Video Article

The Utility of Stage-specific Mid-to-late *Drosophila* Follicle Isolation

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Abstract

Drosophila oogenesis or follicle development has been widely used to advance the understanding of complex developmental and cell biologic processes. This methods paper describes how to isolate mid-to-late stage follicles (Stage 10B-14) and utilize them to provide new insights into the molecular and morphologic events occurring during tight windows of developmental time. Isolated follicles can be used for a variety of experimental techniques, including *in vitro* development assays, live imaging, mRNA expression analysis and western blot analysis of proteins. Follicles at Stage 10B (S10B) or later will complete development in culture; this allows one to combine genetic or pharmacologic perturbations with *in vitro* development to define the effects of such manipulations on the processes occurring during specific periods of development. Additionally, because these follicles develop in culture, they are ideally suited for live imaging studies, which often reveal new mechanisms that mediate morphological events. Isolated follicles can also be used for molecular analyses. For example, changes in gene expression that result from genetic perturbations can be defined for specific developmental windows. Additionally, protein level, stability, and/or posttranslational modification state during a particular stage of follicle development can be examined through western blot analyses. Thus, stage-specific isolation of *Drosophila* follicles provides a rich source of information into widely conserved processes of development and morphogenesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/50493/

Introduction

Each *Drosophila* ovary is composed of ~16 ovarioles, or chains of sequentially maturing egg chambers or follicles. Each follicle is composed of a single oocyte, 15 germ line derived nurse or support cells, and ~650 somatic cells termed follicle cells (**Figure 1A)**. *Drosophila* oogenesis is divided into 14 morphologically defined stages of development[1]. Each stage of follicle development is observed many times within a single fly, making it relatively easy to isolate a substantial number of stage-specific follicles.

The mid-to-late stages of oogenesis (Stages 10B-14) are particularly well suited for stage isolation (**Figure 1**). At Stage 10B (S10B), the follicle is fully elongated (*i.e.* its length is equal to that of a Stage 14 (S14) follicle, see **Figure 1** and **Figure 2H**) and half the length of the follicle is composed of nurse cells while the other half is the oocyte (**Figure 1C**). At this stage the nurse cells undergo dramatic actin remodeling, strengthening the cortical actin and generating parallel bundles of actin filaments[2]. At the same time, a population of follicle cells, termed centripetal cells, migrate in between the nurse cells and the oocyte, and two dorsal groups of follicle cells become specified to undergo migration to form the dorsal appendages, tubular respiratory apparatuses for the embryo[3]. The nurse cells then contract (S11), squeezing their cytoplasmic contents into the oocyte in a process called nurse cell dumping, which provides the oocyte with the factors necessary for it to complete embryogenesis (**Figure 1D**). The nurse cells then undergo cell death (S12-S13)[4], and the follicle cells secrete and pattern the eggshell[5] (**Figures 1E-G**). Thus, the end of oogenesis is rich with important developmental and morphogenetic processes.

Isolated mid-to-late stage follicles (S10B-S14) can be used for a variety of purposes, including molecular analyses. For example, mRNA from staged follicles can be isolated for RT-PCR, microarray, or RNA-seq analyses. This allows one to look at gene expression within a short developmental window, with only a few cell types present, and determine how gene expression is changed by either pharmacologic or genetic perturbations. Stage isolation can also be used to look at proteins by western blotting. Such analysis is important because it allows one to quantify the level of protein expression in wild-type versus mutants at specific stages. While one could use immunofluorescent analyses to achieve similar results, quantification of fluorescence is less robust due to the strict requirements that all of the pixels be within the linear range of detection[6]. Additionally, western blot analysis may provide other information, such as if the protein is posttranslationally modified or is expressed from a specific splice isoform. Isolated stages can also be used for further protein purification, including subcellular fractionation or coimmunoprecipitation.

Stage-specific follicle isolation can also be used for *in vitro* development assays[7] and live-imaging[8]. Isolated S10B-S13 follicles will continue to develop to S14 in simple culture media (see below). It is important to note that S10A follicles will not progress through nurse cell dumping using the culture conditions discussed in this manuscript. We have used S10B *in vitro* development assays to define the role of prostaglandins, both

pharmacologically and genetically, in regulating actin remodeling by using nurse cell dumping and development as read-outs[7,9]. Similarly, the later stages of development can also be isolated to determine the effects of pharmacologic treatments or genetic manipulations on particular processes such as centripetal cell migration, dorsal appendage migration/formation[10], and nurse cell death. Such assays can be used to perform dominant interaction screens or assays; for example, while heterozygosity for mutations in *pxt* or *fascin* alone have no effect on S10B *in vitro* development, follicles from double heterozygotes exhibit nurse cell dumping defects and a block in development[9].

Additionally, because S10B-13 can develop in culture, all of the processes that occur during this time can be observed by live-imaging. Such imaging can be performed simply using transmitted light (if one is only interested in gross changes in morphology) or with confocal microscopy using transgenic flies expressing fluorescent probes or follicles stained with live imaging dyes. Live imaging is being used to substantially advance our understanding of developmental processes. Indeed, live imaging of late stage follicles has expanded the knowledge of dorsal appendage migration, an example of tubulogenesis[10]. We expect that live imaging of additional late stage processes, including actin dynamics during nurse cell dumping, will provide novel insights into these developmental events. It is important to note that while S10A and early stages of follicle development will not continue to develop into a S14 in culture, live-imaging of events occurring during those stages of development is possible using alternative culture conditions[11-14] (see Discussion for more information).

