

Shining light on *Drosophila* oogenesis: live imaging of egg development

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Drosophila oogenesis is a powerful model for the study of numerous questions in cell and developmental biology. In addition to its longstanding value as a genetically tractable model of organogenesis, recently it has emerged as an excellent system in which to combine genetics and live imaging. Rapidly improving *ex vivo* culture conditions, new fluorescent biosensors and photo-manipulation tools, and advances in microscopy have allowed direct observation in real time of processes such as stem cell self-renewal, collective cell migration, and polarized mRNA and protein transport. In addition, entirely new phenomena have been discovered, including revolution of the follicle within the basement membrane and oscillating assembly and disassembly of myosin on a polarized actin network, both of which contribute to elongating this tissue. This review focuses on recent advances in live-cell imaging techniques and the biological insights gleaned from live imaging of egg chamber development.

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Introduction

The development of organs and organisms is a dynamic process, a complete understanding of which requires studying living tissue with the highest possible spatial and temporal resolution. The combination of improved culture systems, light-sensitive proteins, and imaging techniques has revolutionized developmental studies over the past decade. Analysis of mutant phenotypes need no longer be limited to end-point evaluation of developmental failure; now investigators can observe how the end result comes about, by monitoring the dynamic behavior of cells and molecules. The ever-expanding arsenal of genetically

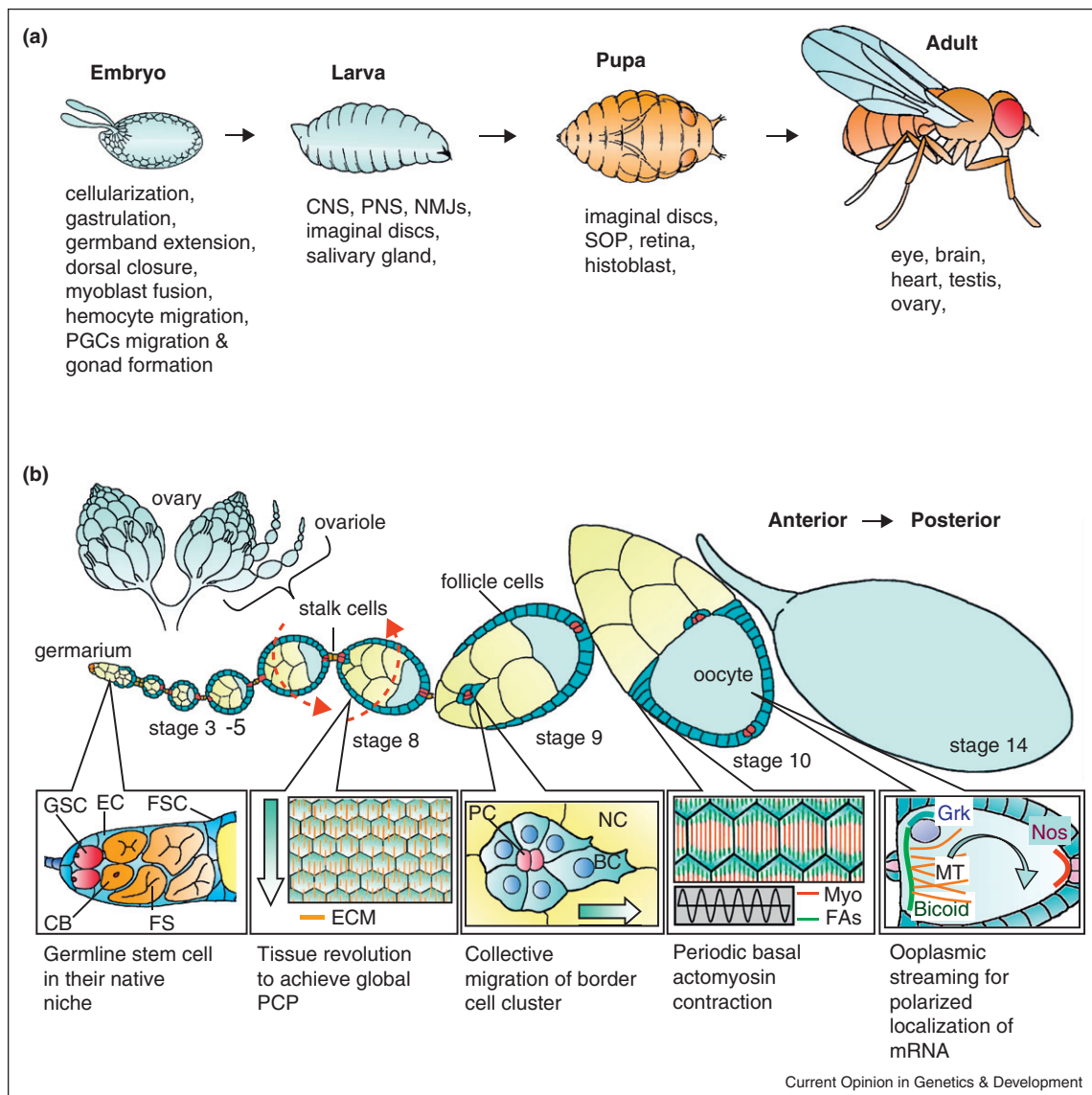
encoded biosensors and caged proteins further provides opportunities to both monitor and manipulate biological processes in real time. Besides being a renowned genetic model for development and disease, *Drosophila melanogaster* is becoming more and more amenable to live imaging, as culture conditions are defined that support *ex vivo* development of larval and adult tissues, most notably the ovary. A few examples of developmental processes studied by live-cell imaging are shown in [Figure 1](#) [1–8]. Here we will review the novel subcellular, cellular, and multicellular dynamics that have been discovered by live imaging studies of egg chamber development in the *Drosophila* ovary.

