

Evaluation of the Asymmetric Division of *Drosophila* Male Germline Stem Cells

Mayu Inaba and Yukiko M. Yamashita

Abstract

Asymmetric cell division (ACD) is utilized in many stem cell systems to produce two daughter cells with different cell fates. Despite the fundamental importance of ACD during development and tissue homeostasis, the nature of ACD is far from being fully understood. Step-by-step observation of events during ACD allows us to understand processes that lead to ACD. Here we describe examples of how we evaluate ACD in vivo using the *Drosophila* male germline stem cell system.

Key words Asymmetric cell division (ACD), *Drosophila* male germline stem cells (GSCs), Centrosome orientation, Spindle orientation, Centrosome orientation checkpoint (COC)

1 Introduction

Drosophila male germline stem cells (GSCs) divide asymmetrically to produce one GSC and one differentiating cell, the gonialblast (GB) (Fig. 1a). Two daughter cells of a GSC division are stereotypically positioned with respect to the niche: only GSCs are attached to the niche component, the hub, but not GBs (Fig. 1a). The hub, a cluster of postmitotic somatic cells, is located at the tip of the testis and surrounded by GSCs. Hub cells secrete ligands that are required for GSC maintenance. Ectopic expression of a niche ligand, Unpaired (Upd), results in an expansion of GSC-like cells outside the niche, indicating that Upd plays a major role in GSC specification. In addition to the extrinsic fate determinants provided by the niche, a number of intrinsic factors and organelles have been reported to be deposited asymmetrically between GSCs and GBs, upon division (Fig. 1a), indicating that these intrinsic factors may also contribute to the asymmetric outcome of GSC divisions. For example, centrosomes, sister chromatids, midbody rings, and histone H3 are reported to segregate asymmetrically during GSC divisions [1–4].

