# Chapter 4

# In Vitro Culturing and Live Imaging of *Drosophila* Egg Chambers: A History and Adaptable Method

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## Abstract

The development of the *Drosophila* egg chamber encompasses a myriad of diverse germline and somatic events, and as such, the egg chamber has become a widely used and influential developmental model. Advantages of this system include physical accessibility, genetic tractability, and amenability to microscopy and live culturing, the last of which is the focus of this chapter. To provide adequate context, we summarize the structure of the *Drosophila* ovary and egg chamber, the morphogenetic events of oogenesis, the history of egg-chamber live culturing, and many of the important discoveries that this culturing has afforded. Subsequently, we discuss various culturing methods that have facilitated analyses of different stages of egg-chamber development and different types of cells within the egg chamber, and we present an optimized protocol for live culturing *Drosophila* egg chambers.

We designed this protocol for culturing late-stage *Drosophila* egg chambers and live imaging epithelial tube morphogenesis, but with appropriate modifications, it can be used to culture egg chambers of any stage. The protocol employs a liquid-permeable, weighted "blanket" to gently hold egg chambers against the coverslip in a glass-bottomed culture dish so the egg chambers can be imaged on an inverted microscope. This setup provides a more buffered, stable, culturing environment than previously published methods by using a larger volume of culture media, but the setup is also compatible with small volumes. This chapter should aid researchers in their efforts to culture and live-image *Drosophila* egg chambers, further augmenting the impressive power of this model system.

**Key words** *Drosophila*, Ovary, Oogenesis, Egg chamber, Germline, Oocyte, Nurse cell, Somatic follicle cell, Live culturing, Live imaging, Confocal microscopy, Epithelial tube morphogenesis

## 1 Introduction

To understand and appreciate the live culturing methods discussed in this chapter, it is first necessary to have a basic knowledge of the *Drosophila* ovary, the *Drosophila* egg chamber, and the process of *Drosophila* oogenesis (Fig. 1). The female fruit fly possesses two artichoke-shaped ovaries, which are joined by a common oviduct and which each contain 15–20 ovarioles (i.e., parallel strings of developing egg chambers). The end of the ovariole distal to the oviduct contains a structure called the germarium, which holds the

Ioannis P. Nezis (ed.), *Oogenesis: Methods and Protocols*, Methods in Molecular Biology, vol. 1457,
DOI 10.1007/978-1-4939-3795-0_4, 

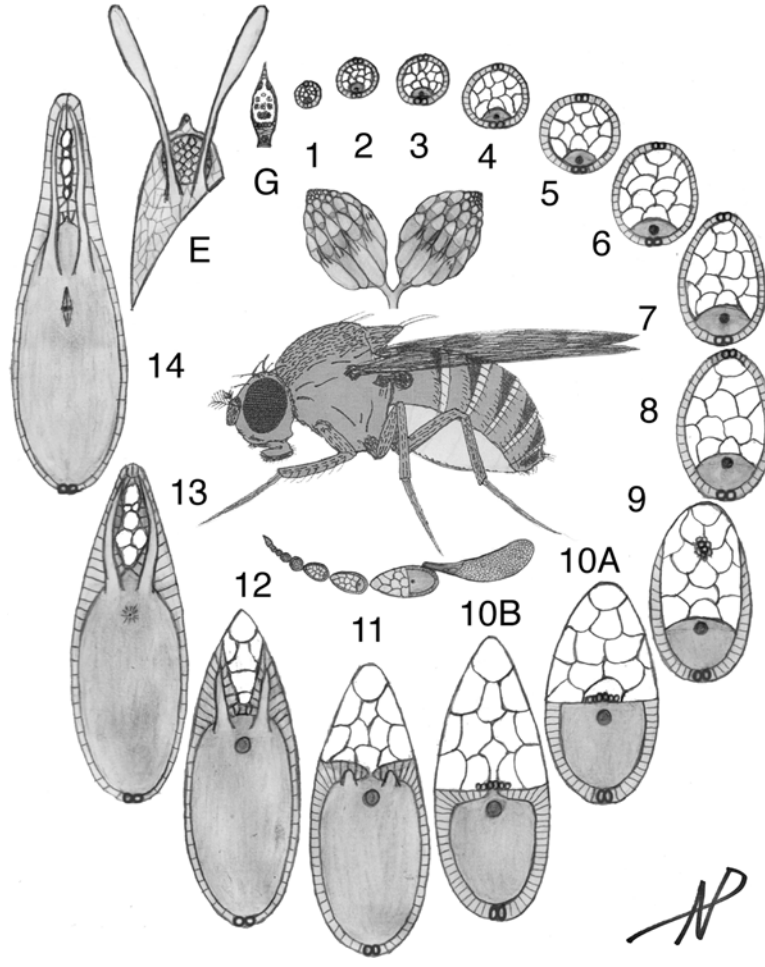


**Fig. 1** Illustrations of the stages of *Drosophila melanogaster* oogenesis, arranged in a clockwise fashion around an adult female (*center*), an ovary pair (*above*), and an individual ovariole (*below*). Egg chambers are created in the germarium (G), which contains the germline and somatic stem cells. Egg-chamber development proceeds through 14 canonical stages (S1–S14) and concludes with the production of a mature egg (E), of which only the anterior portion is shown here. These illustrations are not intended to be absolutely precise in every respect, but are meant to illustrate the most obvious features that a researcher could use to distinguish egg chamber stages in a laboratory setting. These features include egg-chamber shape and relative size, the proportion of oocyte volume to total nurse-cell volume, and the morphology of the anterior end of the egg chamber when the secondary eggshell structures, such as the dorsal appendages, are created late in oogenesis

germline and somatic stem cells and their associated support cells. In the germarium, each germline stem-cell division produces a cystoblast that undergoes four incomplete mitotic divisions to produce an interconnected, 16-cell germline cyst. One cell becomes the oocyte, while the remaining cells become the oocyte's supporting nurse cells. Approximately 80 somatic follicle cells produced by the somatic stem cells of the germarium then encapsulate each germline cyst in a monolayer epithelium (apical faces inward towards the oocyte, basal faces outward); this epithelium will eventually differentiate into distinct cell types and synthesize the eggshell around the oocyte. Each somatically encapsulated germline cyst is subsequently referred to as an egg chamber until it enters the oviduct and becomes a mature egg. The overall purpose of this chapter is to provide a protocol for culturing these egg chambers, one that is adaptable for any stage of oogenesis.

During the course of oogenesis, an egg chamber proceeds through 14 stages (S1–S14) of development based on stereotyped morphological and morphogenetic changes (Fig. 1; *see* refs. 1, 2 for thorough and detailed accounts of egg-chamber development, including genetic analyses). During S1–S5 (~50 h), the growing egg chamber remains spherical in shape, the nurse cells undergo massive endoreplication (~64C), and the cuboidal follicle cells proliferate (i.e., ~80 cells become ~650 cells) and become patterned along the AP axis. During S6–S9 (~24 h), the egg chamber elongates along the AP axis, the nurse cells continue their endoreplication cycles (>500C), the oocyte expands significantly compared to its interconnected nurse-cell siblings (due in large part to uptake of yolk proteins), the follicle cells cease dividing and undergo endoreplication, the vitelline membrane begins to form, and the morphogenetic events of the follicular epithelium begin (i.e., the columnarization of posterior follicle cells contacting the oocyte, the flattening of follicle cells over the nurse cells, and the migration of the border cells). Progression through this period is variable and depends on nutrition. Early-stage S10 (S10A; ~6 h) is marked visually by the oocyte reaching a volume equivalent to that of the entire group of 15 nurse cells, and molecularly by transitions in the DV patterning of the follicular epithelium. Late S10 (S10B; ~4 h) is marked by a dramatic increase in egg-chamber volume, the almost complete separation of the oocyte from the nurse cells by the columnar follicle cells (i.e., centripetal follicle-cell migration), and the apical-basal thickening of the dorsal appendage follicle cells in preparation for their morphogenesis. As egg chambers transition into S11, the shortest of all the stages (~20 min), the nurse cells dump their cytoplasm into the oocyte, and the process of dorsal appendage (DA) tube morphogenesis begins (Fig. 2). S12–S14 (5+ hours) involves the degradation of the nurse-cell remnants following dumping, the completion of DA-tube morphogenesis, the secretion of the eggshell (Fig. 2H), and subsequent death of the