The generation and development of egg chambers provides a good model for the study of a great spectrum of biological processes required for organogenesis in general, including self-renewal of adult stem cells, cell differentiation, pattern formation, axis specification, cell shape change and migration, tissue elongation, cytoskeleton dynamics, RNA biogenesis, transport, localization and function, and even tumorigenesis. Mosaic analysis and RNAi-mediated gene knockdown are highly effective in this tissue that, unlike the embryo, lacks significant maternally provided RNA or protein. The ovary is also readily permeable to drugs and even molecules as large as antibodies, which can diffuse between cell–cell junctions even in living organs. Direct injection of material into the germline cytosol before live imaging has also been successful. Therefore, ovarian development is not only genetically tractable, but also accessible to various treatments that are more commonly used in cell culture. Yet by maintaining the tissue intact, processes that depend upon ensembles of cells and interactions of multiple cell types, which are impossible to study in simple cell culture, can be observed and manipulated.

A brief introduction to the anatomy of the *Drosophila* ovary

Female flies possess a pair of ovaries, each of which is composed of roughly 15 ovarioles. Each ovariole contains a linear sequence of egg chambers of increasing developmental stages. Germline and somatic stem cells reside near the tip of the ovariole in a region called the germarium. Progeny of the germline and somatic stem cells assemble into egg chambers, which then bud off from the germarium and are linked to adjacent chambers by stalk cells, like beads on a string. Each egg chamber produces a single egg and is composed of 16 germline cells (15 nurse cells and one oocyte), surrounded by a monolayer of roughly 600 epithelial follicle cells. The

Figure 1



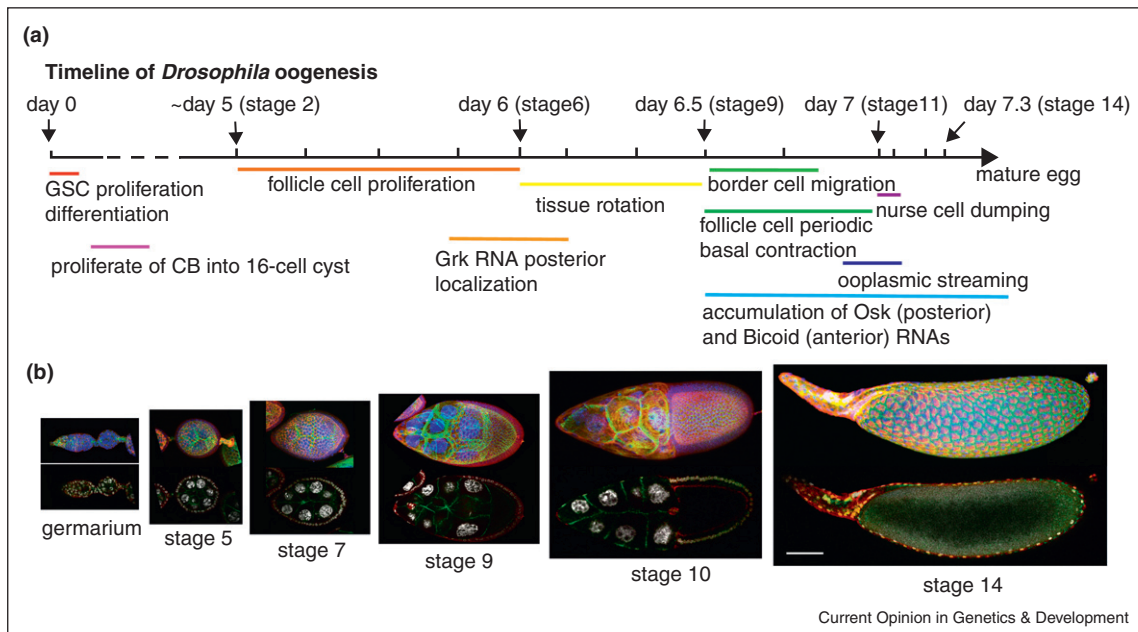
(a) Major stages of the *Drosophila* life cycle with the tissues and developmental events studied by live-cell imaging listed below. **(b)** Anatomy of fruit fly ovary and expanded view of egg chambers in a single ovariole. Germline stem cell self-renewal, follicle rotation, border cell migration, periodic actomyosin contraction, and polarized mRNA localization are further illustrated below. Arrows indicate the direction of movement (PGC: primordial germ cell; CNS: central nervous system; PNS: peripheral nervous system; NMJs: neuromuscular junctions; SOP: sensory organ precursor; GSC: germline stem cell; CB: cystoblast; EC: escort cell; FSC: follicle stem cell; FS: fusome; ECM: extracellular matrix; PC: polar cell; BC: border cell; NC: nurse cell; Myo: myosin; FAs: focal adhesion; MT: microtubule; Nos: nanos; Grk: gurken; and PCP: planar cell polarity).