Here we provide detailed protocols for isolating late stage follicles for either *in vitro* development and live-imaging, or molecular analyses (mRNA and protein isolation).

Protocol

1. Preparing *Drosophila* Prior to Stage Isolation

1. Make wet yeast paste by combining 50 g of active dry yeast with 90 ml of distilled water. Mix with a spatula to combine. Let the mixture stand for ~30 min before assessing consistency. The consistency should be just thick enough to adhere to the side of a fly vial but not run down the side. To achieve the desired consistency it may be necessary to add up to 10 ml of additional water or a small amount of dry yeast. Store in a covered container at 4 °C. The stored yeast can be used for ~1-2 weeks.
2. Collect <24 hr old adult flies (both males and females) of the desired genotypes and put them into a vial with fly food plus wet yeast paste smeared on the side of the vial. The temperature at which the flies are maintained will vary depending upon the experiment. For routine experiments, flies are maintained at room temperature. Whereas experiments in which a gene is being overexpressed using the UAS/GAL4 system[15] may require maintaining the flies at 25 °C or higher, as appropriate.
3. Provide the flies with a fresh smear of yeast paste daily for 2 days. NOTE: As follicle development is tightly regulated by the nutritional status of the female fly, it is essential to provide the fly with the nutrient rich yeast paste for at least 36 hr prior to dissection.

2. Isolating Mid-to-late Staged *Drosophila* Follicles

1. Allow the media to come to room temperature (~30 min). If the staged follicles are going to be used for *in vitro* development assays, freshly prepare IVEM media (Grace's insect media, 10% heat inactivated fetal bovine serum, and 1x penicillin/streptomycin). If the staged follicles are going to be used for mRNA or protein isolation use either Grace's insect or IVEM media. IMPORTANT: Media temperature is critical. If the media is cold it will alter both the actin and microtubule cytoskeletons and block development.
2. Anesthetize the flies in the yeasted vial by injecting CO_2 gas and place 3-5 female flies onto a fly pad under CO_2. IMPORTANT: Do not leave more females than can be dissected in a 10 min time period on the fly pad as extended exposure to CO_2 can cause sterility and death.
3. Fill one well of a 9-spot plate with media and place it on top of a dark surface (a piece of black plexiglass works well). Focus the dissecting scope on the bottom of the well.
4. Using #5 Dumont forceps pick up a single female with one hand (this is best done using the dominant hand). It is often easiest to pick up the female by the wings, legs, or thorax to abdomen transition. Submerge the female in the media filled well. Adjust the focus of the microscope as necessary.
5. Using a second pair of forceps held in the nondominant hand, grab the female at the anterior end of the abdomen so that the posterior end is positioned towards the dominant hand (**Figure 2A**). Make sure to keep the fly submerged in the media.
6. With the dominant hand, use the forceps to grab the cuticle at the 2nd most posterior pigmented segment and rip it away from the rest of the abdomen (**Figures 2B-C**).
7. Using the forceps in the dominant hand, gently squeeze the anterior end of the abdomen until the ovaries emerge **(Figures 2D-E)**. If necessary, place the forceps in between the two ovaries and pull them out of the carcass.
8. Either move the ovaries to a new well with fresh media **(Figure 2F)** or remove the carcass to a Kimwipe or paper towel. This is particularly important if follicles are being isolated for *in vitro* development.
9. Repeat until 3-5 pairs of ovaries have been isolated. NOTE: Each ovary is composed of ~16 ovarioles or chains of sequentially maturing follicles. Each ovariole is contained within a muscle sheath.
10. Using pin vises and the needles supplied with them, gently pull apart the ovary by running the needles between the ovarioles (**Figure 2G**). This will sometimes release the individual follicles as well.
11. Individual ovarioles and the easily distinguishable morphological stages of follicle development should now be visible (**Figure 2G**, teased apart ovary). Refer to **Figures 1** and **2H** for morphological differences that can be used to distinguish the different stages. To isolate follicles that are within intact ovarioles and still contained within a muscle sheath, use a needle to cut across the ovariole at the anterior of the preceding follicle and at the posterior of the follicle immediately following the follicle of interest. This will break the muscle sheath and the follicle of interest away can now be cut away from the neighboring follicles without damaging it.
12. As individual stages are separated, move the follicles of interest, using a glass pipette, to a new well with fresh media. This is particularly important when isolating follicles for *in vitro* development. NOTE: It is important to squeeze the pipette bulb before entering the media to prevent the air bubbles from redistributing the follicles of interest throughout the well. Additionally, if the follicles are sticking to the glass pipette, the pipette can be precoated with 3% bovine serum albumin (BSA) by pipetting the BSA solution up and down several times and rinsing with dissecting media.