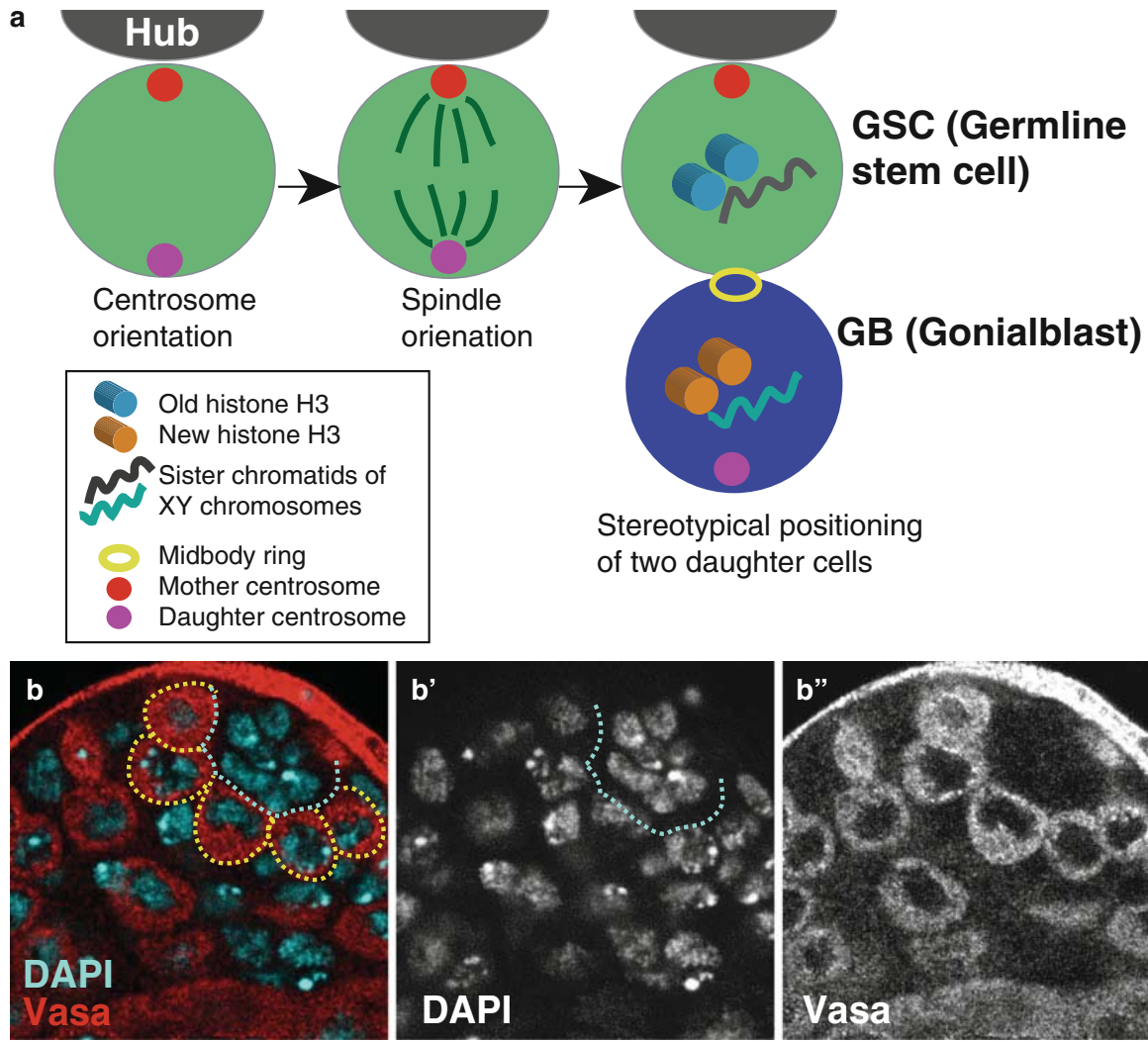


Fig. 1 Asymmetric division of the *Drosophila* male germline stem cells. **(a)** A schematic of asymmetric GSC division and asymmetrically segregating cellular factors. **(b)** A confocal microscope section of a *Drosophila* testis tip region. The hub can be identified as a tightly packed cell cluster as shown using DAPI (encircled by *dotted cyan line* in **b'**). Five germline stem cells (*vasa* positive, **b''**) directly attached to the hub are present in this z-section encircled by *yellow dotted lines* in **(b)**. Scale bar: 10 μ m

For example, the mother centrosome is stereotypically inherited by male GSCs by consistently staying near the hub-GSC junction. Similarly, mouse radial glial progenitor cells inherit the mother centrosome [5]. In contrast, *Drosophila* neuroblasts [6] and female GSCs [3] inherit the daughter centrosomes. These studies indicate the universality of the stereotypical centrosome inheritance during ACD. Asymmetric centrosome inheritance has been reported to correlate with asymmetric cytokinesis. The midbody ring (MR), the remnant of the cytokinetic contractile ring, is segregated to one of the two daughter cells in a stereotypical manner in several cell types that undergo asymmetric cell division. The role of the MR in cell fate specification remains elusive: however, MRs have been shown

to accumulate preferentially in induced pluripotent stem (iPS) cells and cancer stem cells [7, 8], whereas another study showed that stem cells tend to release the MR in various cell lines [9]. In the *Drosophila* testes, the MR is inherited by the differentiating gonialblast, whereas in ovaries, the MR is inherited by the GSCs. In both cases, the cells that inherit the MR are the ones that inherit the daughter centrosomes [3], as opposed to the reported case of MR being inherited by the mother centrosome-containing cells in human embryonic stem cells and cancer cell lines [8]. Although the biological significance of asymmetric MR inheritance is not well understood beyond correlative relationships, it is tempting to speculate that the MR contributes to the asymmetric cell fate directly or indirectly.

Recently, it was reported that the *Drosophila* GSCs selectively inherit preexisting histone 3 (H3), whereas newly synthesized H3 is enriched in the differentiating gonialblasts [4, 10], implying the possibility that distinct epigenetic information might be asymmetrically segregated during male GSC ACD to confer distinct cell fates.

These studies suggest the presence of intricate mechanisms to asymmetrically segregate cellular components, possibly contributing to ACD. Yet, such asymmetries associated with ACD have only begun to be revealed and it remains unclear how critical these asymmetries are in determining asymmetric cell fates. Importantly, it is likely that these asymmetries collectively contribute to asymmetric fates/behaviors of daughter cells (stem cells vs. differentiating cells) with each asymmetry regulating a limited aspect of the cells' fate/behavior. Thus, failure of establishing one particular asymmetry may not result in readily detectable defects such as a stem cell tumor or depletion, and may not be appreciable without specific markers or sensitized background. Accordingly, investigation of ACD involves careful observation to detect subtle changes to fully understand the significance of each asymmetry in ACD. Such subtle changes caused by defective ACD may underlie the complexity of human pathologies, making it difficult to pinpoint a single cause of a disease.

Here, we describe how to assess asymmetries during male GSC ACD, which can serve as a platform to identify potentially many more novel asymmetries. These methods are only to showcase known asymmetries/polarities, and to provide basic methods to detect them. Discovery of new asymmetries and their functions would require the development of new ideas, methods, and reagents. First, we summarize the method of immunofluorescence staining and antibody combinations that can be used to assess distinct aspects of ACD. Later, we also provide a method to observe unfixed testes for live observation. The description of these methods will be accompanied by rationales behind them.

2 Materials

2.1 Preparation of Sample

1. Fixative 4 % formaldehyde in PBS: made from 16 % formaldehyde. PBS: phosphate-buffered saline (NaCl 8 g; KCl 2 g; NaHPO₄ 1.46 g; KH₂PO₄ 0.24 g/1 l water, pH 7.4).
2. PBST: PBS (Phosphate-buffered saline) supplemented with 0.1 % Triton X-100.
3. 3 % Bovine serum albumin heat-shock fraction, pH 7, ≥ 98 %, in PBST.
4. VECTASHIELD (Vector Laboratories #H-1200) with 4',6-diamidino-2-phenylindole (DAPI).
5. Schneider's insect medium with L-glutamine (Sigma-Aldrich #S0146) supplemented with 10 % heat-inactivated fetal bovine serum (FBS).
6. Microscope slides.
7. Cover slips.
8. Gold Seal™ Rite-On™ Micro Slides two etched rings (Live imaging).

