Fluorescent BrdU Labeling and Nuclear Flow Sorting of the Drosophila Ovary

Brian R. Calvi and Mary A. Lilly

1. Introduction

The Drosophila ovary has proven to be an excellent model system for addressing many key questions in biology. Among these are questions relating to the cell cycle control of DNA replication and chromosome structure during development. Early studies of ovarian chromosome dynamics employed various histochemical stains and bright-field microscopy (1). Later, photometric cytometry, ³H-thymidine incorporation, and radioactive in situ DNA hybridization were used to study DNA replication and chromatin organization within the ovary (2–5). Recently, the introduction of fluorescent detection has significantly improved the ability to study chromosome dynamics and DNA replication in the developing ovary (6–8). In this chapter, we describe two techniques based on fluorescent detection, BrdU labeling and nuclear flow sorting, that have recently been applied to the study of oogenesis. These techniques allow visualization of DNA replication with high resolution by epifluorescence microscopy and accurate measurement of DNA copy number during endocycles of the ovary. We first briefly review the current understanding of cell cycle and chromosome modifications during oogenesis to which these techniques have contributed. For more detailed accounts of oogenesis, the interested reader is referred to previous reviews (9–11).

The Drosophila ovary provides several inherent advantages for the study of the developmental control of DNA replication and chromosome dynamics (see ref. 9 for a review). First, both somatic and germ-line cells modify their cell cycles, chromosome structure, and DNA replication patterns in concert with stages of oogenesis. Unlike what occurs in mammals, the entire process of
Drosophila oogenesis, from the division of stem cells to the production of a mature egg, takes place throughout the lifetime of the adult female. Therefore, a second advantage is that a single adult female contains numerous egg chambers representing each developmental stage. Egg chambers are comprised of germ-line and somatic cells. Sixteen germ-line cells, a single oocyte, and 15 sister nurse cells are surrounded by an epithelial sheet of somatic follicle cells (see Fig. 1A). Egg chambers mature within a structure called the ovariole (see Fig. 1A). Each ovary is comprised of approximately 16 ovarioles that typically contain 7 egg chambers at different stages of development. Because egg cham-
bers migrate posteriorly as they mature, a final advantage is that every ovariole contains an anterior to posterior array of successively older egg chambers.

The prelude to forming an egg chamber begins at the anterior tip of the ovariole in a structure known as the germarium, which contains both germ-line and somatic stem cells (see ref. 11 for a review) (see Fig. 1A). A germ-line stem cell division at the anterior tip of the germarium gives rise to a primary cystoblast. This cystoblast undergoes four synchronized mitotic division cycles with incomplete cytokinesis as it migrates posteriorly within the germarium. At the end of these divisions, the 16 cells of this germ-line cyst are connected by intercellular bridges called ring canals. All 16 cells enter the premeiotic S-phase, but only the true oocyte continues meiosis. The other 15 cells become nurse cells and begin endocycles in stage 1 of oogenesis. Endocycles are comprised of alternating S- and G-phases without cell division. The somatic stem cells are located in a posterior lateral position within the germarium (12). These stem cells give rise to a pool of follicle cells that then surround the nurse cell–oocyte complex as it buds off from the germarium to form a stage 1 egg chamber. Fourteen stages of egg chamber development were described by the seminal study of King (13) and have been adopted as the standard nomenclature in the field.