13. Once enough follicles have been isolated, proceed with subsequent experiments.

3. *In vitro* Development of S10B Follicles

1. Isolate S10B follicles using the stage isolation protocol (step 2) in IVEM media. Make sure that the follicles are rapidly moved away from debris. As these follicles will continue to mature, it is important that S10Bs are collected for 30-60 min and then placed into the maturation media of choice. NOTE: S10B need to be carefully distinguished from S10A follicles (**Figure 1C** compared to **1B**), as S10A follicles will not mature in the IVEM culture media. In both S10A and S10B follicles, half of the length is composed of nurse cells and the other half is the oocyte, but in S10B the length of the follicle is equal to that of the S14 follicles.
2. Using a glass pipette, move ~30 S10B follicles into a well of a 24-well tissue culture plate. NOTE: Make sure to verify that all of the follicles being transferred are S10B and have not matured to S11.
3. Prepare 1 µl of maturation media per well, *i.e.* IVEM media plus pharmacological reagents.
4. Using a pulled glass pipette*, remove as much media from the well (step 3.2) as possible, and quickly add maturation media of choice. NOTE: It is essential to use a pulled glass pipette as staged follicles can be easily taken up with either glass pipettes or pipette tips with a Pipetman.
   *To make pulled pipettes: 1) heat the thin portion of long, glass pipettes in the flame of a Bunsen burner just until the glass begins to soften; 2) immediately move the pipette out of the flame and pull horizontally to draw the pipette into a finer tube; 3) break to generate a fine point. CAUTION: Use eye protection as fragments of glass may fly off.
5. Repeat steps 3.1-3.4 for as many wells as the experiment requires.
6. Allow follicles to develop for >10 hr and score the developmental progression under a dissecting scope. NOTE: Experiments are usually scored the next day to allow sufficient time for the follicles to develop. S10B is ~5 hr, S11 is ~30 min, S12 is ~2 hr, and S13 is ~1 hr in duration at 25 °C[1] and development at room temperature will take slightly longer. Similar experiments to that described for S10B follicles can be performed using S11-S13 follicles.

4. Stage Isolation for Live Imaging

1. Isolate late stage follicles (S10B-14) expressing the fluorescent marker of choice using the stage isolation protocol (step 2) in IVEM media, quickly moving follicles away from debris.
2. Move follicles of interest into new media and then transfer by glass pipette to a coverslip bottom Petri plate. Take care to add media so that it bubbles up in the coverslip bottom area but does not spill over.
3. For longer time-lapse movies, it is sometimes necessary to maintain humidity within the Petri plate by adding a rolled up Kimwipe, moistened with water, to the inside edge of the Petri plate. Place the lid on top.
4. Image on an inverted microscope. Detailed resolution will require confocal microscopy. It is necessary to balance frequency of imaging, strength of illumination, and length of imaging; this will need to be independently worked out for each labeling tool and developmental process.

5. Stage Isolation for mRNA Preparation

1. Isolate individual stage follicles using the stage isolation protocol (step 2) with either Grace's or IVEM media.
2. Using a glass pipette, move the individual stages of interest into new wells with media; it is possible to collect multiple stages at once.
3. Keep collection times under 1 hr. Move the follicles, using a glass pipette, to 1.5 ml microfuge tubes.
4. Spin the tube briefly in a mini-microcentrifuge to pellet all of the follicles. Using a pulled glass pipette (see step 3.4) carefully remove all of the media. NOTE: If using a full size microcentrifuge, spin down the follicles at low speed.
5. Add 100 µl of Trizol and grind by hand for ~20 sec using a plastic pestle. Spin down at full-speed in a microcentrifuge and move Trizol to a new 1.5 ml microfuge tube being careful not to disturb any pelleted debris. Store at -80 °C.
6. Repeat steps 5.1-5.5 until enough follicles have been obtained for the experiment. Routinely, ~75 S10B, ~75 S12, and ~100 S14 follicles yield ~10 µg of RNA.
7. Thaw the samples on ice. Combine samples, as appropriate, into one microfuge tube and bring the Trizol volume up to 800 µl. Proceed with RNA isolation as directed by the manufacturer. IMPORTANT: Remember to DNase treat the isolated RNA with RNase free DNase.

6. Stage Isolation for Western Blotting

1. Preheat a heat block to 100 °C.
2. Isolate individual stage follicles using the stage isolation protocol (step 2) with either Grace's or IVEM media. NOTE: It is necessary to empirically determine how many of a particular stage follicle are needed to observe each specific protein. For highly expressed proteins 1-3 S10B follicles/well is enough to see a strong signal on a western blot. However, it is important to make a representative sample, taking follicles from multiple females. Thus, 15-20 follicles of a particular stage are usually collected.
3. Move follicles, using a glass pipette, to a 1.5 ml microfuge tube. Spin briefly in a mini-microcentrifuge to pellet the follicles (see step 5.4). Carefully remove all of the media using a pulled glass pipette (see step 3.4) and add 50 µl of 1x PBS and 50 µl of 2x Laemmli buffer.
4. Grind by hand for ~20 sec with a plastic pestle. NOTE: Plastic pestles can be reused for grinding western samples by washing and autoclaving them.
5. Boil the samples for 10 min in the heat block.
6. Chill briefly on ice and spin at full-speed for 15 sec in a microcentrifuge. Either immediately load onto a SDS-PAGE gel or store at -20 °C. NOTE: If the samples are stored, remember to reboil the samples before loading onto the gel.
7. Perform western blot analysis following standard protocols.