2.2 Useful Antibodies

1. Centrosomal marker; mouse anti- γ -tubulin (GTU-88, 1:500 dilution; Sigma).
2. Germ cell marker; rabbit anti-Vasa (1:200 dilution; Santa Cruz Biotechnology Inc.) or rat anti-Vasa (1:20 dilution; Developmental Studies Hybridoma Bank, DSHB).
3. Hub cell marker; mouse anti-Fasciclin III (FasIII, 7G10) (1:40 dilution; DSHB) (*see Note 1*).
4. Mitosis marker phospho-histone H3 (PH3); rabbit anti-phospho-Histone H3 (Thr3) (1:200 dilution; Upstate, Millipore) or rabbit phospho-Histone H3 (Ser10) (1:500 dilution; Cell Signaling Technology) (*see Note 2*).
5. AlexaFluor-conjugated secondary antibodies (1:400 dilution; Molecular Probes).

3 Methods

3.1 Fly Culture

Flies are raised on standard Bloomington medium at 25 °C and assayed at day 0 to day 2 after eclosion. Low nutrient condition or aging has been shown to result in high frequency of centrosome misorientation [11, 12].

3.2 Whole-Mount Immuno- histochemistry of *Drosophila* Testes

1. Dissect testes in PBS (or Schneider's insect medium if followed by in vitro treatments) and collect them in a tube with 1 ml of fixative at room temperature (RT), within 30 min of dissection.
2. Gently rock the tubes on a nutator for an additional 30 min at RT.
3. Briefly rinse fixed testes three times in 1 ml of PBST.
4. Gently rock testes in 1 ml of PBST for 30 min to 1 h at RT.
5. Remove PBST, add primary antibody solution (antibodies appropriately diluted in a total of 100 μ l 3 % BSA in PBST), and gently rock at 4 °C overnight.
6. Remove antibody solution and add 1 ml of PBST. Gently rock the tubes for 20 min. Repeat three times.
7. Incubate with diluted secondary antibodies in 3 % BSA in PBST and gently rock at RT for 2–4 h or at 4 °C overnight.
8. Remove antibody solution and add 1 ml of PBST. Gently rock the tubes for 20 min. Repeat three times.
9. Mount testes with 1 drop of VECTASHIELD with DAPI. Remove excess amount of VECTASHEILD as much as possible by gently applying paper towel from the top of the cover slip. An excess amount of mounting media will result in low-quality imaging.
10. Scoring has to be performed using a confocal microscope (see below for scoring criteria). We use a Zeiss LSM700 confocal microscope with a 40 \times oil-immersion objective (NA = 1.4) or a Leica TCS SP5 or SP8 confocal microscope with a 63 \times oil-immersion objective (NA = 1.4).

3.3 Scoring Centrosome Orientation

Immunofluorescence staining of testes using primary antibody combinations γ -tubulin, Vasa, and Fas III can be used to score the centrosome orientation in GSCs (*see Note 3*). Throughout the male GSC cell cycle, the mother centrosome remains close to the hub-GSC junction. Once the centrosome is duplicated, the newly formed daughter centrosome moves towards the distal side of the cell [13]. This stereotypical centrosome orientation (the mother being at the apical side) is normally observed in more than 90 % of GSCs during interphase in wild-type testes (Fig. 2a) [13]. We set the criterion for scoring of “centrosome orientation” based on the apical centrosome location relative to the hub-GSC junction as shown in Fig. 2. GSCs are scored as having “oriented centrosomes” when at least one of the two centrosomes is in the pink area (Fig. 2c). When neither of the two centrosomes is closely associated with hub-GSC junction, it is defined as “centrosome misorientation” (Fig. 2b) [11]. Because of this scoring criterion, even “completely random” centrosome orientation will result in only up to 56.25 % “misorientation” (calculated as the probability in which both the centrosomes are