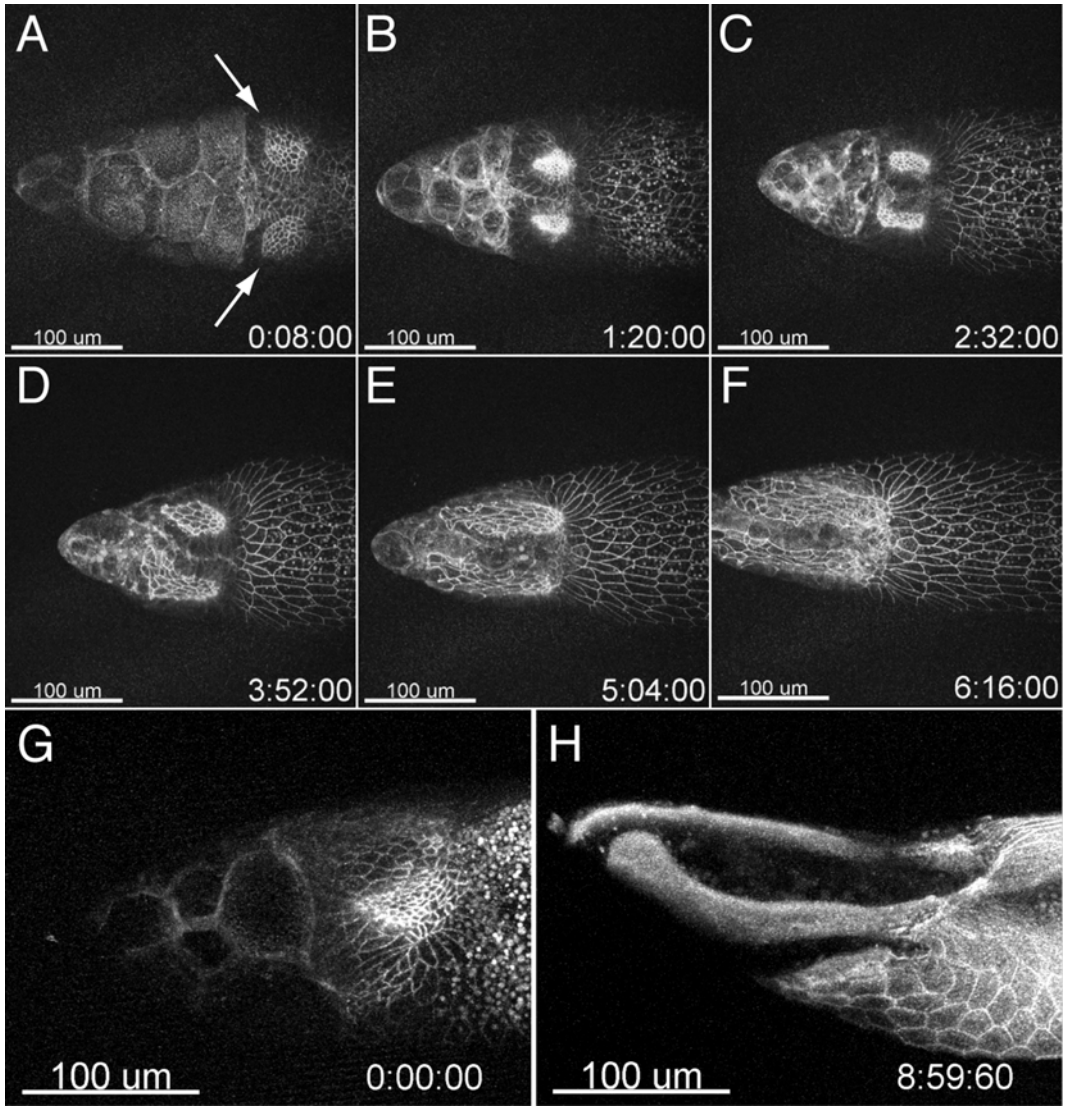


**Fig. 2** Still images from two time-lapse movies of late-stage egg chambers dissected from an E-cadherin::GFP knock-in line [50], which fluorescently labels all E-cadherin with GFP. Since the highest levels of E-cadherin in the egg chamber follicle cells outline the apical surfaces of the follicle cells, this marker serves as an excellent tool for visualizing the morphology and morphogenesis of the dorsal appendage (DA) epithelial tubes between S10B–S14 [34, 47]. In the first movie (**A**–**F**), the egg chamber is homozygous for E-cadherin::GFP and is in a dorsal orientation. *Arrows* in A point to the two populations of cells that are preparing to form the DA tubes. Z-stacks were acquired every 8 min for 10 h, which encompassed both DA-tube formation (**A**, **B**) and expansion (**C**–**F**). In the second movie (**G**, **H**), the egg chamber is heterozygous for E-cadherin::GFP and is in the dorsolateral orientation. Note that the image is noisier because different imaging settings had to be used to detect the weaker signal. Z-stacks were acquired every 15 min for 10 h, which encompassed DA-tube expansion and eggshell secretion (**H**). Still images are adapted from supplementary material in [47] with permission from Elsevier

follicle cells and maturation of the oocyte, including breakdown of the nuclear envelope and movement of the meiosis-I chromosomes onto the metaphase plate. As the finished egg chamber proceeds through the oviduct, the dying follicle cells slough off and expose the eggshell, meiosis in the oocyte concludes, fertilization takes place to form an embryo, and embryogenesis begins.

Since the first detailed descriptions of *Drosophila* oogenesis in 1970 [3], nearly half a century of dedicated research and live-culturing innovation have demonstrated the awesome potential of the *Drosophila* egg chamber as a developmental model (Tables 1, 2, and 3), and these studies revealed a vast diversity of biological questions that the egg chamber can be used to address [4]. Building on the discovery that the abdomen of the fruit fly could act as an in vivo culturing chamber for transplanted tissue such as imaginal discs [5], dissected egg chambers were successfully cultured into mature eggs, even from the earliest stages, by transplanting them into new host abdomens [6]. This technique was used to facilitate development of late-stage egg chambers following ex vivo live imaging of cytoplasmic streaming [7] and mid-stage egg chambers following laser ablation of the oocyte nucleus [8]. It was also possible to culture dissected germaria in this manner, resulting in normal ovarioles and egg chambers even when germaria were transplanted into male flies, and these efforts allowed scientists to ascertain the precise timing of each stage of egg-chamber development [9]. Although this in vivo culturing approach was both elegant and innovative, particularly since it provided such a physiologically ideal environment for egg-chamber development, its primary disadvantage was its inaccessibility and visual obscurity during the culturing process. Fortunately, in vitro culturing of *Drosophila* egg chambers is also possible, enabling live-imaging studies.

Culture protocols vary depending on whether germline or somatic tissue is the focus of the analyses (Tables 1, 2, and 3) or whether the events of interest occur on a short time frame (e.g., less than 90 min) or require long-term imaging (e.g., multiple hours or days). For experiments involving microinjection of the germline (e.g., rhodamine-tubulin) and live culturing for relatively short periods of time (e.g., 100 s—[10]; 30 min—[11]; 90 min—[12]), high-grade halocarbon oil (e.g., Voltalef 10S) can act as a culturing medium. As long as the tissue of interest is not directly exposed to halocarbon oil (e.g., nurse cells, oocyte, migrating border cells), this medium can support normal development and may be preferred due to its amenable characteristics for microscopy ([13–15]). Halocarbon oil, however, is often detrimental to the proper development and morphogenesis of the external somatic follicle cells, and it limits long-term culturing experiments of egg chambers in general (personal observations; [15]). Aqueous media, such as modified Grace's medium [16], Schneider's medium [17],

**Table 1**
**A chronological summary of 40 years of culturing and live-imaging efforts using the *D. melanogaster* ovary, concentrating on the focus/impact of research, as well as the stage(s) and tissue(s) studied**

| References and Date | Focus and impact of research | Stage(s) | Tissue |
|---|---|---|---|
| Srdic and Jacobs-Lorena (1978) | Transplanted egg chambers cultured in vivo can produce mature eggs (no live imaging) | < S1–S14 | Both |
| Petri et al. (1979) | In vitro culturing media can support late-stage epithelial morphogenesis and eggshell synthesis | S10–S14 | Soma |
| Gutzeit and Koppa (1982) | First live imaging of cytoplasmic streaming in the oocyte | S7–S14 | Germline |
| Montell et al. (1991) | Laser ablation of the oocyte nucleus disrupts DV patterning in the oocyte | S6–S14 | Germline |
| Lin and Spradling (1993) | Germaria transplanted and cultured in vivo can develop to the end of oogenesis | < S1–S14 | Both |
| Wang and Hazelrigg (1994) | First live imaging of any fluorescent-labeled protein in *Drosophila*, proposed idea that microtubules could be involved in oocyte mRNA localization | S8–S11 | Germline |
| Theurkauf (1994) | Cytoplasmic streaming in the oocyte is influenced by microtubule organization | S8–S12 | Germline |
| Forrest and Gavis (2003) | First live imaging of fluorescent-labeled mRNA molecules, proposed a diffusion/entrapment model for mRNA localization in the oocyte | S10–S12 | Germline |
| Cox and Spradling (2003) | Live imaging of mitochondrial inheritance in the oocyte, similarity between *Drosophila* and vertebrate mechanisms of germplasm provision | <S1–S11 | Germline |
| Dorman et al. (2004) | First live imaging of late-stage epithelial tube morphogenesis, dorsal-appendage tubes form by wrapping | S10–S14 | Soma |
| Gilliland et al. (2007) | First observation of female meiosis in the oocyte | S13–S14 | Germline |
| Prasad and Montell (2007) | First live imaging of border cell migration, guidance receptors are not required for border cell protrusions | S9 | Soma |
| Bianco et al. (2007) | Live imaging of border cell migration, there are two distinct phases of border cell migration | S9 | Soma |
| Tekotte et al. (2007) | An alternative method for live imaging of border cell migration in halocarbon oil | S9 | Soma |

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|---|---|---|---|
| Tootle and Spradling (2008) | Visualization of actin dynamics during nurse-cell dumping, prostaglandins promote follicle-cell maturation | S10–S12 | Germline |
| Fichelson et al. (2009) | Asymmetric RNP component segregation in single stem cells is required for stem cell renewal | Germarium | Germline |
| Wang et al. (2010) | Light-activated manipulation of border cell migration | S9 | Soma |
| He et al. (2010) | Somatic tissue elongation is driven by a network of basal actomyosin contractions | S9–S10 | Soma |
| Morris and Spradling (2011) | First live imaging of stem cell activity, cyst movement, and cell interactions within the germarium | Germarium | Germline |
| Airoldi et al. (2011) | First live imaging of somatic ring canal formation during follicle-cell mitosis | S10 | Soma |
| Haigo and Bilder (2011) | First evidence of egg-chamber rotation; this rotation is required for egg-chamber elongation | S4–S12 | Soma |
| Zhao et al. (2012) | During nuclear migration, the oocyte nucleus is not pulled, but is pushed via growing microtubules | S7 | Germline |
| Osterfield et al. (2013) | A two-dimensional pattern of line tensions along apical cell-cell edges may explain the events of late-stage epithelial tube formation | S10–S12 | Soma |
| McLean and Cooley (2013) | First evidence that cytoplasmic proteins can equilibrate between follicle cells through somatic ring canals | S10 | Soma |
| Cetera et al. (2014) | Egg-chamber rotation promotes the global alignment of contractile machinery necessary for egg-chamber elongation | S1–S9 | Soma |
| Cai et al. (2014) | E-cadherin-based interactions between border cells and nurse cells is required for direction-sensing during border cell migration | S9 | Both |
| Spracklen et al. (2014) | Evaluation of actin-labeling tools in the germline reveals that each tool has both advantages and disadvantages for live imaging | S10–S11 | Germline |
| Peters and Berg (2016) | Dynamin-mediated endocytosis is required for both late-stage epithelial tube formation and tube elongation | S10–S14 | Soma |