During stage 1 to stage 10 of egg chamber development, the nurse cells execute approx 10-12 endocycles and arrest with enormous nuclei that have a ploidy of greater than 1000C (2,6) (see Fig. 1A). This high ploidy supports their role as nutritive cells that supply the oocyte with protein and RNA for early embryogenesis. The nurse cells within an egg chamber do not cycle in synchrony with each other. Therefore, BrdU labeling yields egg chambers that have some nurse cell nuclei labeled and others are not (see Fig. 1B). Although most euchromatin is replicated during the endocycle, certain heterochromatic satellite sequences are not fully replicated and become progressively under-represented with succeeding endocycles (2–6). In stages 1–4, the nurse cell chromosomes adopt a transient pseudopolytene configuration in which the sister chromatids and homologs are synapsed, but then disperse by stage 6 and individual chromosomes cannot be easily identified thereafter (8). The replicated sisters from each chromosome arm do remain in distinct regions of the nucleus comprising five nuclear domains. In certain mutant strains, the nurse cell chromosomes remain synapsed and form giant polytene chromosomes that have a distinct banded pattern (14–17; also see Chapter 6).

The somatic follicle cells divide mitotically from stage 1 to stage 6. In stage 6, the follicle cells exit the mitotic cycle and enter the endocycle. Nuclear sorting shows that the majority of follicle cells arrest endocycles with a final ploidy of 16C, and BrdU labeling indicates this arrest occurs before stage 10B (6,7) (see Fig. 1A,B). Follicle cells within an egg chamber do not cycle in synchrony
with one another during mitotic cycles and endocycles. Therefore, some follicle cells are seen to incorporate BrdU and others do not. During stage 9, most follicle cells migrate posteriorly to form a columnar epithelial sheet over the enlarging oocyte. A few follicle cells remain over the nurse cells and become thin and squamous. Also during stage 9, special border follicle cells at the anterior of the chamber migrate posteriorly in between the nurse cells. Their arrival at the nurse cell–oocyte border marks the beginning of stage 10A. During stage 10A, only a few follicle cells are completing the last endocycle S-phase and label with BrdU.

Stage 10B begins when the most anterior columnar follicle cells over the oocyte begin to migrate to the interior, centripetal position of the egg chamber, which ultimately separates the nurse cells from the oocyte. At the onset of stage 10B, follicle cells begin what amounts to an extended S-phase during which only a few loci re-replicate (7,18). Two of these loci represent clusters of genes that encode eggshell (chorion) proteins. The cluster on the third chromosome amplifies in copy number to approx 64–100× and the one on the X chromosome to approx 16–20×, above the 16C follicle cell genome (see ref. 10 for review, and ref. 19). The high copy number of these two loci supports rapid biosynthesis of the eggshell later in oogenesis. Because most of the genome is not replicating, BrdU incorporation in these cells appears as four dots (see Fig. 1C,D). The two most intense spots represent the two chorion loci, and the two faint spots represent unknown loci that amplify to only low levels. BrdU incorporation continues at the third chromosome locus until stage 13, but the X chromosome locus and other loci have much diminished BrdU labeling by stage 12. In conjunction with the tools of Drosophila genetics, the ability to monitor the activity of these origins by BrdU has been a useful assay for studying the cell cycle control of replication origin activity (see refs. 20 and 21 for examples).