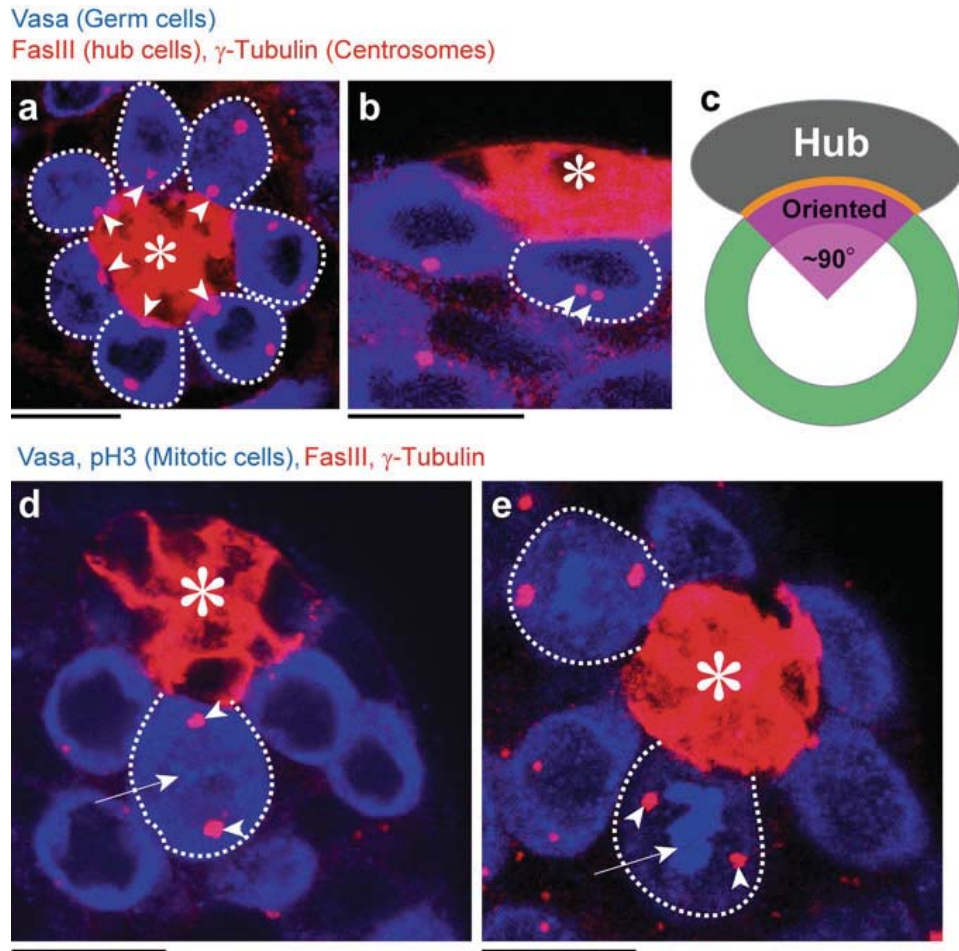


Fig. 2 Centrosome positioning of *Drosophila* male GSCs. (a) Interphase GSCs attached to the hub (white dotted circles) with oriented centrosomes (arrowheads). (b) An example of a GSC (circle) with misoriented centrosomes (arrowheads). (c) Criterion of orientation for scoring. Centrosomes are scored as "oriented" when one of the two centrosomes is in the pink area close to the hub-GSC junction (orange line). (d) An example of a mitotic GSC (circle) with an oriented spindle (arrowheads indicate centrosomes, arrows indicate pH3-positive chromosome). (e) Two mitotic GSCs (circles) with misoriented spindles in a testis. Hub is indicated by an asterisk (*). Scale bar: 10 μ m

located outside of the pink area, $0.75 \times 0.75 = 0.5625$ assuming that the pink area represents $\sim 25\%$ of the entire GSC surface area). In our experience, the highest centrosome misorientation frequency was about 50% of GSCs, which closely matches the highest possible centrosome misorientation frequency based on the model. The low frequency of centrosome misorientation in wild type ($<10\%$) suggests that there are active mechanisms to orient the centrosome close to the hub cells. By scoring centrosome misorientation, mutants defective in this mechanism can be identified.

To find two centrosomes within a GSC, one must scan through the entire depth of the GSC. If two centrosomes are not unambiguously identified, these GSCs are excluded from scoring. This potentially excludes GSCs in G1 phase of the cell cycle prior to

centrosome duplication. However, our previous study has shown that GSCs have an extremely short G1 phase, and thus most GSCs contain two centrosomes [14]. Unless conditions (physiological or mutant) that extend G1 phase are found, and the centrosome orientation under those conditions becomes relevant in the study, discarding GSCs in which two centrosomes cannot be unambiguously identified would not bias the scoring. Typically, in well-stained samples, >70–80 % of GSCs can be scored.

3.4 Scoring Spindle Orientation

Immunofluorescence staining of testes using the primary antibody combination γ -tubulin, Vasa, Fas III, and PH3 can be used to score spindle orientation in GSCs. Mitotic GSCs can be identified by anti-PH3 staining (Fig. 2d, e). Mitotic GSCs are found at a very low frequency under normal conditions (~2 GSCs per 10 testes). Importantly, CO₂ anesthetization longer than 7 min dramatically decreases the mitotic index; therefore it is recommended to dissect testes within 7 min. In addition, it is necessary to let flies recover for at least 2 h before dissection if they must be first sorted based on genotypes. Spindle orientation can be judged by the same criterion as centrosome orientation according to apical centrosome/spindle pole position of mitotic GSCs (Fig. 2c).

3.5 Evaluating the Centrosome Orientation Checkpoint

Centrosome orientation checkpoint (COC) can be scored by immunofluorescence staining of testes using the primary antibody combination Vasa, PH3, and Fas III.