Representative Results

When isolating specific stages of *Drosophila* follicle development it is essential to be able to accurately distinguish the different morphological stages. This is somewhat challenging for S10A and S10B, as the nurse cells and the oocyte each take up half the length of the follicle at these stages (**Figure 1B** compared to **1C**). However, S10A follicles are shorter in length than S10B follicles, as the S10B follicles are fully elongated and thus equal in length to a S14 follicle (**Figure 1C** compared to **1G**). Additionally, a subset of the follicle or somatic cells over the oocyte called centripetal follicle cells begin to migrate in between the nurse cells and the oocyte at S10B. During S10B, which takes ~5 hr, the nurse cells undergo dynamic actin remodeling so that at S11 the nurse cells contract, squeezing their cytoplasmic contents into the oocyte. Thus at S11, the nurse cell region has significantly decreased, while the oocyte has expanded (**Figure 1D**). At S12, the nurse cells begin to undergo death and take on a more opaque appearance (**Figure 1E**); interestingly, in culture, nurse cell remnants curl dorsally in S12 follicles (see S12s in **Figures 3C'-D'**). During S13, the nurse cell region is transparent as cell death is being completed, and dorsal appendage formation is occurring (**Figure 1F**). By S14 only the oocyte and the follicle cells remain, and dorsal appendage formation is complete (**Figure 1G**).

The *in vitro* development assay can be used to assess the consequences of various pharmacological treatments and genetic mutations on S10B development and nurse cell dumping (**Figure 3**). The development of S10Bs in culture is robust, however a number of factors can lead to poor experimental results. In **Figure 3A,** experimental data from both a successful and a failed experiment are provided. A successful experiment is one in which 80-100% of the wild-type follicles in control media complete nurse cell dumping and progress to S12-14 (**Figure 3A**, wt 1). Occasionally wild-type controls will fail, meaning that fewer than 80% of the follicles develop (**Figure 3A**, wt 2). This failure can be caused by a number of issues including: 1) inability to distinguish S10A from S10B follicles (refer to **Figures 1** and **2**), 2) media problems (temperature, age, *etc*.), and/or 3) follicles were exposed to debris for too long.

In vitro development of S10Bs can be used in combination with pharmacologic treatment. For such experiments, it is important that drug controls, *i.e.* treatment of wild-type follicles with the drug, are performed with every experiment because drug effectiveness and/or concentration can change with time. For pharmacologic experiments, it is convenient to analyze the ratio of the percentage of follicles developing in the drug treatment to that in control media; this controls for slight genetic background differences. It is often useful to treat with the IC_{50} concentration of the drug; this is the concentration that blocks 50% of wild-type (*yw*) follicles from undergoing nurse cell dumping and further development. Thus, the ratio for wild-type follicles is expected to be 0.5 (**Figure 3B**, drug 1). **Figure 3B** is an example of how a drug (aspirin) can become too concentrated (drug 2; likely due to solvent evaporation) and block greater than 50% of the wild-type follicles from developing. To further illustrate the *in vitro* development assay, images of two wells of developing S10B follicles are provided. **Figures 3C** and **D** illustrate the S10B follicles at the beginning of the assay, in control or aspirin treated (~2 mM) media. **Figures 3C'** and **D'** illustrate the end of the assay, revealing that the majority of the control treated follicles developed to S14, while the majority of the aspirin treated follicles have not completed nurse cell dumping.

The *in vitro* development assay can also be used to screen for genetic backgrounds that are more sensitive to a particular drug. **Figure 3E** contains two examples of this. In the first example, the genetic background (expt1, blue bar) fails to interact with aspirin as the ratio remains ~0.5; conversely, in the second example, the genetic background (expt2, red bar) enhances the effect of aspirin. Thus, the *in vitro* development assay can be used to define the consequences of mutations and pharmacologic reagents on the developmental and morphological events occurring during late oogenesis; additionally, the assay can be used to assess both pharmacologic and genetic interactions (see[9]).

Stage isolation can also be used for live imaging of developmental processes. **Figure 4** is an example of a time-lapse movie of a S10B follicle expressing Utrophin-GFP, the actin binding domain from human Utrophin fused to green fluorescent protein. Using this imaging tool, actin bundle formation and condensation can be visualized. Many tools are available for live imaging, including protein trap transgenic lines[16,17], UAS driven fluorescently tagged organelle markers (mitochondria, golgi, endoplasmic reticulum, *etc*.), UAS driven fluorescently tagged cytoskeletal markers (actin and actin binding proteins, microtubule and microtubule binding proteins), transgenic lines expressing any fluorescently tagged protein of interest, and vital dyes (Nile Red labels neutral lipids, Edu labels replicating DNA, FM4-64 labels membranes).

Molecular analyses can be performed using isolated stages of *Drosophila* follicles. For protein analysis by western blotting, it is necessary to empirically determine how many follicles, of a particular stage of development, are required to observe the protein of interest. **Figure 5** is an example of how to sequentially dilute a concentrated protein lysate to determine the number of S10B follicles needed to observe a particular protein, Fascin. In this case one S10B follicle is sufficient to observe this protein by western blotting.

