for intact arm cohesion (two arm probe spots, one per homolog) and for disrupted arm cohesion (three to four arm probe spots). DNA is blue, the arm probe signal is yellow, and the pericentric probe signal is red. Images correspond to maximum intensity projections of Z series after deconvolution and contrast enhancement. Scale bar = 2 µm. This figure was adapted from reference[19]. Please click here to view a larger version of this figure.

Discussion

The use of FISH probes to assess the state of arm cohesion in prometaphase I and metaphase I *Drosophila* oocytes is a significant advancement in the field of *Drosophila* meiosis. Historically, *Drosophila* researchers have been limited to genetic tests to infer premature loss of arm cohesion in mature oocytes[11,18,19]. Now, with the methods presented here, the state of arm cohesion can be assayed directly using FISH. The ability to obtain physical evidence for premature loss of arm cohesion greatly expands the repertoire of approaches available to study the mechanisms that lead to premature loss of arm cohesion and chromosome missegregation in *Drosophila* oocytes.

Critical Steps

Although we have not performed a systematic analysis of critical parameters necessary for successful visualization of a fluorescent probe that hybridizes to single copy sequences along the chromosome arm in mature *Drosophila* oocytes, we offer the following list of factors that may have contributed to the success of this technique. To generate the arm probe, we used a combination of six overlapping BAC probes that cover an interval of approximately 0.8 Mb on the *X*-chromosome arm. Our initial attempts using fewer BACs that spanned a smaller region were not successful. In addition, we have used a method to fragment and amplify the BAC DNA before end-labeling with Alexa-647. The majority of restriction fragments that were labeled were 100 - 200 bp long (**Figure 2**), which agrees well with the target size of 150 nucleotides that is necessary for efficient diffusion through thick tissues[21]. Fixation conditions that preserve morphology of the large *Drosophila* oocyte but also allow probe penetration are essential. We have modified our previously used fixation method[23] by adding room temperature heptane to a fix solution preheated to 37 °C. We think it possible that both the addition of heptane to increase penetration through the vitelline membrane as well as the lowered temperature for fixation contributed to successful fixation conditions for visualization of arm cohesion.

When assaying for premature loss of cohesion, one requires a sample size that allows quantification of defects that are present at low to moderate levels. To obtain large numbers of mature oocytes, others have used blender disruption of adult females combined with sieving [26,27]. Here we describe a method to hand dissect ovaries from 20 - 25 females, with manual separation of late stage oocytes by pipetting. While the blender/sieving method should also work with this protocol, one advantage of hand dissection is that without the sieving step, oocytes spend less time in buffer before fixation, which decreases the chance of anaphase I onset due to artificial egg activation. In addition, this method requires

fewer females as starting material, uses smaller volumes of reagents, and has a simpler workflow, all of which facilitate processing multiple genotypes.

Hand dissection and manual separation of mature oocytes provides sufficient quantities of oocytes to "roll" for chorion and vitelline membrane removal and results in approximately 75 - 150 oocytes at the end of the hybridization washes. One trick to maximize the yield of metaphase I arrested oocytes is to hold virgin females in yeasted vials in the absence of males before dissection[25]. Oocyte rolling to remove chorions and vitelline membranes must be performed with a gentle touch in order to avoid destroying oocytes. However, removing chorions and vitelline membranes from 100% of the oocytes is not a realistic goal and excessive rolling only results in loss of oocytes. Therefore, each rolling cycle should be stopped when approximately 75 - 85% of oocytes lack chorions and vitelline membranes.

Limitations

Despite our success at generating and using arm probes to monitor the state of cohesion in mature *Drosophila* oocytes, the procedure still suffers from limitations. While we rarely failed to detect a signal for the pericentromeric probe, the arm probe signal was weak or absent in approximately 15 - 20% of the oocytes that we imaged. One contributing factor may be differential penetration of the pericentric oligonucleotide probe (22 - 28 nucleotides) versus the larger arm probe (100 - 200 nucleotides). In addition, the large repetitive sequence recognized by the pericentric probe results in a signal that is brighter than that for the arm probe. The orientation of the oocyte and placement of meiotic chromosomes within the oocyte also present a challenge when imaging. Although the meiotic chromosomes are relatively close to the cell cortex, it is impossible to control the orientation of the oocyte when mounting on the coverslip. Therefore, in some fraction of the oocytes, the meiotic chromosomes may be 100 - 200 µm from the coverslip and the signal intensity and quality may be negatively impacted when trying to image through the bulk of the oocyte. These limitations mean that the number of oocytes included in the final quantitative analysis is considerably lower than the number of oocytes processed in the protocol. Determining the state of arm cohesion in 100 oocytes will most likely require two independent preparations. An additional limiting factor in quantifying cohesion defects using a laser scanning confocal microscope is the time required to capture a typical Z series (approximately 5 min), even when the captured area is confined to 1,024 x 512 pixels. This means that comparison of cohesion in control and experimental genotypes (100 oocytes each) will necessitate approximately 16 h of image collection time.