Additional steps:

1. Dissect testes and transfer to Schneider's insect medium. After sample collection, add colcemid (100 μ M final concentration, Calbiochem) and incubate at 25 °C for 4.5 h.
2. Briefly rinse with PBS three times remove PBS and add 1 ml of fixative.
3. Continue to **step 2** in Subheading 3.2.
4. Score the frequency of mitotic GSCs, gonialblasts (GBs), and spermatogonia (SG) (number of mitotic GSC, GB, or SG per testis). SGs are scored per stage (i.e., 2-cell, 4-cell, 8-cell SG). Although the mitotic index defined as "mitotic GSCs/total GSCs" (and mitotic 2-cell SG/total 2-cell SG, and so on) would be more accurate, this requires scoring total numbers of GSCs, GBs, and SGs for each testis, which is very time consuming. Thus, we typically count the number of GSCs/GBs/SGs using fewer testes (than required for scoring mitotic indexes), and if the numbers are not dramatically changed, we use mitotic cells/testis as the mitotic index.

We have shown that colcemid-induced depolymerization of microtubules leads to centrosome misorientation, which causes COC activation and thus G2 cell cycle arrest in GSCs [15].

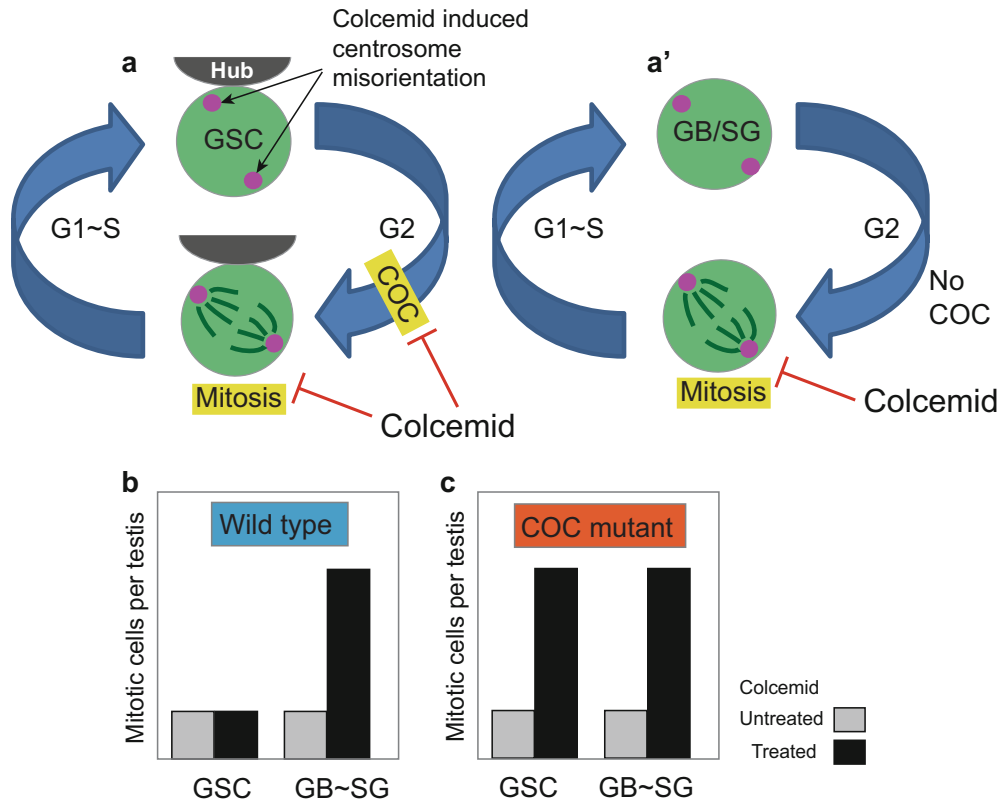


Fig. 3 A technique to detect defective COC activity of GSC by colcemid treatment. (a) Colcemid treatment induces centrosome misorientation in GSC. (a') GBs or SG does not possess COC. (b, c) A typical mitotic index pattern of colcemid-treated testes. Number of mitotic cells per testes increases under the presence of colcemid when COC is inactive (wild-type GB ~ SG in b or mutant GSC defective of COC in c)

In contrast, GSCs defective in COC, as well as spermatogonia, which do not possess COC, enter mitosis. Gonialblasts (GBs), and are then arrested due to activation of the spindle assembly checkpoint (Fig. 3a, a'). Since colcemid treatment artificially increases centrosome misorientation, and arrests COC-proficient GSCs in G2 phase, and COC-defective GSCs in mitosis, respectively (Fig. 3a), it provides a sensitive and robust method to detect defective COC. For example, after 4.5 h of colcemid treatment of testes defective in COC (such as those from *par-1* or *cnn* mutants) >1 GSC per testis was found to be arrested in mitosis (Fig. 3c). This is in stark contrast to wild-type GSCs, which shows about 0.2 mitotic GSCs per testis (Fig. 3b). The mitotic index of SG from the same testes serves as a positive control of spindle assembly checkpoint-dependent mitotic arrest.

3.6 Generation of Heat-Shock-Induced Germline Clones

1. Cross *hs-FLP, nos-FRT-stop-polyA-FRT-gal4, UAS-GFP* [16] females with *UAS-RNAi* males for experimental or wild type males for control crosses.
2. Heat shock progeny at days 0–2 after eclosion.