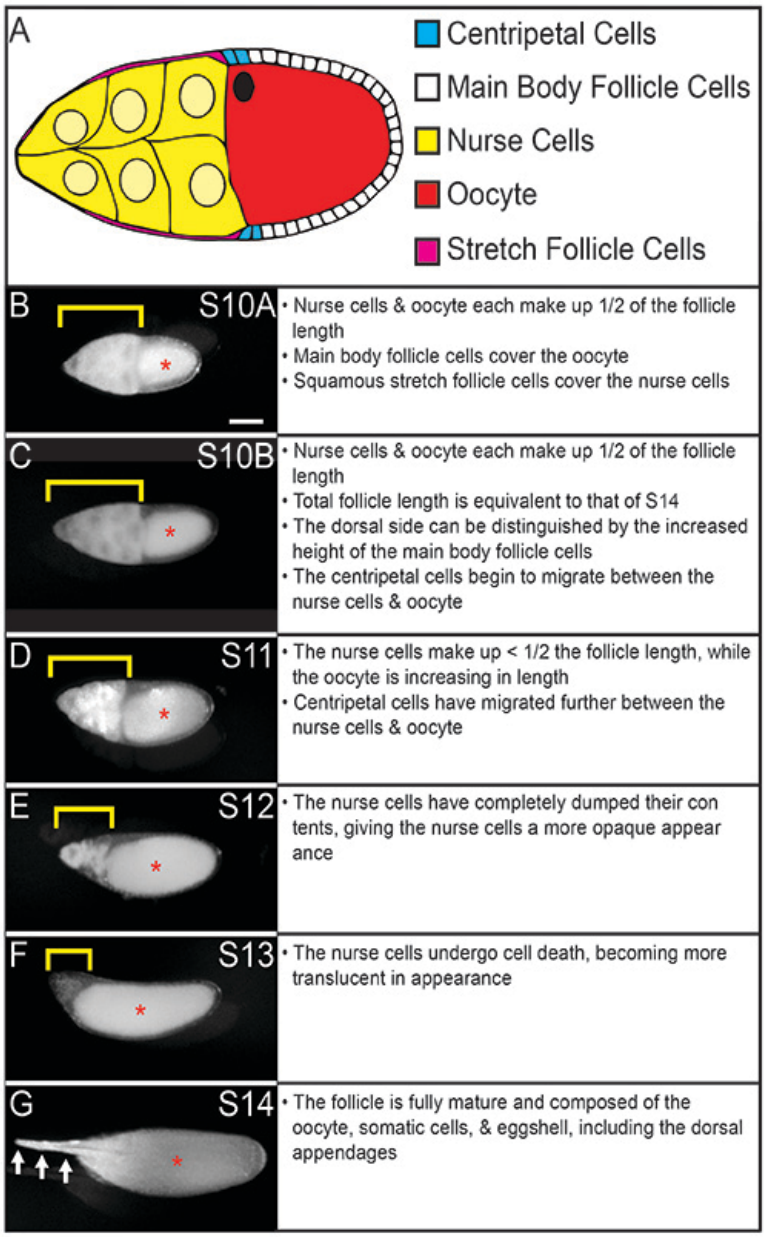

Figure 1. Diagram describing the cellular composition of *Drosophila* follicles and images illustrating the morphological difference of mid-to-late stage *Drosophila* follicles. A. Diagram depicting the cellular composition of a S10A follicle. **B-G.** Representative images of S10A-S14 *Drosophila* follicles taken using a stereo dissecting scope. The *Drosophila* follicle consists of 16 germline-derived cells: 1 oocyte (red, **A**) and 15 nurse or support cells (yellow, **A**), which are surrounded by ~650 somatically-derived epithelial cells (white, magenta, and cyan, **A**). At S10A, one half of the length of the follicle is composed of the nurse cells (yellow bracket, **B**), which are covered by the stretch follicle cells (magenta in **A**), and the other half is composed of the oocyte (red asterisk, **B**), which is covered by the main body follicle cells (white in **A**). At S10B, the nurse cells (yellow bracket, **C**) and oocyte (red asterisk, **C**) each compose half of the length of the follicle, however the overall length of the follicle is now equal to that of a S14 follicle (compare **C** to **G**). During S11, the nurse cells (yellow bracket, **D**) rapidly squeeze their cytoplasmic contents into the elongating oocyte (red asterisk, **D**) in an actin/myosin-dependent process termed nurse cell dumping. By S12, the oocyte (red asterisk, **E**) has fully elongated as nurse cell dumping is complete and only nurse cell remnants remain (yellow bracket, **E**). The nurse cell remnants (yellow bracket, **F**) complete cell death at S13. S14 represents the fully mature follicle, which is composed of the oocyte (red asterisk, **G**), somatic cells, and eggshell, including the dorsal appendages (white arrows, **G**). **B.** Scale bar = 0.1 mm.