2. Materials

2.1. BrdU Labeling

1. 10 mM BrdU (Sigma) in dH₂O. Store at –80°C. These aliquots are good for several freeze–thaw cycles. Surprisingly, BrdU stock goes off over time at –80°C and should be remade every couple of months. BrdU solution made fresh works best. BrdU is a mutagen and should be handled with care.
2. Mouse anti-BrdU monoclonal antibody (Becton Dickinson).
3. Cy3-Conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories).
4. Glycerol-based antifade solution for mounting ovaries onto microscope slides such as Vectashield (Vector Laboratories).
5. Grace’s insect culture medium (Gibco-BRL) brought to room temperature.
6. 2 N HCl (8.6 mL concentrated 12 N HCl stock per 50 mL H₂O).
7. 100 mM Borax neutralization solution: 1.907 g Na₂B₄O₇·10H₂O/50 mL H₂O. Sterilize by filtration and keep stock at 4°C to prevent mold growth.
8. Phosphate-buffered saline (PBS): 130 mM NaCl, 3 mM NaH₂PO₄, 7 mM Na₂HPO₄, pH 7.2. Sterilize by filtration or autoclaving.
9. Phosphate-buffered saline + Triton (PBT): PBS + 0.1% (v/v) Triton X-100.
10. PBT + BSA: PBT + 0.2% (w/v) bovine serum albumin (BSA).
11. PBT + normal goat serum (NGS): PBT + 5% (v/v) NGS. Heat-inactivate NGS at 55°C, 20 min, and store in aliquots at –20°C.
12. PBS + DAPI: PBS + 1 µg/mL DAPI (Roche).
13. Buffer B for fixation: 100 mM KH₂PO₄/K₂HPO₄, pH 6.8, 450 mM KCl, 150 mM NaCl, 20 mM MgCl₂.
14. 37% Formaldehyde stock (methanol stabilized). Fresh EM-grade formaldehyde (16%) without methanol can be substituted and volumes should be adjusted accordingly.
15. 5% BSA in dH₂O (distilled water) for pretreating Eppendorf tubes and pipets to discourage sticking of the ovaries.
16. Two fine-tipped dissecting forceps, such as Inox 5 biologie tip (Fine Science Tools).
17. A deep glass dissecting dish such as a 9-well dish with wells that hold 1 mL of solution (Fisher).
18. Short-nose Pasteur pipets.
19. Standard microscope slides, 22 × 22-mm² #1 cover slips, and nail polish for sealing.
20. Low-power stereomicroscope for dissections.

2.2. Nuclear Flow Sorting

1. Ultra centrifuge equipped with swinging-bucket rotor.
2. Ephruzzi–Beadle ringers (EBR): 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 10 mM HEPES, pH 6.9; autoclave and store at 4°C.
3. 5 mg/mL Collagenase (Type 1A Sigma) diluted in 1X EBR.
4. Nuclear isolation buffer: 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 250 mM sucrose, 1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine. Sterilize with a 0.2-µm filter and store at room temperature.
5. 1X Nuclear isolation buffer with 1.5% NP-40.
6. 100-µm Nitex mesh (Tetko Inc).
7. Three solutions of 0.8 M, 1.5 M, and 2.5 M sucrose dissolved in nuclear isolation buffer.
8. 5-mL plastic pipet cut into 1.5-cm sections.
9. 2-mL Dounce homogenizer (clearance 0.0005–0.0025 in.) (Kontes).
10. 1-mg/mL stock solution of DAPI (4’6-diamidino-2-phenylindole). DAPI should be diluted in 70% ethanol. If the DAPI is diluted in water, it will precipitate over time.
11. 5% BSA in dH₂O for pretreating pipets and tubes.
12. 10-mg/mL Stock solution of RNase A (Sigma-Aldrich) diluted in 0.01 M sodium acetate (pH 5.2). Heat to 100°C for 15 min to inactivate DNases.
13. 1 mg/mL Propidium iodide in water.
3. Methods
3.1. BrdU Labeling

1. Three days before dissection, condition *Drosophila* females with males on wet yeast for 2 d, followed by fresh wet yeast for 1 d (see Note 1).

2. On the day of the experiment, bring enough Grace’s medium to room temperature for dissection, incubation, and washes (approx 4 mL per sample). The tissue must not be chilled before or during BrdU incubation because this inhibits incorporation. Pretreat tubes and Pasteur pipets with 5% BSA to discourage the sticking of ovaries (see Note 2).

3. Dissect ovaries in 400 µL of room-temperature Grace’s medium in the dissecting dish. After dissecting approx six pairs of ovaries, puncture the outer ovary sheath and partially tease apart the ovarioles at their anterior ends. At the end of this process, most ovarioles should be separated at their anterior tip but remain attached at the posterior near the common duct and uterus. Some mature egg chambers will rupture from the ovariole and break free. Do not be concerned by this.