follicle cells serve several important functions including patterning the oocyte, synthesizing and transporting yolk polypeptides to the oocyte, and secreting the protective layers of the egg shell [9]. The nurse cells produce and transport cytoplasm into the oocyte, which is mostly transcriptionally quiescent. *Drosophila* ovarian development has been recently reviewed by Horne-Badovinac and Bilder [10], and Bastock and St Johnston [11]. An illustrated developmental timeline of *Drosophila* oogenesis is shown in Figure 2.

Ex vivo culture of fly ovaries for live-cell imaging

Ex vivo culture and observation of egg chambers at stage 10B and later has been possible since the founding work of Petri *et al.* [12], and Gutzeit and Koppa [13[•]]. It was also known that egg chambers could develop normally following removal of the muscular sheath that normally encases each ovariole, followed by injection into a host female [13[•],14]. However long-term *ex vivo* culture of earlier stage egg chambers took another 16 years to achieve [15^{••}]. To

Figure 2



(a) Timeline of *Drosophila* oogenesis with major developmental events labeled below. The beginning of each developmental stage is indicated by a mark on the line. The interval between stages was drawn in proportion to estimated development time. **(b)** Micrographs of major developmental stages of *Drosophila* oogenesis. Top panels show three-dimensional projections of z-stacks of confocal images with nuclei labeled in blue, E-cadherin labeled in green, and myosin labeled in red. Bottom panels show single-plane confocal images through the middle of the tissue with nuclei labeled in white, E-cadherin in green, and myosin in red. Scale bar is 50 μm .

approximate normal development, it is crucial to maintain the tissue under physiological conditions, which requires precise control of environmental temperature, nutrients, oxygen, pH, hormones, and growth factors. Techniques to isolate egg chambers for live imaging have been described in detail in several articles [15^{••},16–18,19^{••}]. The key breakthrough was the discovery that pH of at least 6.9 and insulin supplementation are crucial for egg chamber growth. With these modifications and addition of fetal calf serum, stages 6–9 egg chambers can be cultured in Schneider's or Grace's medium for up to 6 h of continuous observation of tissue growth and cell movements [15^{••}]. More recently, proliferation of germline stem cells and production of cysts in the germarium have also been observed in living cultures for up to 14 h [20^{••}]. While tremendous progress has been made, further improvements are still needed since the egg chambers cultured under current best conditions cannot as yet progress from stage 9 to stage 10. To achieve full development of egg chambers *ex vivo* may require specific combinations or pulses of juvenile hormone, 20-hydroxyecdysone, insulin or perhaps unknown factors.

Live imaging of germarium development

Imaging the dynamics of stem cells within their native niches has the potential to reveal information inaccessible in fixed samples. Short-term imaging (~ 30 min) of the

Drosophila germarium during stem cell division was used to study asymmetric distribution of Wicked, a component of the U3 snoRNP complex that is important for maintenance of stem cell fate [21]. Long-term imaging (~ 14 h) of living germaria has recently been achieved by Morris and Spradling [20^{••}]. In their study, full cycles of division and differentiation of germline stem cells were recorded. Interestingly, these live imaging studies revealed that the escort cells, which were thought possibly to migrate along with the germline stem cell daughters and be replenished by escort stem cells, in fact remain largely stationary and are mitotically quiescent. This finding emphasizes the necessity of observing developmental events as they actually occur and the hazards of inferring dynamic behavior from fixed samples.