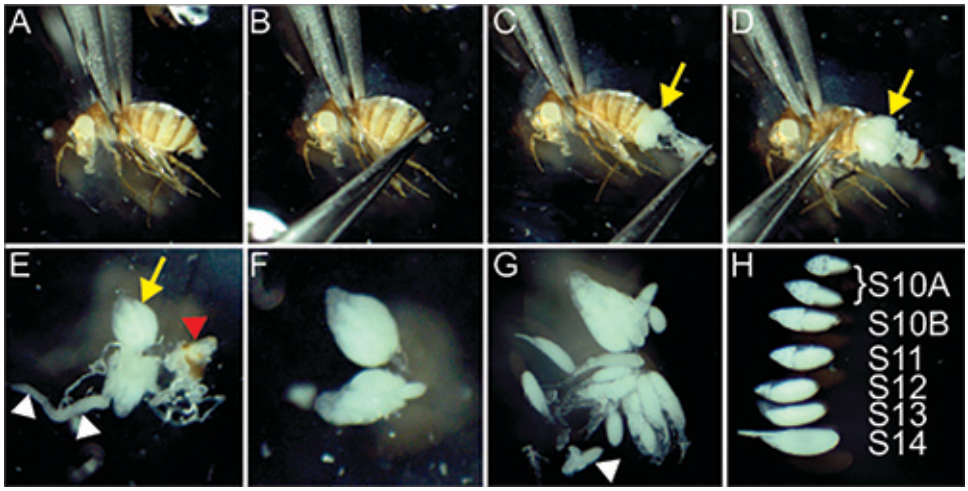

Figure 2. Images providing an overview of ovary dissection and follicle isolation. A. Submerge the fly in the dissecting media and orient it so that it is held, by forceps, in the nondominant hand. **B.** Using the dominant hand, grab the cuticle at the posterior of the abdomen. **C.** Pull the cuticle off, exposing the ovaries (yellow arrow). **D.** Working from the anterior of the abdomen toward the posterior, gently squeeze the abdomen releasing the ovaries (yellow arrow). **E.** Separate the ovaries (yellow arrow) from any remaining cuticle (red arrowhead) and/or internal organs (white arrowheads). **F.** Transfer the isolated ovaries to a fresh well containing dissecting media. **G.** Tease apart the ovaries, using dissecting needles, to expose individual follicles (compare intact ovary (top) to separated ovary (bottom). White arrowhead points to a stabbed S10B follicle, an example of a follicle that should not be used for subsequent experiments. **H.** Select the morphological stages of interest (Stages 10A-14 (S10A-S14) shown).

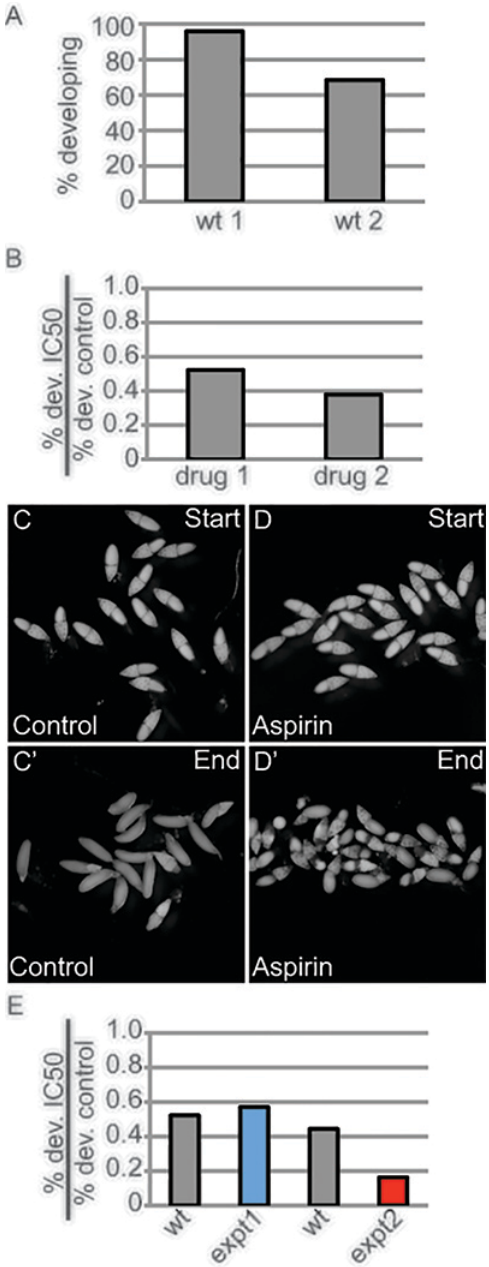

Figure 3. Examples of *in vitro* development assays. A. Chart of the percentage of S10B follicles that complete development in culture. wt 1 is an example of expected development of wild-type S10B follicles (96% developing), while wt 2 is an example of poor development in culture (68% developing). We would typically discard the whole experiment if the development of wild-type S10B follicles in control media is below 80%. **B.** Chart of the ratio of the percentage of S10B follicles developing (dev.) in media containing the IC_{50} for aspirin to the percentage developing in control media. The IC_{50} is the concentration that blocks 50% of the S10B follicles from developing; this means the wild-type value should be ~0.5. Drug 1 is an example of the expected wild-type ratio (0.52), while drug 2 is an example of what happens when the drug concentration is too strong (0.37). **C-C'.** Images of *in vitro* development wells. **C-C'.** Control treated. **D-D'.** Aspirin treated (~2 mM). **C-D.** Image of the S10Bs at the start of the assay. **C'-D'.** Image of the follicles at the end point of the assay. Control treated S10B follicles develop to S14s in culture (**C'**), while the majority of aspirin treated follicles fail to complete nurse cell dumping (**D'**). **E.** Chart of the ratio of the percentage of S10B follicles developing (dev.) in media containing the IC_{50} for aspirin to the percentage developing in control media. This is an example of two experiments looking for pharmaco-interactions. The expt1 (blue bar) mutant does not alter the effect of the IC_{50} for aspirin (0.57), while the expt2 (red bar) mutant enhances the effect of aspirin (0.16).