3. Dissect testes at various time points (for example, 3, 7, 14, 21 days) after heat shock. To identify the GSC clones, immunofluorescence staining of the testes using the primary antibody combination Vasa and Fas III can be used together with GFP.

Although other genetic schemes to induce GSC clones can be used for the same purpose, the use of *hs-FLP*, *nos-FRT-stop-polyA-FRT-gal4*, *UAS-GFP* has two unique advantages: (1) clonal induction is limited to germ cells, and thus there will be no complication due to clonal induction in somatic gonadal cells (cyst cells and hub cells). (2) Any *UAS* transgenes can be introduced into this genetic scheme easily, and thus the effect of gene overexpression or RNAi-mediated knockdown on ACD can be easily tested (see below for more detail) [14].

Generation of mosaic clones in an otherwise wild-type background has served to investigate cell autonomy of gene function. Also, mosaic clones can be used to assess gene function in adult tissues, even when homozygous animals are lethal at earlier developmental stages. It has been shown that GSCs compete for niche occupancy, which can generate apparently inconsistent results, depending on whether a mutant exists as a mosaic clone or homogeneous population of GSCs. For example, GSC clones mutant for JAK-STAT signaling components (such as *Hop*, *Dome*, *STAT92E*) are quickly lost from the niche, leading the authors to suggest that these genes are essential for GSC self-renewal [17, 18]. However, it was later shown that GSCs mutant for *STAT* can be well maintained if all of GSCs are mutant for *STAT* [19]. These results revealed the necessity of addressing gene functions using multiple experimental paradigms. In this context, an experimental caveat is the fact that the GSC clones are typically generated by FLP-mediated mitotic recombination of homologous chromosomes using mutant alleles, whereas *nos-gal4*-mediated RNAi is used to deplete the gene product from all GSCs. When inconsistent results are obtained between FRT clones of mutant alleles and *nos-gal4 > UAS-RNAi*, it is unclear whether it is due to the gene's requirement in GSC competition (but not self-renewal) or different strengths of the allele vs. RNAi used. By using *hs-FLP*, *nos-FRT-stop-polyA-FRT-gal4*, *UAS-GFP*, the maintenance of RNAi clones can be directly compared to "global knockdown" of the gene (*nos-gal4 > UAS-RNAi*), and the gene's requirement in GSC competition can be unambiguously tested.

3.7 Assessment of Asymmetric vs. Symmetric Stem Cell Division

Although spindle misorientation may lead to symmetric GSC divisions to generate two GSCs, spindle misorientation is not an accurate predictor of symmetric outcome. It has been reported that gonialblast can crawl back to the niche, acquiring hub attachment to yield two GSCs, even after GSCs have undergone mitosis with correctly oriented spindles [20]. Conversely, spindle misorientation

does not necessarily lead to symmetric self-renewal, since one daughter of GSC division may eventually lose hub attachment and leave the niche. Therefore, it is important to assess asymmetric vs. symmetric GSC divisions, independently of spindle orientation. By following marked GSC clones, the frequency of symmetric division can be inferred. By using the transgenic line described above (*hs-FLP, nos-FRT-stop-polyA-FRT-gal4, UAS-GFP* [19]), the asymmetric vs. symmetric outcome of GSC division can be assessed. Upon heat-shock treatment, only a subset of GSCs undergo “FLP out” of the “FRT-stop-polyA-FRT” cassette, thereby expressing *nos-gal4, UAS-GFP*. Heat-shock treatments can be adjusted so that only one GSC per testis is a clone on average. Such a singlet clone can convert to a doublet if GSCs undergo symmetric self-renewal (Fig. 4). By using this method, we estimated that wild-type GSCs undergo symmetric self-renewal about 2 % of the time [16]. Although accurate frequency of symmetric self-renewal is obtained via mathematical modeling [16] by taking other parameters (such as the probability of two neighboring GSCs undergoing clonal induction independently) into account [16], high frequency of symmetric divisions can be easily detected by this method [14]. Prolonged chase time (>24 h) often leads to more variation of clone frequency possibly due to other parameters than symmetric division (e.g., cell adhesion, cell cycle difference). Thus, for detection of symmetric self-renewal, data collection at 24 h after heat-shock treatment is recommended.

Immunofluorescence staining of testes with primary antibody Vasa and Fas III together with detecting GFP-positive clone can be used to score the symmetric outcome of GSC division.