**Table 2**
**A chronological summary of 40 years of culturing efforts using the *D. melanogaster* ovary, concentrating on the culture media and apparati used, as well as the maximum duration of culturing attempted**

| References | Culture medium | Culture apparatus | Max duration |
|---|---|---|---|
| [6] | Female abdomen | Female abdomen | 9 days |
| [20] | Schneider's [17], Robb's [18], Grace's [16], D-22/ Echalier [55] | Covered glass depression slides | 11 h |
| [7] | Imaged in Robb's, cultured in the female abdomen | Covered glass depression slides or flow-through chamber [56] | 45 min |
| [8] | Ablations in Schneider's + 10% FBS, culturing in the female abdomen | Female abdomen | 1–2 day in vivo |
| [9] | Short culturing in Schneider's + 10% FBS, culturing in male or female abdomens | Coverslip with dabs of vacuum grease, male or female abdomens | 1 h in vitro, 1–3 day in vivo |
| [23] | Imaged in modified Robb's [22], treated in Schneider's + 10% FBS | Undescribed | Undescribed |
| [10] | Halocarbon oil (Voltalef 10S) | Oil on coverslip | 100 s |
| [30] | Schneider's | Coverslip-bottomed culture dish with coverslip | 8 h |
| [27] | Grace's | Greiner Lumox[a] culture dish and coverslip | 3 h |
| [21] | Schneider's or Robb's | Machined aluminum culture slides with gas-permeable membranes [46] | 14 h |
| [11] | Halocarbon oil (700) | Oil on coverslip | 77 min |
| [35] | Schneider's + 200 μg/mL insulin, 10% FBS, and 0.6× Pen/Strep, pH ~6.9 | Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil | 6 h |
| [37] | Schneider's + 5 μg/mL insulin, 2.5% FBS, etc. [37] | Glass culture dishes | 2.5 h |
| [13] | Halocarbon oil (Series 95) | Oil on coverslip | 160 min |
| [28] | Modified Grace's + 10% FBS and 1× Pen/Strep | 24 well tissue culture plates | 10 h |

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|---|---|---|---|
| [25] | Halocarbon oil (Voltalef 10S) | Oil on coverslip | 40 min |
| [39] | Schneider's + 100 μg/mL insulin, 20% FBS, and 0.6× Pen/Strep, pH 6.95–7.0 | Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil | 1 h |
| [40] | Schneider's + 200 μg/mL insulin, 20% FBS, and 0.6× Pen/Strep, pH 6.95–7.0 | Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil | 20 min |
| [19] | Schneider's + 200 μg/mL insulin, 15% FBS, and 0.6× Pen/Strep, pH 6.95–7.0 | Coverslip-bottomed culture dish, humidification KimWipe | >14 h |
| [32] | Schneider's + 200 μg/mL insulin | Slide and coverslip supported by vacuum grease | 30 min |
| [43] | Schneider's + 200 μg/mL insulin and 10% FBS | Greiner Lumox[a] culture dish, coverslip supported by vacuum grease, sealed with oil | 4 h |
| [26] | Halocarbon oil (Voltalef 10S) or Schneider's + 5 μg/mL insulin and 2.5% FBS | Oil on coverslip or poly-L-lysine coated imaging chamber | 5 h |
| [34] | Schneider's + 10% FBS and 0.6× Pen/Strep | Coverslip-bottomed culture dish | 60 min |
| [33] | Schneider's + 200 μg/mL insulin | Coverslip and slide separated by vacuum grease | 60 min |
| [44] | Schneider's + 200 μg/mL insulin, 15% FBS, and 0.6× Pen/Strep, pH 6.95–7.0 | Greiner Lumox[a] culture dish, coverslip supported by culture medium in low-melt agarose, sealed with oil | 60 min |
| [42] | Schneider's + 200 μg/mL insulin, 15% FBS, and 0.6× Pen/Strep, pH 6.95–7.0 | Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil | 6 h |
| [29] | Modified Grace's + 10% FBS and 1× Pen/Strep | Coverslip-bottomed culture dish with low-melt agarose in modified Grace's | 70 min |
| [47] | Schneider's | Coverslip-bottomed culture dish with weighted immobilization blanket | 10 h |

[a]Greiner Lumox culture dishes were previously known as petriPerm plates

**Table 3**
**A chronological summary of 40 years of live-imaging efforts using the *D. melanogaster* ovary, concentrating on fluorescent marker(s) and imaging system(s) used, as well as the time interval(s) between data acquisition**

| References | Fluorescent marker(s) used | Live imaging system(s) | Interval |
|---|---|---|---|
| [6] | NA | No imaging attempted | NA |
| [20] | NA | Undescribed light microscope (no live imaging) | NA |
| [7] | NA (brightfield only) | Leitz microscope and Bolex H16 Reflex Camera | 100 s |
| [8] | NA (brightfield only) | Zeiss 4FL fluorescence microscope | NA |
| [9] | NA | Laser ablations on Olympus BH2 | NA |
| [23] | GFP::Exu | BioRad MRC600 laser confocal | Unspecified |
| [10] | Rhodamine-labeled tubulin | BioRad MRC600 confocal on Nikon DiaPhot | 10 s |
| [30] | GFP-labeled *nanos* mRNA | Zeiss 510 Meta LSM confocal | 1 min |
| [27] | mito-GFP, mitoTracker GreenFM | Leica DM IRE with Ultraview spinning disk | 2–3 s |
| [21] | *CY2*-Gal4 > UAS-GFP::Moesin | BioRad MRC600 or Zeiss 510 Meta LSM confocals | 3–20 min |
| [11] | rhodamine-labeled tubulin, Oli-Green DNA dye; Polo::GFP | Deltavision deconvolution microscope | 54 s |
| [35] | *slbo*-, *Actin < flip out >* -, or *upd*-Gal4 > UAS-GFP::Moesin, UAS-actin::GFP, UAS-mCD8::GFP, UAS-dsRed::N; *slbo*-actin::GFP | Zeiss Axioplan2 with Axiocam MRm camera | 2 min |
| [37] | *slbo*-Gal4 > UAS-mCD8::GFP; *Ubi*-NLS::GFP | Zeiss 510 Meta LSM confocal | 5 min |
| [13] | *Ubi*-NLS::GFP, *Ubi*-EB1::GFP, *α4-tub*-Tau::GFP | Deltavision deconvolution microscope (Olympus IX70 with Coolsnap HQ CCD camera) | 20 min |
| [28] | NA (brightfield only) | Zeiss Stereolumar (live) or Zeiss Axiophot with QImaging RETIGA 1300 camera (DIC) | 10 min |
| [25] | *tubP*-Gal4 > UAS-Wcd::GFP or UAS-RFP::Wcd; H2B::RFP, Armadillo::GFP | Leica DM IRBE with Ultraview spinning disk (CSU10) | 5 s |

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|---|---|---|---|
| [39] | *slbo*-Gal4 > UAS-GFP::Moesin, UAS-mCD8::GFP, UAS-cherry-PA-RacQ61L, UAS-Rac-FRET | Zeiss 510 Meta LSM (photoactivation) and Zeiss 710 NLO-Meta (FRET) confocals | 80 s |
| [40] | *Ay-Gal4* > UAS-Moesin::GFP, UAS-GFP::Paxillin, UAS-mCD8::GFP, UAS-NLS::GFP; *Ubi*-E-cadherin::GFP and *Sqh*-Sqh::mCherry | Zeiss 710 NLO-Meta confocal | 10–60 s |
| [19] | *11A12*-Gal4 > UAS-Tubulin::GFP; Df31::GFP, Jupiter::GFP, His2Av::mRFP | Yokogawa CSU10 spinning disk confocal or Leica DMIRE2 | ≥10 min |
| [32] | *Act5c*- or *tubp*-Gal4 > UAS-PA::GFP, UAS-2xGFP; Vsg-GFP, Oda-GFP, Rpl30-GFP, Yps-GFP; His2Av::RFP, *Ubi*-GFP::Pav-KLP, Yps::mRFP | Zeiss Axiovert 200 with CARVII confocal imager and CoolSnap HQ2 camera or Zeiss 510 Meta LSM confocal (photoactivation) | 90 s |
| [43] | *e22c*- or *GR1*-Gal4 > UAS-Indy::GFP, UAS-His2Av::mRFP, UAS-CollagenIV::GFP, UAS-my::mRFP; *Ubi*-NLS::mRFP | Leica TCS SL or Zeiss 510 Meta LSM confocals, Zeiss Axio M1 | 2–15 min |
| [26] | *mat-α4tub*-Gal4 > UASp-EB1::GFP; *Ubi*-EB1::GFP, *Ubi*-Cnn::GFP, *Ubi*-PACT::GFP, *Ubi*-Sas4::GFP, *Ubi*-Dlic::GFP | Olympus IX81 with Yokogawa CSU22 spinning disk and iXon DV855 camera | Variable |
| [34] | E-cadherin::GFP | Leica SP5 or Nikon A1 confocals | 2.25 min |
| [33] | *c855a*- or *tubp*-Gal4 > UASt-PA::GFP, UAS-2xEGFP, 20XUAS-IVS-Syn21-mC3PAGFP, 10xUAS-IVS-myr::tdTomato; *Ubi*-RFP::LacI, *Ubi*-GFP::Pav-KLP | Zeiss 510 Meta LSM confocal | 20 s–5 min |
| [44] | *slbo*-, *Act5c*-, *GR1*-, *tj*-, *e22c*-, or *mirr*-GAL4 > UAS-dsRed, UAS-mCD8::GFP, UAS-GFP, UAS-RFP, UAS-MoeABD::mCherry, UAS-UtrophinABD::GFP, UAS-Paxillin::GFP; Nrg-GFP, Indy-GFP, Vkg-GFP; His2Av::mRFP, *Sqh*-Sqh::mCherry | Zeiss 510 Meta LSM confocal, Nikon Ti-E with Yokogawa CSUX spinning disk confocal and HQ2 or Rolera em-c[2] cameras, or Nikon Ti-E with Andor iXon3 897 EM-CCD camera (TIRF) | 30–60 s |
| [42] | *slbo*-, *upd*-, *triple*-, or *nos*-Gal4 > UAS-Rac-FRET, UAS-PA-RacT17N, UAS-dsRed, UAS-mCD8::GFP; *slbo*-Lifeact::GFP | Zeiss 510 Meta LSM (photoactivation, time-lapse) or Zeiss 710 LSM(FRET) confocals | 2 min |
| [29] | *mat3*-, *c355*-, or *oskar*-Gal4 > UASp-GFP::Utrophin, UASp-Lifeact::mEGFP, UASp-F-tractin::tdTomato | Zeiss Axio Observer.Z1 with Zeiss 700 LSM confocal | 10 min |
| [47] | E-cadherin::GFP | Zeiss 510 Meta LSM confocal | 15 min |

and Robb's medium [18], have facilitated the majority of egg-chamber culturing experiments, including experiments of the longest attempted duration (e.g., 14+ hour germarium culturing [19]), and all external, somatic follicle-cell culturing experiments have used aqueous media (e.g., dorsal-appendage epithelial tube morphogenesis and eggshell synthesis; 6–11 h [20, 21]).