Modifications and Troubleshooting

We have been able to monitor the state of arm cohesion using a probe that recognizes the *X* chromosome at cytological position 6E-7B[19]. A probe that hybridizes to a more distal location on the *X* chromosome may be more desirable for detecting the failure of chiasma maintenance. In addition, other researchers may wish to analyze arm cohesion on other chromosomes. While we have not attempted to probe the autosomes, the preliminary data indicate that a probe that recognizes the 2F-3C region of the *X* chromosome also results in a detectable signal. However, based on limited preliminary data, the signal of the 2F-3C probe may be less "compact" than that for the 6E-7B probe. Although the FISH signals are tight and crisp for some oocyte chromosomes, the hybridization signal on the chromosomes of other oocytes is considerably more diffuse. Whether these differences in signal reflect genuine differences in chromosome morphology between the two cytological locations, variability in the hybridization efficiency of the two probes, or just stochastic variability between different oocytes will require a more thorough analysis.

Importantly, even for the 6E-7B probe, we observed a diffuse chromosomal signal in some oocytes, and this often necessitated their exclusion from analysis. In addition, we have noticed variation in signal quality and brightness between different preparations of the 6E-7B probe and this required that imaging parameters be adjusted accordingly. Raising the hybridization temperature to 42 °C did not reduce the number of oocytes with diffuse signal, but it did severely impact the signal for the pericentric oligonucleotide probe. In an effort to better preserve chromosome morphology[24], we also tried a longer denaturation step at a lower temperature (80 °C), but found that this treatment neither increased the signal intensity nor reduced the number of oocytes with diffuse chromosomal FISH signal. We also attempted to obtain a brighter arm signal by using 12 overlapping BACs corresponding to an approximately 1.5 Mb interval that spanned cytological region 5E-7B. When using a 12 BAC probe, the degree of variation in signal intensity as well as the percentage of oocytes lacking signal was no different than when six BACs were used to make the arm probe. It was also more challenging to score for cohesion defects when using the 12 BAC probe because the FISH signals were larger, making it more difficult to resolve spots corresponding to individual chromatids. In cases in which no signal is obtained with a newly prepared probe, researchers should check the size of the amplified and restriction digested BAC DNA to confirm that the fragments used for end labeling are primarily within the 100 - 200 bp range. This is likely to be one of the most critical factors in successful probe preparation.

Future Directions

Future work to enhance image acquisition as well as adapting the protocol to include immunostaining would be major improvements that would benefit other researchers. During image collection, collecting a greater number of images in the axial dimension as well as faster image acquisition would be advantageous for quantifying cohesion defects. In optimizing the imaging parameters for the laser scanning confocal, we found that 0.25 µm was the smallest step size that could be used for the Z series in order to avoid appreciable bleaching of the FISH signals. However, for FISH signals that are separated by less than 0.25 µm in the Z stack, this step size may result in failure to capture the "space" between the foci and may therefore underestimate the number of cohesion defects. The faster image acquisition speed of a spinning disc confocal may allow smaller step sizes to be used without signal bleaching. This would provide more accurate image reconstruction in the axial dimension as well as permit larger number of oocytes to be analyzed for cohesion defects. In addition, the ability to combine immunostaining with FISH visualization of the state of arm cohesion would significantly enhance the protocol. For a number of preparations, we tried to perform spindle immunostaining following the hybridization procedure. However, robust spindle staining was visible in only a small fraction of the oocytes. Moreover, for reasons that are not clear, higher levels of spurious FISH signals surrounded the meiotic chromosomes when FISH and immunostaining were combined. Further work to optimize the protocol to allow immunostaining either before or after the FISH procedure would greatly expand the capability of this technique.

Disclosures

The authors declare no competing financial interests.