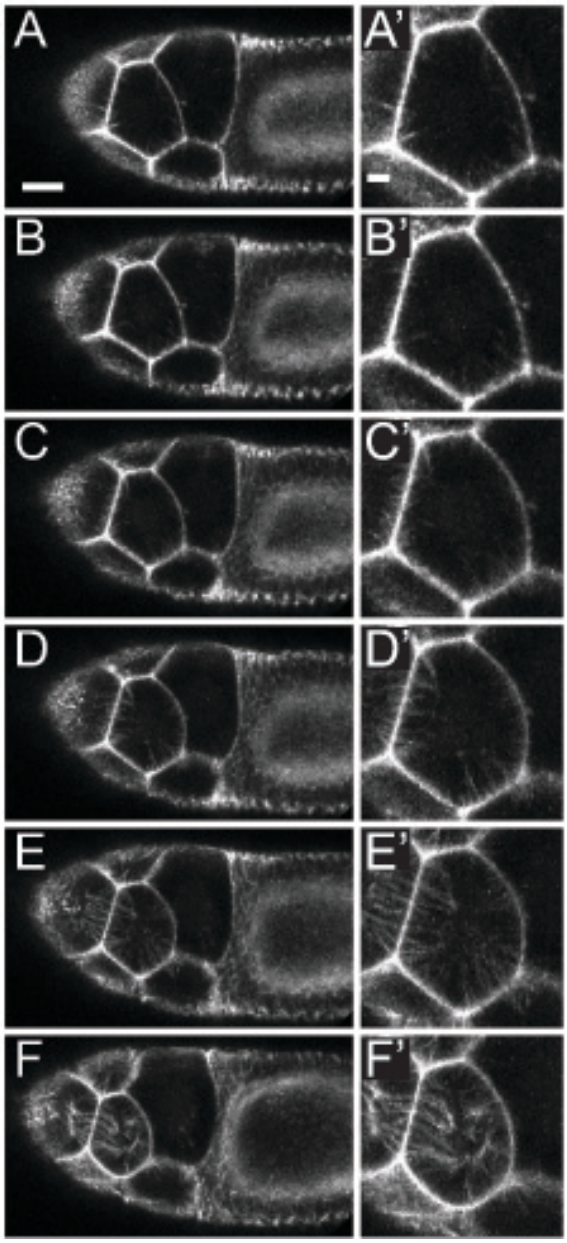

Figure 4. Example showing the use of isolated S10B follicles for live imaging. A-F. Maximum projections of 3 slices from time-lapse z-stacks of a S10B follicle isolated from Utrophin::GFP expressing transgenic flies (*sqh-Utrophin::GFP*). **A'-F'.** Magnified insets highlight a single nurse cell from **A-F. A-F'.** F-actin (Utrophin::GFP), white. **A, A'.** time (t) = 0 min. **B, B'.** t = 20 min. **C, C'.** t = 40 min. **D, D'.** t = 60 min. **E, E'.** t = 80 min. **F, F'.** t = 100 min. At the initial time point, short actin filaments can be observed extending inwards from the nurse cell membranes (**A, A'**). These actin filaments elongate throughout the early time points (**B-C'** compared to **A, A'**) until they are fully elongated (**D, D'**). In later time points, these fully elongated actin filaments then shorten and condense throughout nurse cell dumping (**E-F'**) **A.** Scale bar = 50 µm. **A'.** Scale bar = 10 µm.

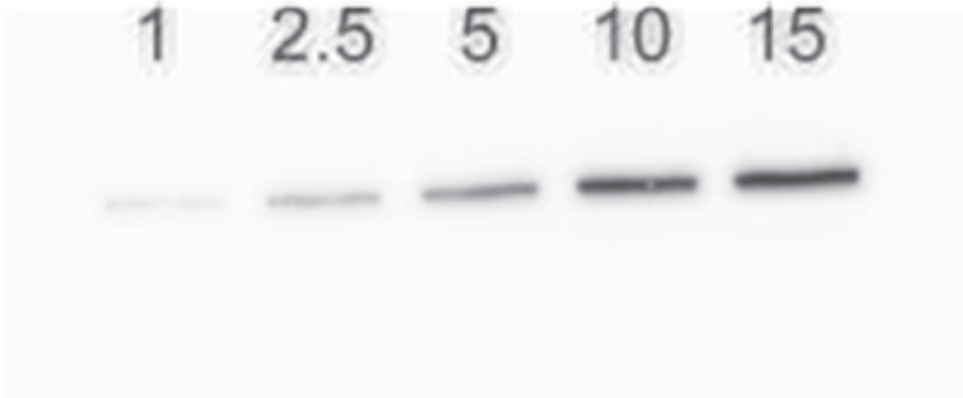

Figure 5. Illustration of how to determine the number of follicles needed to examine a particular protein by western blot analysis. This is a western blot looking at Fascin expression in S10B follicles (1:20, sn 7C, Cooley, L.; Developmental Studies Hybridoma Bank (DSHB), developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA, 52242). The numbers across the top represent the approximate number of S10B follicles loaded per lane.