The description of media that could support egg-chamber development and the advent of *in vitro* culturing methods resulted in a slew of influential discoveries (Tables 1, 2, and 3). In the germline, cytoplasmic streaming within the oocyte was first documented and imaged in egg chambers cultured in Robb's medium [7]. Using an optimized version of Robb's medium (composition described in [22]), the first GFP-reporter experiments ever performed in *Drosophila* allowed scientists to culture and live-image egg chambers expressing GFP::Exu in the germline [23]! This work, along with live imaging of egg chambers injected with rhodamine-tubulin and cultured in halocarbon oil [10], suggested a novel mechanism for establishing mRNA gradients via trafficking of ribonucleoprotein (RNP) complexes along microtubules (reviewed in [24]). Halocarbon-oil culturing also allowed the first live observations of female meiosis in the oocyte [11], the visualization of asymmetrical RNP component segregation within single stem cells [25], and the discovery that the oocyte nucleus is pushed by growing microtubules during DV axis specification [26]. Grace's medium facilitated analyses of organellar transport within the germ cells, including movement of mitochondria from the nurse cells to the oocyte [27]. Modified Grace's medium allowed characterization of prostaglandin signals that regulate actin dynamics during nurse-cell dumping [28], and this medium was used to show that not all actin-labeling tools available for live imaging are created equal [29]. Schneider's medium facilitated the first live imaging of fluorescently labeled mRNA molecules in the oocyte ([30]; reviewed in [31]), the discovery that somatic ring canals form through incomplete mitoses during follicle-cell proliferation [32], the observation that certain proteins can equilibrate between sibling follicle cells through somatic ring canals [33], and the first live imaging of follicle-cell morphogenesis (both Schneider's medium and Robb's medium were used successfully in [21]). Each of these culturing media has facilitated studies that provided important insights into the cell and molecular biology underlying egg-chamber development; nevertheless, most researchers now prefer aqueous culturing media for egg-chamber culturing and live imaging.

When selecting an aqueous medium for culturing egg chambers, important factors to consider are ease-of-use and availability, chemical definition, and reliability. Both modified Grace's medium and Schneider's medium are commercially available, but they are only partially defined because they contain yeastolate (i.e., yeast

medium extract). Robb's medium, which is chemically defined, must be assembled fresh and requires 56 separate ingredients, making it the most difficult of the aqueous media to use. Modified Grace's medium or Schneider's medium are used alone or with minimal supplementation, depending on the context, and are therefore preferable if complete knowledge of chemical composition is not essential. Of these aqueous culturing medium options, Schneider's medium is the most widely used and reliable and has become the preferred live-culturing medium for *Drosophila* egg chambers.

All efforts to study external, somatic follicle-cell events have utilized Schneider's medium (Tables 1 and 2). The culturing of late-stage egg chambers (i.e., S10+) is possible in unaltered, sterile Schneider's medium ([20, 21]) or in Schneider's medium supplemented with fetal bovine serum and, to suppress bacterial growth, penicillin streptomycin ([34]). For culturing younger egg chambers (i.e., prior to S10), however, supplementation of Schneider's is absolutely required. The addition of insulin and fetal bovine serum, and the adjustment of the pH to an optimal value of ~6.9, facilitated the first culturing of S9 egg chambers and the live imaging of border cell migration (S9) ([35]; method described in [36]). An alternative blend of Schneider's medium supplements, including insulin and fetal bovine serum, also enabled culturing during S9 ([37]; method described in [38] and reviewed in [36]). These culture conditions made possible the manipulation of collective cell movements via light-activated molecules in mid-stage egg chambers [39], the visualization of tissue elongation in late-stage egg chambers ([40]; method reviewed in [41]), and the use of an E-cadherin tension sensor to evaluate directional cues during collective cell migration [42]. The same conditions allowed visualization of egg-chamber rotations at mid-stages (S5–S9 [43]) and early stages (beginning at S1 [44]) and facilitated studies of the regulation of stem cells and germline–soma interactions in germaria [19]. In summary, Schneider's medium is the preferred vehicle for live culturing and analysis of the egg-chamber soma, and supplementation with insulin and fetal bovine serum as well as careful attention to pH are essential if the intent is to culture an egg chamber younger than S10 [45].

In addition to variations in culturing media, there have been numerous types of devices used for culturing *Drosophila* egg chambers (Table 2). The devices described below accommodate differences in microscope architecture, most importantly, by allowing imaging using both upright and inverted platforms. Machined aluminum culture chambers with gas-permeable Teflon membranes [46] have been used to live culture S10B–S14 egg chambers, to visualize the epithelial morphogenesis of the tubes that form the DA filaments of the mature eggshell [21]. Culturing devices constructed from gas-permeable plates, small droplets of Schneider's

medium, coverslips, risers made from coverslip fragments, and halocarbon oil have been used to culture S9 egg chambers ([35, 36, 39, 40]. Replacement of coverslip risers with vacuum grease [43] or 0.4% low-melt agarose pads and vacuum grease [44] facilitated culturing of smaller S1–S6 egg chambers.

Despite the success of previously described culturing devices, we sought to devise a culturing apparatus that would be logistically simpler to assemble than previous apparati, would hold the egg chamber securely but also gently against the coverslip with a flexible material, and would allow the egg chamber to contact a greater volume of media, to avoid complications from restricted gas exchange, temperature fluctuations, and evaporation-induced changes in ion concentration and pH. To achieve these goals, however, our method does require the use of an inverted microscope. As others have before us ([19, 30, 34]), we employ readily available and affordable glass-bottomed culture dishes. We then simply fill the culture dish with Schneider's medium; position the egg chambers in the center of the coverslip at the bottom of the dish; cover them with a small, flexible "blanket" made from a square of lab tissue (e.g., KimWipe); and weigh the lab wipe down with a brass washer. When positioned correctly, the washer applies indirect pressure to the egg chamber and holds it gently against the coverslip. Not only is assembly of this culturing device rapid and easy, but it also allows one to immobilize egg chambers during late-stage development, when the morphogenetic movements of the somatic follicle cells are most robust [47]. We see no reason why this device could not be readily used for culturing early or mid-stage egg chambers, as the immobilization "blanket" is flexible. If supplements to the culturing medium meant that using a large volume of medium is prohibitively expensive, the device could utilize a smaller volume of conditioned medium in a humidity chamber (*see* ref. 19). If the intended egg chambers were too small to be held down with the weighted lab wipe "blanket" alone, an agar pad could provide further support and immobilization (*see* ref. 44). Thus, our device, along with readily available Schneider's medium (supplemented if necessary) and minimal modifications, can provide an easy-to-assemble and cost-minimal tool for culturing and imaging living *Drosophila* egg chambers of any stage.

## 2 Materials

The materials described below are for the dissection, culturing, and live imaging of late-stage *Drosophila* egg chambers on an inverted confocal microscope. Notes, where appropriate, discuss details of, and reasoning for, specific steps in the protocol. Notes also indicate where the protocol can be altered to facilitate culturing of different egg-chamber stages or for use with an upright confocal microscope system.

### 2.1 Media and Solutions

1. Schneider's medium ([17])
   - For somatic and germline applications
   - Storage instructions (*see* **Note 1**).
   - Medium supplementation (*see* **Note 2**).
   - **-or-**
   - High-grade halocarbon oil (e.g., Voltalef 10S).
   - For internal somatic or germline applications.
2. 5× gelatin (nonstick) stock solution for coating glass transfer devices: 0.25 g (0.5%) Knox unflavored gelatin, 250 µL formalin (0.19% formaldehyde), 50 mL $dH_2O$.
   - Storage and coating instructions (*see* **Note 3**).
   - Alternative nonstick solutions (*see* **Note 4**).

### 2.2 Fly Stocks

1. Stocks for live culturing and imaging are numerous and depend on the needs of the user (Table 3; *see* **Note 5**).
2. E-cadherin::GFP (Fig. 2; *see* **Note 6**).
3. *CY2*-GAL4 > UAS-GFP::Moesin (*see* **Note 7**).

### 2.3 Dissection Station

1. Stereomicroscope with a dark stage (for contrast).
2. External, adjustable white-light illumination (*see* **Note 8**).
3. $CO_2$ source, pad, and blowgun for anesthetizing flies.
4. Fine brush or alternative implement for sorting flies.
5. Tissue wiper and tape for making a carcass disposal wipe; tissue wiper and $dH_2O$ for cleaning forceps after dissection.
6. Three glass dissection dishes: One for dissecting ovaries from the fly, one for dissecting egg chambers from the ovaries, one for holding liberated egg chambers of the desired stage until ready for imaging (*see* **Note 9**).
7. Dissecting forceps (Dumont #5; *see* **Note 10**).
8. Pipette controller for egg-chamber transfer (e.g., Assistent micro-classic 558; *see* **Note 11**).
9. Glass transfer device (Pasteur pipette or capillary tube; *see* **Note 11**) pre-coated with nonstick gelatin solution (*see* **Note 3**).