3.8 Microscopy of Living Tissue

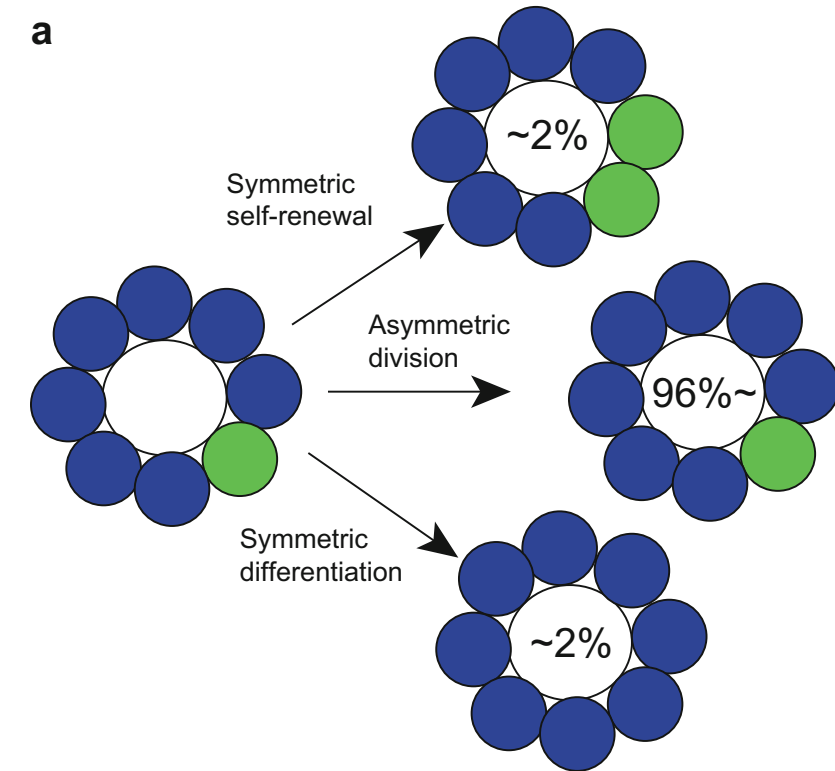
1. Dissect testes and transfer to Schneider’s insect medium.
2. Add vital dyes as necessary and incubate for desired time.

If using the membrane dye, FM4-64FX Lipophilic Styryl Dye (5 µg/ml, Molecular Probes), add the dye 1 min prior to analysis and perform imaging within 15 min. (Sample can be rinsed with PBS after dye incorporation if longer imaging is necessary. FM4-64FX is also fixable.)

If using the organelle dye LysoTracker or the DNA dye Hoechst 33342, then dissect the testes in PBS, incubate with LysoTracker (DND-99; Invitrogen) or Hoechst 33342 (2 µg/ml) for 30 min, and rinse with PBS twice prior to imaging.

3. Mount on the slide glasses with two etched rings (*see* Subheading 2) and cover with a cover slip.
4. Start imaging immediately. In most cases, we perform imaging within 30 min. *See* [21] for prolonged live imaging.

Whole living *Drosophila* testis can be observed under the conventional confocal microscope without isolating or fixing the cells.



Vasa (Germ cells), GFP

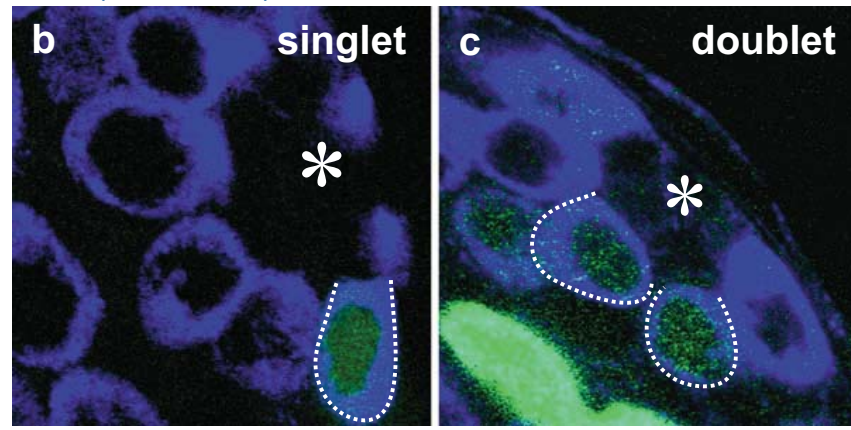


Fig. 4 Examining symmetric GSC divisions. (a) GFP⁺ clone is induced at low frequency (<single clone/testis) using hs-FLP, nos-FRT-stop-polyA-FRT-gal4, UAS-GFP following a 20–30-min heat-shock treatment. GFP clones are examined 24 h post-heat shock. 2 % of GSCs undergo symmetric self-renewal or symmetric differentiation. The remainder undergo asymmetric division (96 %). Symmetric self-renewal will generate doublet clones (*upper*, two GFP⁺ GSCs are juxtaposed to each other). (b, c) Representative images of singlet (b) and doublet (c) GSC clones

Fluorescent proteins (GFP, RFP, mCherry, etc.) and various vital dyes (e.g., styryl dyes, LysoTracker, Hoechst) are often combined. Figure 5a shows the architecture of hub-GSC area in which membrane lipids are visualized by FM4-64 styryl dye.