4. Using a BSA-treated Pasteur pipet, transfer ovaries and free egg chambers from the dissecting dish to a BSA-treated Eppendorf tube. Allow ovaries to settle to

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**Fig. 2. Flow cytometry (FACS) profile of ovarian nuclei from mature wild-type females.** The numbers over the peaks correspond to the DNA copy number (C) of euchromatic sequences. The 2C-32C peaks are composed predominantly of follicle cell nuclei, but the less abundant nurse cell nuclei are also present in these populations. In contrast, the 64C–512C peaks represent only nurse cell nuclei. A blowup of these higher ploidy nurse cell peaks is presented in the right-hand corner of the graph. The 16C follicle cells have been gated (dotted lines) to be collected by FACS.
bottom of the tube for 30 s. Remove Grace’s medium with a p1000 Pipetteman and add 1 mL Grace’s medium containing 10 µM BrdU. This is prepared by adding 1 µL of 10 mM BrdU stock to 1 mL of Grace’s. Incubate for 1 h with rocking (as short as 15 min of incubation will yield detectable incorporation).  
5. At end of incubation, remove Grace’s/BrdU to hazardous waste and rinse the ovaries twice for 3 min (per rinse) in Grace’s medium to remove unincorporated label.
6. Fix the ovaries in 1 mL of 6% formaldehyde/buffer B/dH₂O (1 : 1 : 4 using 37% formaldehyde : Buffer B stock : dH₂O) at room temperature for 20 min with rocking.
7. Wash with 1 mL PBT three times, 5 min each rinse with rocking.
8. Acid-treat the ovaries with 1 mL of 2 N HCl at room temperature for 30 min with rocking to denature DNA. Alternatively, DNA can be denatured by treating with DNase (see Note 3).
9. Remove the acid and neutralize the ovaries in 1 mL of 100 mM borax (sodium tetraborate) for 2 min.
10. Wash with 1 mL PBT three times, 10 min each wash with rocking.
11. Block the ovaries in 10 µL PBT/5% NGS for 30 min without rocking.
12. Remove the blocking solution and add 100 µL PBT/NGS with 1 : 20 mouse anti-BrdU monoclonal antibody (Becton Dickinson cat. no. 7580). Incubate at 4°C overnight without rocking.
13. The next day, wash with 1 mL PBT/BSA 0.2%, three times for 10 min each wash, followed by three times for 30 min each wash, with rocking.
14. Block the ovaries again with 100 µL PBT/NGS for 30 min.
15. Label with secondary antibody. Remove block and add 200 µL PBT/NGS containing a 1 : 400 dilution of Cy3 goat anti-mouse antibody or other secondary antibody of choice. Mix gently. Incubate in the dark at room temperature for 2 h without rocking.
17. Remove as much PBT as possible and counterstain the nuclei in 100 µL of PBS containing 1 µg/mL of DAPI. Mix gently and let sit at room temperature for 7 min.
18. Remove the PBS/DAPI solution and add 80 µL Vectashield (Vector Laboratories) with a cutoff pipet tip. Gently mix by flicking the tube. Spin full speed in an Eppendorf centrifuge for 3 s. Mix again by flicking the tube but make sure the ovaries remain in the Vectashield. If the ovaries stick on the wall of the tube, spin again briefly. Allow the ovaries to equilibrate in Vectashield for at least 30 min before mounting. At this point, ovaries can be stored at 4°C.
19. Mount the ovaries on microscope slide for observation. With a cut off p200 pipet tip, pipet 23 µL of fresh Vectashield to the middle of the slide. With the same tip, pipet ovaries to one end of slide. Using forceps, pick desired ovarioles and egg chambers into the fresh Vectashield. It is important to transfer ovaries into fresh Vectashield to ensure protection from photobleaching in the microscope. Mount approximately one pair of ovaries per slide. Separate ovarioles completely at this point and remove excess ovary sheath. Spread out and align ovarioles in parallel
by raking forceps through Vectashield. Pipet unused ovaries back into the Eppendorf tube and clean up excess Vectashield that remains on the end of the slide with a kimwipe. Place a 22 × 22-mm², #1 cover slip on ovaries. If desired, place a light weight (approx 12 g) on top of cover slip for 3–10 min. This flattens the egg chambers so that more cells are in a single focal plane. Seal the edges of the slide with nail polish and allow to dry for several minutes. Optimal storage of the slides is in the dark at –20°C.