### 2.4 Imaging Station

1. Inverted scanning confocal microscope with a stage adaptor that is compatible with glass-bottomed culture dishes (*see* **Note 12**).
2. Glass-bottomed 35-mm culture dish (MatTek) with lid (*see* **Note 13**).
3. Light-Duty Tissue Wipers for creating the egg-chamber immobilization "blanket" (*see* **Note 14**).

4. Brass washer for weighing down the immobilization "blanket" (*see* **Note 15**).
5. Dissecting forceps (Dumont #5; *see* **Note 10**).

## 3 Methods

The methods described below are for the dissection, culturing, and live imaging of late-stage *Drosophila* egg chambers on an inverted confocal microscope. Notes, where appropriate, discuss details of, and reasoning for, specific steps in the protocol. Notes also indicate where the protocol can be altered to facilitate culturing of different egg-chamber stages or for use with an upright confocal microscope system.

### 3.1 Care of Female Flies Prior to Dissection

1. To maximize egg-chamber production in the ovary, transfer 2–4-day-old female flies (5–15 individuals) into fresh fly food vials with several males and wet-yeast paste (*see* **Note 16**).
2. Incubate vials at 25 °C for ~24 h; these conditions should provide large numbers of S10B–S11 egg chambers (*see* **Note 17**). Female flies that are actively producing eggs will have swollen abdomens following this incubation.

### 3.2 Ovary Dissection

1. Equilibrate an aliquot of Schneider's medium to room temperature or desired culturing temperature (*see* **Note 18**). Fill the three dissecting dishes with Schneider's medium (*see* **Note 19**), placing the first dish on the dark surface directly under the stereomicroscope and the other two dishes off to one side.
2. Adjust the light source to illuminate the centered dissecting dish from the side (*see* **Note 8**) and focus the stereomicroscope on the bottom surface of the dish.
3. Use tape to anchor a tissue wiper near the nondominant hand for disposing of fly carcasses during dissection.
4. When the dissection station is fully assembled, turn the vial upside down and anesthetize flies with a $CO_2$ blowgun. Gently tip the flies onto a pad with actively flowing $CO_2$ and use a brush to separate males and any females with non-swollen abdomens from females with swollen abdomens (*see* **Note 20**). Position the females with swollen abdomens in a vertical column with their heads oriented towards the user's nondominant hand.
5. Take hold of a pair of forceps with the thumb and forefinger of each hand so that a pinching motion will close the forceps. During the dissection itself, the wrists should be placed on a steady surface (e.g., benchtop or stereomicroscope stage), and the forceps can be steadied against the middle finger and/or the side of the dissecting dish.

6. With the forceps in the nondominant hand, firmly grasp a female by the thorax and anterior end of the abdomen (this process will crush that part of the fly) and immerse the body in the first dissecting dish filled with Schneider's medium. Do not let go! With the forceps in the dominant hand held open, pierce the posterior cuticle of the abdomen with one tip (Fig. 3A–C), close the forceps, and remove the ovary pair (Fig. 3D) (*see* **Note 21**) while maintaining a hold on the body with the nondominant-hand forceps. Once the ovary pair is liberated with the dominant hand, use the nondominant hand to remove the body of the female from the dissecting dish, and discard the carcass by wiping the forceps across the nearby anchored tissue wiper. Return the nondominant-hand forceps to the medium and, if necessary, gently remove any non-ovarian tissue (e.g., gut, Malpighian tubules) from the ovaries (Fig. 3E) (*see* **Note 22**).
7. Use the dominant-hand forceps to gently transfer the ovary pair to the second dissecting dish with Schneider's medium (*see* **Note 23**).
8. When several females have been dissected (*see* **Note 24**), wipe both forceps clean, move the first dissecting dish to the side, and place the second dissection dish containing the clean ovaries directly under the stereomicroscope.

### *3.3 Ovariole Dissection and Individual Egg-Chamber Isolation*

1. With the nondominant-hand forceps, grasp an individual ovary near the oviduct (potentially crushing some late-stage egg chambers). Holding the dominant-hand forceps open, use the tips to gently comb apart the ovarioles with a brushing motion (Fig. 3G); this process sometimes frees individual egg chambers (*see* **Note 25**). When an individual ovariole containing an egg chamber of the desired stage is visible, grasp it near the germarium with the dominant-hand forceps and gently tug it until it is free from the ovary (Fig. 3H) (*see* **Note 26**).
2. To isolate a late-stage egg chamber from the transparent muscle sheath of an individual ovariole (*see* **Note 27**), grasp the ovariole with the nondominant-hand forceps just past the anterior end of the desired egg chamber and hold it firmly against the base of the dissecting dish (Fig. 3J). Using the dominant-hand forceps, close the tips around the anterior end of the desired egg chamber so that they are just narrower than the maximum diameter of the egg chamber and then slowly and steadily draw those forceps away from the ovariole. If done correctly, there will be some initial resistance, and the egg chamber will deform as it passes through the narrow opening of the muscle sheath, but the egg chamber will immediately return to its normal shape as it pops out of the muscle sheath into the medium (Fig. 3J, K) (*see* **Note 28**).

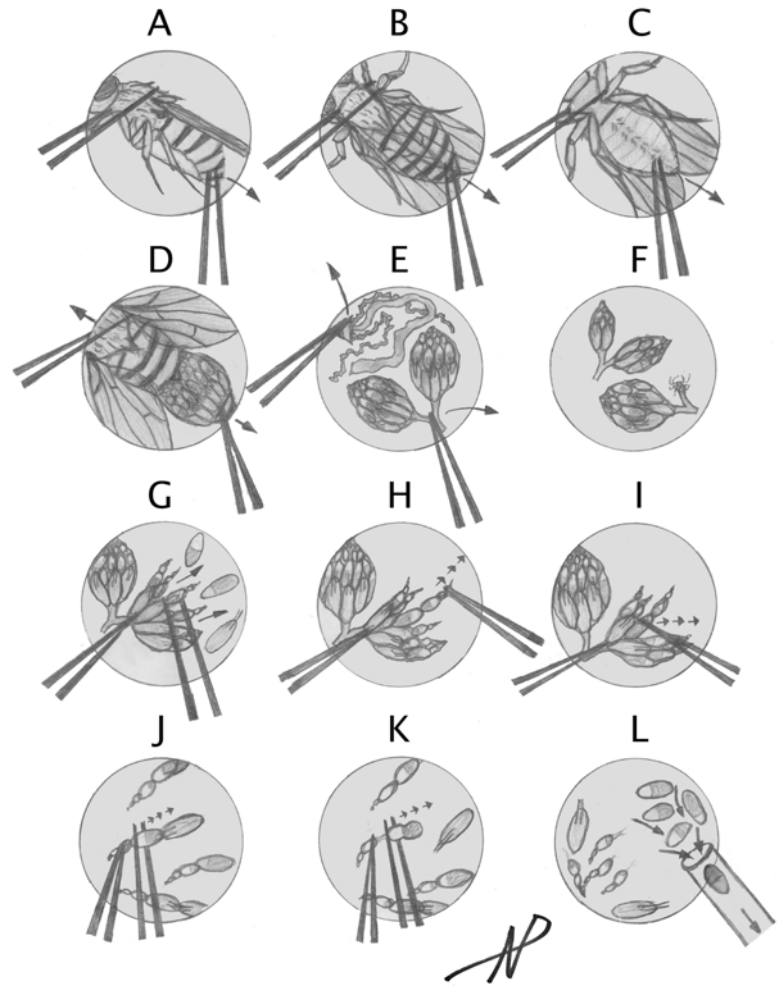


**Fig. 3** Illustrated instructions for dissecting ovaries from *D. melanogaster* females, removing individual ovarioles, and isolating individual, late-stage egg chambers. In all illustrations, the dominant-hand forceps are shown on the *right*, and the nondominant-hand forceps are shown on the *left*. (**A**–**C**) illustrate three possible orientations (**A**-lateral, **B**-dorsal, **C**-ventral) for the body of the female during ovary removal and approximately where the opening in the female abdomen should be made. (**D**) illustrates the ovary pair being removed from the abdomen. When the ovaries are removed from the abdomen, the gut and Malpighian tubules are often attached, and these tissues need to be separated from the ovary pair (*top* in **E**), removed from the culturing dish, and discarded prior to any further dissection. Once isolated, the ovary pair (*bottom* in **E**) is moved to a new dissecting dish with fresh culturing medium. (**F**) illustrates how the ovaries of a very young or undernourished female might appear (*top* in **F**) or how an ovary pair might appear if one of the ovaries fails to develop (*bottom* in **F**). (**G**–**I**) illustrate the process of combing open an ovary to separate the ovarioles (**G**) and removing individual ovarioles for culturing either late-stage (**H**) or early-stage (**I**) egg chambers. (**J**, **K**) illustrate how an individual late-stage egg chamber can be removed from the muscle sheath of the ovariole by anchoring the distal end of the ovariole (nondominant forceps) and squeezing egg chambers out the proximal end with the dominant-hand forceps. This process will often deform the egg chamber as it moves out of the muscle sheath (**K**), but the egg chamber will quickly return to its normal morphology once liberated. (**L**) illustrates how egg chambers of the desired stage(s) (*right* in **L**) should be moved away from ovarioles, debris, and non-desirable egg chambers (*left* in **L**) and then moved via pipette to a glass-bottomed culture dish with fresh culturing medium

3. When a sufficient number (5–10) of late-stage egg chambers have been isolated in this manner, gently brush them into a group on one side of the dissecting dish with the dominant-hand forceps (Fig. 3L).
4. If desired, use the pipette controller and a nonstick glass pipette to transfer the group of late-stage egg chambers to the third dissecting dish with fresh Schneider's medium for observation prior to imaging (*see* **Note 29**).
5. Observe egg chambers for at least 5 min; remove and discard any egg chambers that are visibly damaged (*see* **Note 30**).