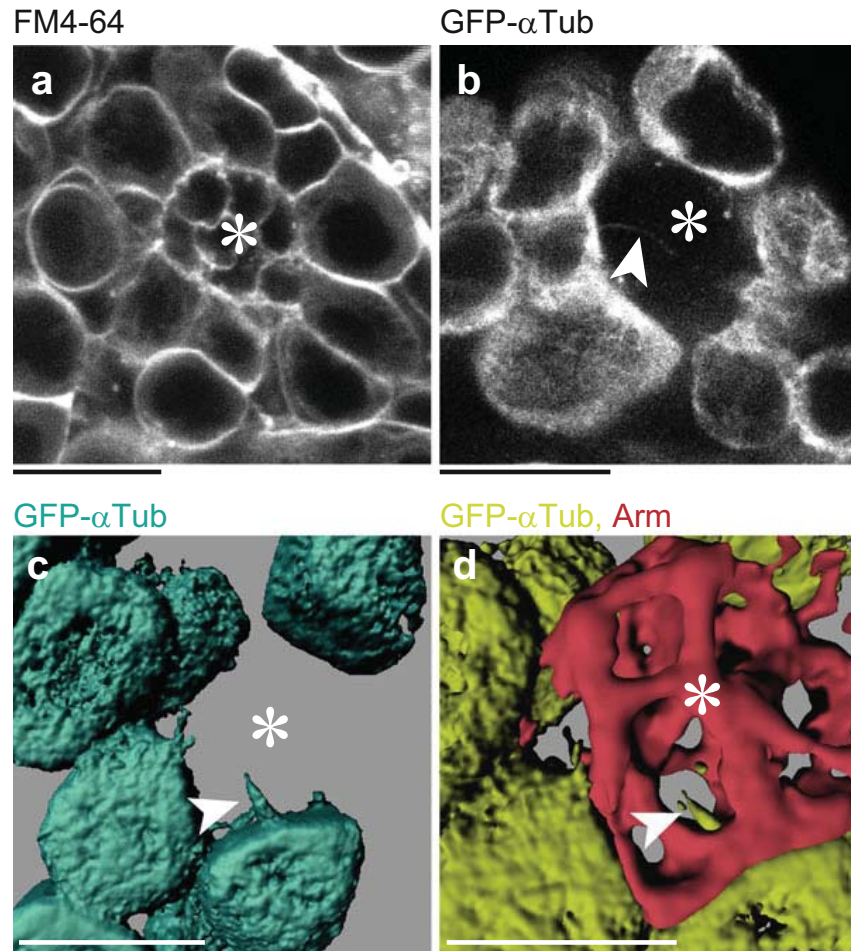


Fig. 5 Examples of live imaging. (a) FM4-64 dye clearly visualizes cell membranes in the testes. The image focuses on the hub-GSC area. (b) *UAS-GFP- α Tubulin* expressed under the germline-specific driver, *nos-gal4*, showed GSC-specific fixation-sensitive protrusions extended from the GSC towards the hub area (*arrowhead*). (c, d) Images of preserved MT-nanotubes in fixed tissue. (d) Arm (Armadillo, *red*) stains adherens junction of hub cells. 3D reconstitutions were performed by Imaris software. The hub is indicated by an asterisk (*). Scale bar: 10 μ m

Although long-term live observation (~ 20 h) requires additional equipment and care [21], short-term live observation (~ 30 min) can be conducted relatively easily (with regular confocal microscopy settings used for imaging of immunofluorescent staining) and enables the detection of fixation-sensitive structures. Recently, we reported microtubule-based cellular protrusions (MT-nanotubes) that extend from GSC towards the hub, using the GFP- α Tubulin transgene specifically expressed in germ cells (*nos-gal4* > *UAS-GFP- α Tubulin*) (Fig. 5b-d) [22]. MT-nanotubes are fragile structures and have escaped our detection in

fixed samples. As revealed by this example, microscopic structures can be missed due to sample processing, and the imaging of unfixed tissue will help identify such structures (*see* **Notes 4** and **5**).

4 Notes

1. FasIII usage is optional. Once eyes are trained, the hub is easily distinguishable by using DAPI staining as a cluster of ~15–20 cells with tightly packed nuclei and small amount of cytoplasm at the testis apical tip (Fig. 1b).
2. Histone H3 is phosphorylated at Thr-3 by haspin during prophase and dephosphorylated during anaphase. Phosphorylation at Ser-10 is maintained until anaphase/telophase and thus more suitable as a marker to detect all stages of mitosis.
3. GSCs can be identified as Vasa-positive germ cells directly attached to the hub (Figs. 1b and 2a). Typically 8–10 GSCs are found in each testis. GSCs are only ~7 μm in diameter, and the study of ACD involves detection of subcellular structure within GSCs. Furthermore, the study of ACD in the tissue context inevitably involves imaging of multiple cells (with many cells being positioned along the z -axis). In particular, for scoring of centrosome orientation (*see* below), the signal from neighboring cells (below/above the cells of interest) can be confusing. Accordingly, a microscope with sufficient resolution is required, and it will take some training to be able to judge localization of subcellular structures within cells, distinguishing them from signals from neighboring cells.
4. Later, we developed a fixation technique to preserve MT-nanotubes by adding a low concentration of taxol (1 μM) to the fixative (Fig. 5d).
5. 3D rendering data processing further helps understand geometric relationships among cells and subcellular structures. Figure 5c, d shows examples of MT-nanotubes extended from a GSC invaginate into hub cells. Images were processed with Imaris software (Bitplane) to reconstitute z -stacks.

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