20. Observe incorporation on an epifluorescence microscope equipped with bandpass filters that allow the visualization of DAPI and Cy3 (excitation/emission wavelength (nm): DAPI approx 330/450; Cy3 approx 550/570). BrdU incorporation during mitotic, endocycles, and chorion gene amplification should be visible using a 10× objective (see Fig. 1A–D). Incorporation will be more apparent under higher-power, 20–100× objective, and an oil immersion lens is recommended. Nuclei should be brightly stained with DAPI, and a focus of more intense DAPI staining should be seen in the heterochromatic chromocenter of polyploid cells (see Fig. 1C).

### 3.2. Nuclear Flow Sorting

1. Condition 30–50 females on wet yeast as described in Subheading 3.1. (see Note 1). Processing more than 50 females at a time may result in significantly increased background during the FACS analysis.

2. It is important to pretreat all materials that will come in contact with the nuclei with a 5% solution of BSA to prevent the nuclei from sticking. This includes Pasteur pipets, Eppendorf tubes, 2-mL Dounce homogenizer, step gradient tube, Pipetman tips, and the 100-µm Nitex filters. This treatment will greatly increase your yields.

3. Dissect ovaries in 500 µL of cold EBR in a dissection dish. After dissecting approximately five females, transfer the group of ovaries to an Eppendorf tube on ice containing 1 mL EBR. Continue dissecting in groups of five until all the females have been dissected.

4. Digest the ovaries at room temperature in 1 mL of 5 mg/mL collagenase diluted in EBR with rocking for 15 min. After the incubation is complete, rinse the ovaries several times with EBR to remove residual collagenase. This is best accomplished with a Pasteur pipet (see Note 4).

5. Resuspend the ovaries in 300–500 µL of 1X nuclear isolation buffer with 1.5% NP-40. Using a Pasteur pipet, transfer the ovaries to a 2-ml Dounce homogenizer. Dounce the ovaries for 10–30 times with a pestle that has a clearance of 0.0005–0.0025 inches. It is not necessary to disrupt the eggshell of older egg chambers.

6. To remove large particulate matter from the solution, including eggshells, filter the homogenate twice through a 100-µm Nitex mesh (see Note 5).

7. Add DAPI to a concentration of approx 1 µg/mL and incubate for 5 min at room temperature. For a 500 µL sample, this will involve adding to the homogenate 0.5 µL of the 1-mg/mL DAPI stock solution. The DAPI is used to follow the nuclei in the next steps.
8. Build a step gradient with the 0.8-\(M\), 1.6-\(M\), and 2.5-\(M\) sucrose solutions in nuclear isolation buffer, starting with 2.5 \(M\) and finishing with the 0.8-\(M\) solution on top. We use 300–500 \(\mu L\) for each step in Beckman 11 \(\times\) 34-mm polyallomar centrifuge tubes. It is important not to disturb the layers when making the gradient. After making the step gradient, three defined layers should be clearly visible when holding the tube up to a light.

9. Gently layer the homogenate on top of the sucrose step gradient, taking care not to disrupt the layers. Centrifuge for 20 min at 20,000 \(g\) in a swinging-bucket rotor. We use a Beckman Optima TLX Ultracentrifuge. Check that the nuclei have pelleted by examining the tube under an ultraviolet (UV) light source. You should see a small pellet of DAPI-bright material at the bottom of the tube.