Discussion

The *Drosophila* follicle is composed of only a small number of cell types, making it ideal for both morphologic and molecular analyses. Furthermore, due to the structure of the ovary, it is relatively easy to obtain large numbers of specific stages of follicle development with a common dissecting scope and minimal training. As each stage represents a short temporal window, stage isolation can provide significant molecular insights into the developmental processes occurring during that stage. For example, we have used mid-to-late stage follicle isolation to characterize the gene expression changes occurring during S10B, S12, and S14[18]. This analysis suggests a number of previously uncharacterized genes are likely to contribute to follicle development and, in particular, eggshell formation.

Stage isolation can provide dramatic insights into morphological events through live imaging. Such imaging can be performed on all stages of *Drosophila* follicle development. While the focus of this work is S10B-14, readers are referred to the following articles for live-imaging of earlier stages: germarium[13], follicle elongation[19], and stage 9[11,12,14]. The culture conditions utilized in these studies are distinct from those discussed in this work. Specifically, these studies used Schneider's Insect Media with variable levels of FBS (2.5-15%) as well as the addition of insulin[11-13,19] and, in some cases, the further addition of trehalose, methoprene, 20-hydroxyecdysone, and adenosine deamidase[14]. It is important to point out that these live imaging studies have significantly advanced our understanding of the events occurring during these earlier stages of development. However, these early stage follicles cannot progress all the way to the final stage of follicle development, S14. Conversely, S10B-13 will develop to S14 in culture.

During S10B-S14 many morphological processes occur that can be studied by live imaging. For example, live imaging can be used to examine the process of nurse cell dumping, the process by which the nurse cells squeeze their cytoplasmic contents into the oocyte to provide it with everything it needs to complete embryogenesis. Nurse cell dumping can be observed by time-lapse imaging using transmitted light 7 or by confocal imaging using transgenic lines expressing fluorescent markers (see **Figure 4**). Continued use of live imaging is expected to provide novel insights into the cytoskeletal dynamics necessary for nurse cell dumping. Live-imaging of later stages has also advanced our understanding of dorsal appendage primordia migration and tubulogenesis[10].

In vitro development of S10B-S13 follicles can be used to define the effects of both pharmacologic reagents and genetic manipulations on the processes occurring during this time. We have used *in vitro* development of S10B follicles to establish the role of prostaglandins in regulating nurse cell dumping[7]. Subsequently we have been using this assay to perform a pharmacologic-interaction screen; specifically, we have been testing if loss of one copy of an actin regulator enhances or suppresses the nurse cell dumping defects due to the loss of prostaglandins. This screen has revealed a number of putative downstream targets ([9] and Spracklen, Meyer, and Tootle, unpublished data). The assay can also be used to examine genetic interactions by assessing developmental defects, such as a block in nurse cell dumping, due to heterozygosity for two different factors (for an example of this see[9]).

While isolating mid-to-late stage *Drosophila* follicles is a fairly simple process, there are a number of critical factors for optimum success. First, the preparation of the flies is very important. It is best to maintain different genotypes of flies in as similar a condition as possible, *i.e.* the number of flies per vial, the ratio of females to males (2:1 ratio is ideal), and provide fresh, wet yeast consistently. The feeding (wet yeast) and dissection time will alter the prevalence of the different stages of development. We have found that when the flies are consistently fed in the morning, more S10Bs can be isolated in the morning, while more S12s are present in the afternoon. A second critical factor is the media. For *in vitro* development and live imaging it is essential to prepare fresh IVEM media. Additionally, the media must come to room temperature as cold media will alter the cytoskeletons, both actin and microtubules, and therefore, disrupt further development. It is also important to keep the follicles away from debris. Sometimes, during the dissection, the gut will come out with the ovaries. We have found that if the gut is ruptured and the follicles are kept in the contaminated media, the follicles are unlikely to develop in culture. As the follicles continue to develop in culture, it is essential to reverify the staging after dissection before proceeding with either *in vitro* development or molecular analyses. Lastly, it is important to have proper controls for the experiments. For *in vitro* development, it is necessary to use wild-type follicles to test that the media allows for normal development (80-100% complete nurse cell dumping), and to test that pharmacologic reagents act as expected (see **Figure 3**).

In conclusion, isolation of mid-to-late stage *Drosophila* follicles can provide significant insight into developmental processes through a variety of experimental techniques.

Table of Specific Reagents and Equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
Active Dry Yeast	Genesee Scientific	62-103	Any source of Active Dry Yeast is fine
Grace's Insect Media	Lonza	04-457F	
Heat Inactivated Fetal Bovine Serum	Atlanta Biologicals	S11050H	Any Heat Inactivated FBS should work
10x Pen/Strep	Gibco/Invitrogen	15140-122	
Pin Vises and Needles	Ted Pella, Inc.	13561-10	
Spot Plate, Nine Well	Corning	7220-85	
#5 Dumont forceps	Fine Science Tools	11252-20	
24 multi-well plates	Becton Dickinson	35 3226	Any 24-well tissue culture dish should work
Coverslip Bottom Dishes (35 mm)	MatTek Corporation	P35G-1.0-14-C	Coverslip thickness will depend on the microscope/objective being used
Glass pipettes	Corning	7095B-5x (for transferring follicles) 7095B-9 (for producing pulled pipettes)	
Sample pestle (1.5 µl; RNase/ DNase free)	Research Products International	199228	Any plastic pestle that fits 1.5 µl microfuge tubes can be used
Trizol	Invitrogen	15596-018	

Disclosures

There is nothing to disclose.

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