### 3.4 Assembly of the Culturing Chamber

1. The culturing chamber should be assembled on the stage of the inverted confocal microscope that will be used for live imaging (*see* **Note 31**).
2. Place a CLEAN, 35-mm, glass-bottomed culture dish on the stage of the confocal microscope and fill it with ~5 mL of Schneider's medium (*see* **Note 32**).
3. Using the pipette controller and a nonstick glass pipette, slowly transfer the desired egg chambers from the final dissecting dish to the center of the culture well in the bottom of the culture dish and gently group them together with the tip of the forceps (Fig. 4A) (*see* **Note 33**).
4. To make the immobilization "blanket", wet the tips of the forceps in the Schneider's medium, pinch them twice at right angles around an untouched corner of a tissue wiper to make a 1-cm X 1-cm square, and tear off the square with the forceps (*see* **Note 34**).
5. Release the immobilization "blanket" onto the surface of the Schneider's medium in the culture dish, taking care that it does not stick to the forceps; prod it until it begins to sink; carefully guide it with the forceps so that it comes to rest over the culture well and egg chambers (Fig. 4B) (*see* **Note 35**).
6. When the immobilization "blanket" is in place, grasp the clean brass washer with the forceps so that it is stable and horizontal, rinse it in the Schneider's medium in the last dissecting dish, lower it *SLOWLY* into the culture dish, and *CAREFULLY* lay it on top of the immobilization blanket so that it lies inside the culture well and against the coverslip (Fig. 4C) (*see* **Note 36**).
7. Under the microscope (widefield, 10× or 20×) confirm that the egg chambers have not been flattened (i.e., they still have a similar shape as when they were isolated from the ovarioles) and are immobile (i.e., they do not sway when the side of the culturing dish is tapped) (*see* **Note 37**). Be sure that one or preferably several egg chambers are in the desired orientation (*see* **Note 38**). If any of these criteria is not met, make any

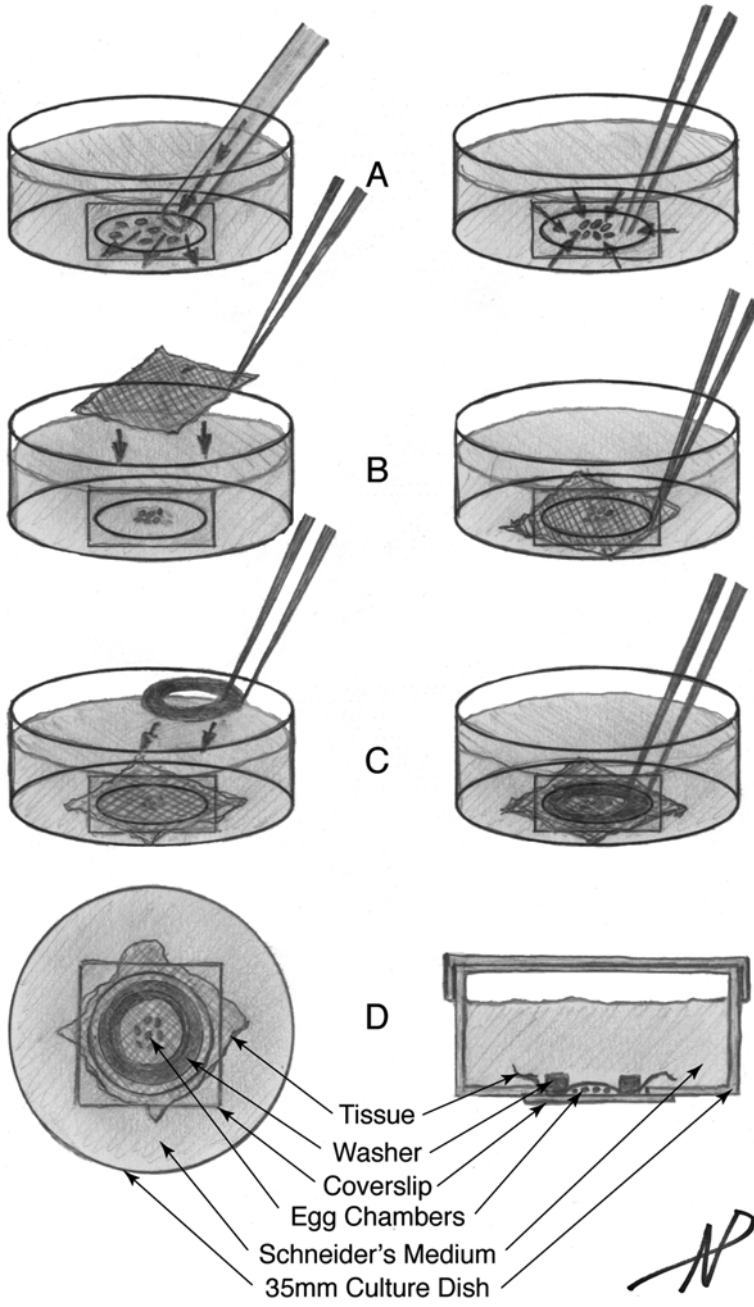


**Fig. 4** Illustrated instructions for assembling an apparatus for culturing and live imaging *D. melanogaster* egg chambers. (**A**) illustrates egg chambers being dispensed from a pipette into a glass-bottomed culturing dish filled with culturing medium of the appropriate temperature and composition (*left*) and gently moved into a tight group in the center of the culturing dish (*right*). (**B**) illustrates how a small (~1 cm × 1 cm) square of tissue is first placed on the surface of the culturing medium (*left*) and then guided down to the bottom of the culturing dish so that it rests over the egg chambers (*right*). (**C**) illustrates how a brass washer, which is slightly smallerFig. 4 (Contined) than the culturing well at the bottom of the culturing dish, is first carefully lowered into the culturing medium so that it is centered over the egg chambers under the tissue (*left*) and then *VERY* gently released so that no egg chambers are crushed (*right*). (**D**) illustrates a fully-assembled culturing apparatus, viewed from the top (*left*) and from the side (*right*). Note that in the side view the culturing dish lid has been replaced, to prevent evaporation of culturing medium

necessary adjustments before proceeding to the next step (*see* **Note 39**).

8. When egg chambers are properly positioned and immobilized, place the lid on the culture dish to prevent evaporation during imaging (*see* **Note 40**). The culture chamber is now fully assembled and ready for imaging (Fig. 4D), and the forceps should be rinsed, dried, and sheathed.

### *3.5 Live Imaging of Egg-Chamber Development*

1. Using widefield illumination (brightfield or standard fluorescence), locate a suitably oriented, late-stage egg chamber (*see* **Note 38**), and move an appropriate objective for live imaging into place (*see* **Note 41**).
2. Using the confocal microscope's time-lapse function (four dimensions: XYZT), select appropriate imaging parameters (e.g., resolution, scanning speed, *Z* parameters, laser power, gain, offset, time interval, duration) (*see* **Note 42**).
3. Upon the initiation of time-lapse imaging, monitor image acquisition during the first two time points to confirm that the microscope is imaging under the intended parameters (*see* **Note 43**).
4. If possible, monitor the sample every 30–60 min during live imaging to make sure that the egg chamber has remained in place and in focus and that development is progressing normally (Fig. 2). If necessary, pause the time-lapse imaging to make appropriate adjustments or to select a new egg chamber (*see* **Note 44**).
5. When time-lapse imaging is complete, immediately *SAVE THE EXPERIMENT* (*see* **Note 45**). The culturing chamber should then be disassembled, and the culturing dish and brass washer washed thoroughly, dried, and saved for future use.

## 4 Notes

1. Schneider's medium should be aliquoted under sterile conditions and can be stored at 4 °C for several months. Before use, the pH of an aliquot of Schneider's medium should be tested at room temperature to make sure that it is between 6.95 and 7.00. Aliquots should be checked for contamination by swirling prior to use, and, if the solution is cloudy, it should not be used. If salt precipitates form in the medium, the medium should be heated to 37 °C to dissolve the crystals and then brought down to the desired culturing temperature, or a new batch of medium should be used.
2. For late-stage egg-chamber culturing (>S10), we have found that no medium supplementation is necessary ([21, 47],

Fig. 2). If desired, however, 10% fetal bovine serum and 0.6× penicillin streptomycin can be added [34]. For early- or mid-stage egg-chamber culturing (<S10), a combination of 15% fetal bovine serum, 0.6× penicillin streptomycin, and 200 μg/mL insulin is optimal ([19, 36, 48].

3. The 5× gelatin (nonstick) stock solution can be stored at room temperature for several months. Prior to use, a 1× working solution should be made with $dH_2O$. For coating a glass Pasteur transfer pipette (or capillary tube), simply draw the 1× gelatin solution into the pipette, expel all liquid (may require several light flushings of air), allow to air dry, and rinse several times with $dH_2O$. Pipettes can be prepared and stored for several months or more in this manner. For more information on this method and other tips on handling tissues, see the MBL Embryology Course protocol for *Tool Making and Handling of Marine Invertebrate Embryos and Larvae* [49].
4. As alternatives for making nonstick glass transfer devices, we have successfully used 10% bovine serum albumin (BSA) or 10% normal goat serum (NGS) solutions, preparing the devices in a manner similar to that described in **Note 3**. In a rush, we have found that simply flushing a nonstick solution through a glass transfer device and rinsing with $dH_2O$ immediately prior to use is sufficient to make the device nonstick.
5. For an additional list of strains that have been used for live imaging in egg chambers, *see* Table 3 in [48].
6. E-cadherin::GFP replaces the endogenous E-cadherin locus [50] and allows tight visualization of follicle-cell apices and DA-tube morphology (Fig. 2). Because the expression of E-cadherin::GFP is under endogenous control and is relatively lower than the expression of fluorescent reporters in many other strains that we have used for live imaging, some care is needed to avoid harming the egg chambers during imaging. Minimal laser exposure and careful tweaking of imaging parameters are required when using this strain.
7. The combination of *CY2*-GAL4 [51] and UAS-GFP::Moesin [52] can be maintained stably in a strain and allows visualization of follicle-cell outlines and actin dynamics. Since this strain illuminates all F-actin within the follicle cells, it is easier to visualize the overall movements of the follicle cells but harder to visualize the apices of the follicle cells during DA-tube morphogenesis. The expression of GFP::Moesin in this strain is very strong, requiring low-level laser power while also allowing fast image collection, making it relatively easy to image these egg chambers compared with those expressing E-cadherin::GFP.
8. We recommend using external, gooseneck, fiber-optic light sources to illuminate the glass dissection dishes from the side

and to prevent heating of the tissue. When such lighting is used in combination with a dark stage, it provides optimal illumination and contrast for dissecting ovaries, staging egg chambers, and isolating egg chambers of the desired stage.