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References

1. Peters, J. M., & Nishiyama, T. Sister chromatid cohesion. *CSH Perspect Biol.* **4** (11) (2012).
2. McNicoll, F., Stevense, M., & Jessberger, R. Cohesin in gametogenesis. *Curr Top Dev Biol.* **102** 1-34 (2013).
3. Buonomo, S. B. *et al.* Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. *Cell.* **103** (3), 387-398 (2000).
4. Bickel, S. E., Orr-Weaver, T., & Balicky, E. M. The sister-chromatid cohesion protein ORD is required for chiasma maintenance in Drosophila oocytes. *Curr. Biol.* **12** (11), 925-929 (2002).
5. Hodges, C. A., Revenkova, E., Jessberger, R., Hassold, T. J., & Hunt, P. A. SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction. *Nat Genet.* **37** (12), 1351-1355 (2005).
6. Freeman, S. B. *et al.* The National Down Syndrome Project: design and implementation. *Public Health Rep.* **122** (1), 62-72 (2007).
7. Nagaoka, S. I., Hassold, T. J., & Hunt, P. A. Human aneuploidy: mechanisms and new insights into an age-old problem. *Nat Rev Genet.* **13** (7), 493-504 (2012).
8. Angell, R. R. First-meiotic-division nondisjunction in human oocytes. *Am. J. Hum. Genet.* **61** 23-32 (1997).
9. Tsutsumi, M. *et al.* Age-related decrease of meiotic cohesins in human oocytes. *PLoS One.* **9** (5), e96710 (2014).
10. Liu, L., & Keefe, D. L. Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes. *Reprod Biomed Online.* **16** (1), 103-112 (2008).
11. Subramanian, V. V., & Bickel, S. E. Aging predisposes oocytes to meiotic nondisjunction when the cohesin subunit SMC1 is reduced. *PLoS Genet.* **4** (11), e1000263 (Electronic), (2008).
12. Chiang, T., Duncan, F. E., Schindler, K., Schultz, R. M., & Lampson, M. A. Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes. *Curr. Biol.* **20** (17), 1522-1528 (2010).
13. Lister, L. M. *et al.* Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2. *Curr. Biol.* **20** (17), 1511-1521 (2010).
14. Duncan, F. E. *et al.* Chromosome cohesion decreases in human eggs with advanced maternal age. *Aging Cell.* **11** (6), 1121-1124 (2012).
15. Yun, Y., Lane, S. I., & Jones, K. T. Premature dyad separation in meiosis II is the major segregation error with maternal age in mouse oocytes. *Development.* **141** (1), 199-208 (2014).
16. Subramanian, V. V., & Bickel, S. E. Heterochromatin-mediated association of achiasmate homologues declines with age when cohesion is compromised. *Genetics.* **181** 1207-1218 (2009).
17. Jeffreys, C. A., Burrage, P. S., & Bickel, S. E. A model system for increased meiotic nondisjunction in older oocytes. *Curr. Biol.* **13** (6), 498-503 (2003).
18. Weng, K. A., Jeffreys, C. A., & Bickel, S. E. Rejuvenation of Meiotic Cohesion in Oocytes during Prophase I Is Required for Chiasma Maintenance and Accurate Chromosome Segregation. *PLoS Genetics.* **10** (9), e1004607 (2014).
19. Perkins, A. T., Das, T. M., Panzera, L. C., & Bickel, S. E. Oxidative stress in oocytes during midprophase induces premature loss of cohesion and chromosome segregation errors. *Proc Natl Acad Sci U S A.* **113** (44), E6823-E6830 (2016).
20. Dernburg, A. F., Sedat, J. W., & Hawley, R. S. Direct evidence of a role for heterochromatin in meiotic chromosome segregation. *Cell.* **86** 135-146 (1996).
21. Dernburg, A. F., & Sedat, J. W. in *Method Cell Biol.* Vol. 53 187-233 Academic Press (1998).
22. Guo, Z., Batiha, O., Bourouh, M., Fifield, E., & Swan, A. Role of Securin, Separase and Cohesins in female meiosis and polar body formation in Drosophila. *J Cell Sci.* **129** (3), 531-542 (2016).
23. Giauque, C. C., & Bickel, S. E. Heterochromatin-Associated Proteins HP1a and Piwi Collaborate to Maintain the Association of Achiasmate Homologs in Drosophila Oocytes. *Genetics.* **203** (1), 173-189 (2016).
24. Joyce, E. F., Apostolopoulos, N., Beliveau, B. J., & Wu, C. T. Germline progenitors escape the widespread phenomenon of homolog pairing during Drosophila development. *PLoS Genet.* **9** (12), e1004013 (2013).
25. Gilliland, W. D., Hughes, S. F., Vietti, D. R., & Hawley, R. S. Congression of achiasmate chromosomes to the metaphase plate in Drosophila melanogaster oocytes. *Dev Biol.* **325** (1), 122-128 (2009).
26. Gyuricza, M. R. *et al.* Dynamic and Stable Cohesins Regulate Synaptonemal Complex Assembly and Chromosome Segregation. *Curr Biol.* **26** (13), 1688-1698 (2016).
27. Radford, S. J., & McKim, K. S. Techniques for Imaging Prometaphase and Metaphase of Meiosis I in Fixed Drosophila Oocytes. *J Vis Exp.* (116) (2016).