10. Remove all but 50–100 \(\mu L\) of the sucrose gradient, being careful not to disrupt the nuclei. Resuspend the pellet in 500 \(\mu L\) of nuclear isolation buffer. Disrupt the pellet by pipetting the nuclear isolation buffer up and down several times using a 1 mL Pipetteman. To ensure that the pellet has been resuspended, examine the tube with a UV light source.

11. Add 0.5 \(\mu L\) of the 1-mg/mL DAPI stock solution to obtain a final concentration of 1 \(\mu g/mL\). Alternatively, nuclei can be stained with propidium iodide (see Note 6).

12. Store nuclei on ice before sorting. If necessary, nuclei can be stored overnight at 4\(^\circ\)C.

13. To obtain a flow cytometry profile, examine the DAPI-stained nuclei excited with a krypton laser with a multiline UV (337–356 nm) source. DAPI emission is collected through a 450/465 bandpass filter. Specifically, we use a Coulter EPICS 752 flow cytometer.

4. Notes

1. *Drosophila* oogenesis is dependent on environment, age, and mating status of the female. Therefore, for both BrdU labeling and nuclear flow sorting, it is critical to use mated females of optimal age that are well fed and watered. This entails conditioning females on wet baker’s yeast (the consistency of creamy peanut butter) for 2 d, followed by one more day on fresh wet yeast (a total of 3 d of conditioning). The females should have eclosed from the pupal case at least 3 d and no more than 8 d before the day of dissection (4–6 d post eclosion is optimal in most cases). Because oogenesis proceeds apace only if females have recently mated, males should be present during conditioning.

2. Treat eppendorf tubes and Pasteur pipets with 5% BSA solution to discourage sticking of the ovaries. Pipet 1 mL of 5% BSA into a tube, close the cap and invert, remove the BSA to the next tube to be treated, and so on. Treat pipets by pipetting the solution up and down once.

3. DNase I denaturation is preferred if ovaries are to be labeled for BrdU and an antibody to a protein because most epitopes are not detected after HCl treatment. After formaldehyde fixation in Subheading 3.1., step 6, substitute the following for steps 7–9. Wash twice, 15 min each, in PBS + 0.6% Triton-X; wash twice, 15 min each, in DNase buffer (66 mM Tris-HCl, pH 7.5, 5 mM MgCl\(_2\), 1 mM of 2-mercaptoethanol), added fresh; incubate ovaries in 100 \(\mu L\) of DNase I.
(12.5 units/mL DNase buffer) at 37°C for 30 min (DNase I: Roche cat. no. 776-785). Proceed to Subheading 3.1., step 10.

4. After collagenase treatment, the EBR may become slightly viscous, making it difficult for the ovaries to sink to the bottom of the Eppendorf tube. If this occurs, gently centrifuge the ovaries in a microfuge for 2–3 s to bring them to the bottom of the tube.

5. Crude but effective filters can be easily generated by supergluing a small section of Nitex mesh onto the end of a 1.5-cm section from a 5-mL plastic pipet that has been cut for this purpose. These sieves fit snugly into 1.5-mL Eppendorf tubes.

6. As an alternative to DAPI, the nuclei can be stained with the nucleic acid dye propidium iodide. However, because propidium iodide (PI) stains both RNA and DNA, it is necessary to remove the RNA before analyzing the nuclei. After resuspending the nuclei in 500 µL of 1X nuclear isolation buffer in Subheading 3.2., step 10, add 5 µL of a 10-mg/mL RNase A solution to obtain a final concentration of 100 µg/mL. Incubate for 15 min at room temperature. Next add 2.5 µL of a 1-mg/mL PI stock solution to the nuclei. Let the nuclei stain for at least 15 min at room temperature before the analysis. The RNase A digestion and PI staining can be done concurrently. To obtain a flow cytometry profile, examine the propidium iodide stained nuclei using an argon 488 nm laser collected through a 545/642 bandpass filter.

References

Ovarian BrdU Labeling and Nuclear Sorting