9. We find that three dissecting dishes are optimal for egg-chamber dissection and isolation, but that at least two are required. Dissection will be easier if the dissection dishes are filled with several mL of dissecting medium so that the female can be completely submerged, but if the volume of dissecting medium is limiting, glass depression slides can be used instead. In the first dish, the transfer of dissected ovaries away from the fly carcasses, particularly the gut, is the most important step, as gut enzymes can easily disrupt egg-chamber development. In the second dissection dish, egg chambers are inevitably crushed as one separates ovarioles from each other and removes the desired egg chambers from the ovarioles. For this reason, we prefer to transfer the isolated egg chambers of the desired stage to a third dissecting dish to provide an additional rinse and for observation prior to transferring them to the culturing device.
10. We find that sharp and well-aligned dissecting forceps are essential for ovary dissection and intact egg-chamber isolation, and we prefer Dumont #5 forceps made from Dumostar alloy. Great care should be taken to protect the tips of these forceps, for even the smallest impact can blunt or bend the tips. Forceps should be rinsed with $dH_2O$ immediately after use and stored clean and dry with a tip protector (a 20 μL pipette tip works very well for this). Prior to dissection, the forceps should be held closed and examined under the stereomicroscope from both the side and from above; the tips should align precisely from both perspectives or it will be difficult to isolate individual egg chambers without damaging them. Minimally bent or blunted tips can usually be realigned and sharpened with a wet sharpening stone.
11. We find that the Assistent-micro-classic pipette controller, when coupled with a glass, nonstick transfer device, works beautifully for transferring isolated egg chambers. The controller's thumb-controlled roller wheel enables easy, precise, one-handed operation. The controller is compatible with either Pasteur pipettes or capillary tubes, though we prefer Pasteur pipettes because they are more durable, provide a greater working volume, and are easier to coat, store, and use.
12. Our protocol is designed for imaging on an inverted scanning confocal microscope, and we have successfully used both Ziess 510 Meta and Leica SP8X confocal microscopes to this end. Spinning disk confocals allow faster image acquisition, more frequent time intervals, and lower phototoxicity, but usually

have lower resolution than scanning confocals. If only upright confocal microscopes are available, a different culturing approach using machined aluminum culture slides ([21, 46]) or gas-permeable membrane plates ([36, 48]), or a significant modification to our approach, will be required. We use adjustable stage adaptors that allow us to securely hold our 35-mm glass-bottomed culture dishes. If culturing and live imaging must be performed at a temperature other than room temperature, we use a temperature-controlled stage that is compatible with 35-mm culture dishes.

13. We use 35-mm MatTek P35G-1.5-10-C culture dishes for live culturing and imaging of late-stage egg chambers. These dishes use a No.-1.5 thickness coverslip, with a circular culturing well of 10-mm diameter. Other dish diameters, coverslip thicknesses, and culturing well diameters are also available (http://glass-bottom-dishes.com/pages/product.html). We have reused these culturing dishes several times by rinsing them thoroughly with $dH_2O$. Alternatively, a light detergent solution and ethanol can be used for cleaning the dishes, but all traces of detergent must be removed prior to culturing. These culture dishes require the use of an adjustable stage, or stage adaptor, and are also compatible with certain temperature-controlled stages.
14. We use VWR 82003-822 Light-Duty Tissue Wipers for creating our egg-chamber immobilization "blankets", and we find that contact with this material has no adverse effects on egg-chamber development. Alternatively, we have successfully used Kimtech Science KimWipes Delicate Task Wipers (Kimberly-Clark Professional, 34120). Since impurities in paper products might affect culture conditions, we recommend testing your brand of tissue before carrying out a critical experiment.
15. We use brass washers for weighing down our immobilization blankets because brass will not oxidize in the culture medium; oxidation could adversely affect egg-chamber viability. Because our culture dishes have a 10-mm diameter culturing well, we use a brass washer with an outer diameter of ~8 mm, so that it can lie flat against the coverslip inside the culturing well. The inner diameter of this washer is ~4 mm, a diameter that provides just enough space to cluster the egg chambers in its center without crushing them. Depending on how much compression of the tissue-immobilization "blanket" is necessary, and which glass-bottomed culture dish is selected, the outer and inner diameter of the brass washer can be altered accordingly.
16. We highly recommend the use of young, 2–4-day-old females for several reasons. First, their ovaries respond rapidly to the

presence of wet-yeast paste, which stimulates egg-chamber production. Second, a greater proportion of the ovarioles will become active at the same time, which provides greater numbers of synchronously developing egg chambers. Third, there will not be an excess of fully developed egg chambers; S14 egg chambers held within the ovary can negatively feedback on egg-chamber development, making it slower and asynchronous. Additionally, overcrowding of the vials or a lack of male flies can negatively influence the rate of egg-chamber production, so no more than 15 females should be maintained in a vial, and males should always be present. If yeast-fattened females cannot be used on a given day but can be used the following day, they should be transferred to a fresh food vial with fresh wet yeast.

17. In addition to the age of the female fly, the density of flies in a given vial, the presence of males, and the availability of good nutrition, the temperature and duration of incubation can dramatically influence the rate of egg-chamber development and the relative proportions of egg-chamber stages in a given ovary. Incubation of females for ~24 h at 25 °C usually provides large numbers of S10B–S11 egg chambers as well as some fully developed egg chambers, which provide sacrificial material for the forceps to grasp during egg-chamber isolation. Shorter incubation times at 25 °C will yield greater proportions of younger egg chambers, and longer times will yield greater proportions of older egg chambers. For experiments that utilize the GAL4-UAS system, we find that incubation at 30 °C is helpful to maximize GAL4 expression ([47, 53]). The rate of egg-chamber development is directly correlated with temperature, but incubating at temperatures higher than 30 °C will risk killing the flies. Incubating flies at temperatures lower than 25 °C will slow development proportionately, for example, 18 °C requires twice as long for egg chambers to develop. We *STRONGLY* encourage individual users to experiment with the temperature and duration of wet-yeast incubation and empirically determine the optimal incubation conditions for their experiments. This preliminary analysis will almost certainly minimize time, effort, and frustration!

18. We typically dissect flies in Schneider's medium equilibrated to 25 °C. If the flies have been raised at another temperature, such as 30 °C, and the user wishes to maintain that temperature for culturing, then the aliquot of Schneider's medium should be equilibrated to the desired temperature and poured into the dissection dishes immediately prior to dissection. Ovary dissection, egg-chamber isolation, and transfer to the culturing device should then be performed as quickly as

possible, and the culturing device should be immediately placed on a temperature-controlled microscope stage.

19. We typically fill our dissecting dishes with several mL of Schneider's medium because we find that it is easier to remove the ovaries from the female if the body of the fly is fully immersed. However, a lower volume can be used if necessary. If the user needs to use supplemented Schneider's medium for culturing and the cost of the medium is a concern, then the ovaries can be removed from the fly in non-supplemented Schneider's medium, and the ovary pair can then be transferred to a small volume of supplemented Schneider's medium in the second dissecting dish. Additionally, though we prefer to separate isolated egg chambers from ovary debris by transferring them to a third dissecting dish, a third dissecting dish is not required.
20. If the desired egg chambers for culturing are S10B–S11, as they are for our studies, the ovaries will be very large and the abdomens of the females will be swollen. In this case, females with non-swollen abdomens are not worth dissecting. If the intent is to obtain younger egg chambers and the incubation conditions have been appropriately adjusted, the ovaries may be much smaller (Fig. 2F) and the abdomens of the females may not be swollen.
21. Depending on the preference of the dissector, the female fly can be held back up, on its side, or belly up for dissection. The *Drosophila* female abdomen is divided into eight segments (A1–A8, anterior to posterior), and each of these segments is covered in a cuticular plate called a sclerite. A female's dorsal sclerites (i.e., tergites) are pigmented, relatively rigid, and have a black band at each posterior margin. A female's ventral sclerites (i.e., sternites) are nonpigmented, relatively soft, and somewhat transparent (Fig. 1). In all orientations, the goal should be to pierce the abdominal cuticle in the vicinity of A6, grab the oviduct that connects the two ovaries, and remove the ovary pair, intact, with a single motion (Fig. 3A–D). We prefer to hold the female back up and to pierce the A6 tergite with one tip of the forceps just anterior of its black band, which serves as an excellent dissection landmark. If this positioning is done correctly (Fig. 3B), the tip of the forceps will hook the "Y" of the oviduct and, when closed and pulled, will remove the posterior end of the abdomen (A6, A7, and A8), the ovary pair, and the intact gut and Malpighian tubules (Fig. 3E). If the female is held on its side, it can be harder to hook the oviduct, but the dorsal tergites can still serve as a useful dissection landmark (Fig. 3A). If the female is held belly up, the white ovaries can often be distinguished through the sternites. Although this orientation offers a better view of the ovaries

through the body of the fly, there is very little definition between the sternites to serve as a dissection landmark, and the relative softness of the sternites can resist piercing by the forceps (Fig. 3C). If, during the dissection, the oviduct separates from the posterior end of the abdomen and the ovary pair remains in the abdomen, retain the hold on the body of the fly with the nondominant-hand forceps and use the dominant-hand forceps to gently squeeze the abdomen from anterior to posterior and coax out the ovary pair. The same technique can be used if one of the ovaries separates from the oviduct and remains in the abdomen. If the abdomen separates from the thorax before the ovaries are removed, hold onto the abdomen with the dominant-hand forceps and discard the anterior of the fly on the tissue wiper. Then, either regrasp the anterior end of the abdomen with the nondominant-hand forceps and proceed as before or rotate the abdomen 180°, grasp the posterior end with the nondominant-hand forceps, and use the dominant-hand forceps to coax the ovaries out of the anterior end of the abdomen. For a useful instructional movie on removing ovaries from female flies, including what to do in these alternative situations, refer to our movie in the supplemental material of [54].

22. If the gut of a dissected fly is pierced during ovary dissection, the surrounding media will become cloudy. The user should still remove the ovaries, dip them in the cleaner medium away from the pierced gut, and transfer them to the second dissecting dish. Before dissecting any more females, the first dissecting dish should be rinsed and refilled with fresh Schneider's medium.
23. If the dissected ovary pair is still attached to the common oviduct (an ideal situation), then the dissector can grasp the oviduct with the dominant-hand forceps for transferring, as this location avoids damaging the ovaries. If an ovary has become detached from the oviduct, carefully and gently close the dominant-hand forceps around the ovary but do not close the tips all the way. The middle finger can be held against the side of the forceps to steady them and keep them from closing entirely. As the nearly closed forceps are lifted from the Schneider's medium, a small droplet of medium will remain suspended between the tips of the forceps with the ovary inside. The forceps can then be moved to the second dish and allowed to open, which will release the ovary. In this manner, even an individual egg chamber can be transferred without damaging it.
24. If females are young, well fed, and incubated for an optimal period of time, just one ovary pair can yield numerous egg chambers of a desired stage and will allow successful culturing.

To be confident that we will obtain the material we need, we usually dissect 2–3 females at a time, but this effort is not always necessary. The more ovaries that are dissected, the longer it will take to sort through the ovaries and isolate egg chambers of the desired stage. The goal of the dissector should be to dissect ovaries and isolate egg chambers as quickly and cleanly as possible, so the number of females necessary for a culturing experiment will ultimately depend on the preference and skill of the dissector and on the strain being dissected.

25. Sometimes the act of combing apart the ovarioles will liberate late-stage egg chambers on its own (Fig. 3G). While this liberation can be a fortunate situation, these egg chambers should be examined carefully to make sure the forceps have not damaged them before using them for live imaging (*see* **Note 29**).
26. If the intention is to image the germarium or very early egg chambers, do not grasp ovarioles near the germarium end. Instead, after gently combing apart the ovarioles, grasp the ovariole around a mid- or late-stage egg chamber and peel the ovariole away from the rest of the ovary (Fig. 3I).
27. Because the transparent muscle sheath of the ovariole will often continue its rhythmic contractions even after it is isolated from the ovary, and because this sheath can easily adhere to dissecting and transferring implements, we recommend the removal of individual egg chambers from the muscle sheath prior to live imaging. This removal, however, is not absolutely essential, and egg chambers can be successfully imaged while still in the transparent muscle sheath.
28. If it is unclear whether an egg chamber is still in its transparent muscle sheath, move a tip of the dominant-hand forceps in a circle around the egg chamber and see if the egg chamber catches and follows the tip. If an egg chamber ever sticks to a tip of the forceps or the dissecting dish, that is a good sign that it is still in its muscle sheath. A liberated egg chamber will not normally stick to the forceps or dissecting dish.
29. If the user opts to use only two dissecting dishes, the pipette manipulator should be used to remove all extra ovarioles, ovariole remnants, and other material from the second dissecting dish, as well as the majority, but not all, of the Schneider's medium. Never let the egg chambers dry out! Fresh Schneider's medium should then be added back to this dish, providing the desired egg chambers with an additional wash while they are held for observation.
30. Damage to egg chambers during dissection (e.g., breaking, tearing, piercing) can perturb aspects of egg-chamber development, and such damage is usually visible under widefield conditions (e.g., brightfield, fluorescence) at 10× or 20×, or even

under the stereomicroscope. The most obvious sign of egg-chamber damage is the leakage of yolk from the oocyte. While we have observed that follicle-cell morphogenesis will proceed even if all the yolk is drained from the oocyte, this situation is usually not desirable, and we typically remove the damaged egg chamber. We do not typically add dyes to our culture medium to indicate more subtle egg-chamber damage and instead rely just on visual, widefield inspection at 10× or 20×. However, incubation with a membrane marker, such as FM 4–64, or a nuclear stain, such as DAPI or Hoechst 33342, can be a useful technique for detecting subtle egg-chamber damage [48].

31. Since the culturing chamber we describe is not fixed and contains a relatively large volume of culture medium, transporting the fully assembled culturing chamber is inadvisable as it can lead to movement of the egg chambers and to egg-chamber damage.
32. We typically use ~5 mL of Schneider's medium for culturing, which fills the 35-mm culture dish ~2/3 full and leaves room to add, manipulate, and secure the egg chambers (Fig. 4). If a temperature-controlled stage is being used, the temperature of the medium should be allowed to equilibrate to the intended temperature before proceeding. If a small volume of supplemented medium is being used, the culturing dish can be made into a humidity chamber by adding a damp tissue wiper around the perimeter of the inside of the culture dish [19].
33. When transferring the egg chambers using the pipette manipulator, hold the pipette as vertically as possible to minimize contact between the walls of the pipette tip and the egg chambers. Transfer the egg chambers as quickly as possible to further minimize any contact between glass and egg chamber, but do not do so at the expense of accuracy and precision. When expelling the egg chambers into the culture dish, do so in a steady, slow motion so that the egg chambers remain in the culturing well and do not disperse throughout the culture dish (Fig. 4A). If the egg chambers need to be regrouped into the center of the culture well following transfer, make small motions in the medium with the tips of the forceps to group the egg chambers and minimize actual contact between egg chambers and forceps (Fig. 4A).
34. When making the immobilization "blanket", do not use a piece of tissue wiper that has come into contact with your hand. Wetting the forceps and pinching at the intended tear sites is not essential, but such effort greatly helps the user in making a "blanket" of the intended size. The size of the "blanket" can be altered to accommodate different sizes of culture dish and washer or to satisfy the preference of the user.

35. The immobilization "blanket" will sometimes curl and try to wrap around the tip of the forceps. If this happens, it is easier to discard that "blanket" and make a new one rather than attempt to flatten the blanket. If the user must reposition the blanket when it has sunk to the bottom of the culture dish, take care not to drag the egg chambers off to the side of the culture well, where the brass washer might crush them.
36. If the brass washer is not held horizontally, it will be very difficult to move it into place without crushing the egg chambers. It should only be released when it is just above its intended position. In order to put pressure on the immobilization "blanket" and the egg chambers, the brass washer must fit inside the culture well and lie flat against the coverslip.
37. If egg chambers are crushed and have popped, dissect new egg chambers and reassemble the culturing chamber. If the egg chambers are overly compressed and have deformed, reassemble the culturing chamber or use a different size of brass washer or culture well. To determine if the egg chambers are immobile, gently tap the side of the culture dish. Unsecured egg chambers will sway in the medium, while secured egg chambers will remain in a fixed location.
38. The optimal orientation of the egg chamber will vary depending on the goals of the user. To image DA-tube morphogenesis, the egg chambers must contact the coverslip with their dorsal or dorsolateral surfaces. These surfaces can be distinguished by the thickened placode of follicle cells that will form the DA tubes, by the initiation of apical constriction in the DA-tube cells, and by the location of the germinal vesicle (i.e., oocyte nucleus) along the dorsal midline.
39. If the egg chambers are overly compressed or unsecured, gently lift the brass washer off the immobilization "blanket", replace it, and recheck the egg chambers. Alternatively, the user can gently tug on the edges of the immobilization "blanket" with the forceps while the brass washer is in place to increase the tension on the egg chambers. If the egg chambers are in the wrong orientation, or if simply replacing the washer doesn't relieve over-compression or secure the egg chambers, remove the brass washer and immobilization "blanket", move the egg chambers around with the forceps, and reassemble the culturing chamber.
40. We find that using the culture dish lid dramatically limits evaporation of the medium, especially if the egg chambers are being cultured at a temperature higher than room temperature (*see* **Note 32**).
41. For live imaging, we have used 20× dry and 40× water or oil immersion objectives. We prefer to use at least 40× magnifica-

tion for detail, and depending on the microscope, this level of magnification does not require repositioning of the egg chamber (stage) during live imaging. Higher magnification is possible (e.g., 63×), but may require repositioning of the egg chamber during live imaging.

42. For live imaging of developing late-stage egg chambers on a scanning confocal, we typically use the following parameters: 512×512 pixel resolution, maximum or near-maximum scan speed, 3–4 line averaging, near-optimal Z-sectioning for the chosen objective, a balance of minimal laser power, minimal gain, and appropriate offset to achieve the desired level of detail, 10-min time intervals, and 10-h duration. Spinning disk confocals will usually have lower resolution than scanning confocals, but they allow for much faster Z-stack acquisition, more frequent time points and/or more recovery time between Z-stacks, and overall lower risk of phototoxicity. The imaging parameters will depend on the needs of the user, but the most important factors for minimizing phototoxicity are laser power, time interval (i.e., acquisition time vs. resting time), and duration of imaging.
43. Observing the first several time points closely is important to make sure that the egg chamber remains immobile, that the proper *Z* parameters are met, that the appropriate time interval is observed, and that the imaging software's prediction of the experimental duration is as expected.
44. Most imaging software allows the experiment to be paused, readjusted, and restarted. Alternatively, the experiment can be ended, readjustments made, a new experiment started, and the movies stitched together at a later time. Possible readjustments include changing the *Z* parameters, changing the position of the egg chamber in XY, changing exposure settings, or selecting a new egg chamber. If no development is observed after 30 min, a new egg chamber should be selected.
45. Always *SAVE THE EXPERIMENT* immediately after live imaging, especially if the imaging software does not automatically do so! Nothing is more frustrating than realizing that you have just lost an exhaustively prepared time-lapse due to user error.

## Acknowledgements

We are grateful for support from the National Institutes of Health, 2 R01 GM079433 to CAB and 1S10 OD016240 to the W. M. Keck Imaging Center at the University of Washington. We thank the Bloomington *Drosophila* Stock Center and Trudi Schüpbach, Dan Kiehart, and Miriam Osterfield for fly strains.

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