Another live imaging study in the germarium focused on the fusome, a complex structure of endomembranes, membrane-associated cytoskeleton, and microtubules, which ramifies into the interconnected cells of each germline cyst [22]. For a long time, it was unclear whether the fusome lumen was shared between all the cells of the cyst. Snapp *et al.* found that photobleaching one portion of the fusome present in one cyst cell caused a rapid depletion of fluorescence in the whole structure, suggesting that the fusome endomembranes are part of a single continuous endoplasmic reticulum (ER) [23[•]].

Live imaging of egg chamber rotation

What controls the overall shape of an organ? In principle, it could be achieved simply by the sum of the shapes of its component cells. Alternatively, the overall shape of an organ might emerge dynamically from mechanical interactions between different cells and extracellular components. The latter turns out to be the case in *Drosophila* ovary. Early stage egg chambers are spherical but they gradually elongate as they grow, ultimately producing eggs that are 2.5-fold longer than they are wide. This change of tissue shape coincides with development of dramatically polarized arrays of basal F-actin bundles oriented perpendicular to the long axis of the egg chamber [24]. At the same time, the ECM proteins within the basement membrane surrounding the egg chamber become aligned in the same direction [25]. The polarized basal F-actin and ECM fibers have been proposed to function as a ‘molecular corset’ to constrain radial growth of egg chamber. However, images of fixed tissue did not reveal how the F-actin and ECM polarization was achieved. Using live imaging techniques, Haigo and Bilder made the astonishing observation that follicles rotate within the basement membrane and that this rotation provides a novel mechanism to achieve global alignment and orientation of the F-actin and basement membrane fibers required for elongation of the tissue [26^{••}]. During stages 5–8, egg chambers rotate clockwise or counterclockwise around their long axis at a speed of $\sim 0.5 \mu\text{m}/\text{min}$ and thereby produce circumferentially polarized tracks of collagen IV. This directional rotation depends on integrin-mediated interactions of follicle cells with the ECM, and disruption of collagen IV or integrin expression prevents follicle rotation and results in round eggs. Interestingly, this rotation is similar to follicle rotation found in gall midges by Went in 1977, suggesting that it may be a general phenomenon [27].

Many fascinating questions arise from the observation of egg chamber rotation including for example how this directional movement is achieved. One suggestion is that the follicle cells crawl upon the ECM in a coordinated manner. Since the direction of rotation is random from one follicle to the next, it is probably selected through a stochastic mechanism. The nature of the mechanism is unclear, as is the mechanism by which all the cells within a single egg chamber choose the same direction. What initiates the movement is another mystery. How the correct rotation axis is selected is also an open question. There are multiple possible explanations for how directional rotation might lead to elongation of the tissue. One model is that alignment of the ECM and actin fibers creates a corset that constrains increases in egg chamber volume toward the poles. Another possibility, which is not mutually exclusive, is that the rotation itself creates an anisotropic force. Finally, the polarized actin cytoskeleton and integrin-mediated adhesion to the ECM that occurs as a consequence of the follicle rotation serve as the

substrate for periodic myosin contractions, which also contribute to egg chamber elongation, as described in the next section.

Live imaging of follicle cell basal contractions

Immediately after egg chambers stop rotating, they start to grow dramatically and continue to elongate. Within 10–12 h, the egg chamber increases its volume by 10-fold and elongates 1.6-fold in the absence of cell division. Achieving this elongation in the presence of such dramatic tissue expansion requires anisotropic mechanical forces to constrain the radial volume increase. One of the major generators of forces in tissues is actomyosin contractility. Using time-lapse imaging with fluorescently tagged myosin, we found that contractile myosin began to accumulate at the basal surfaces of a subset of follicle cells beginning in early stage 9. We also observed that the basal surfaces of follicle cells actively contract and relax, shrinking specifically along the short axis. The contractions were strikingly cyclical with an average period of 6.5 min [28^{••}]. These myosin-mediated basal contractions require the Rho–ROCK pathway, cytosolic calcium, integrin-mediated cell–ECM interactions, and E-cadherin-mediated cell–cell adhesion. Both pharmacological and genetic approaches show that interfering with the contractions results in rounder eggs whereas enhancing the contractions leads to longer eggs. Since these contractions do not occur in all follicle cells but rather are mostly confined to follicle cells near the center of the egg chamber, we postulate that the effect of the contractions is to constrain the tremendous increase in tissue volume to the two ends. We envision that this is somewhat similar to squeezing a ball of dough to make it longer, although obviously the elastic properties of cells differ from those of dough. Nevertheless, exertion of an anisotropic force near the middle of the tissue as it expands in volume over the course of 10 h does affect its shape, although it is not clear precisely which cellular and extracellular elements respond to the force and create the lasting change in shape. Live imaging reveals that elongation of this tissue involves much more dynamic molecular and cellular behaviors compared to the static view of a corset that was developed from analysis of fixed tissue. It will be important in the future to decipher the mechanisms that initiate, sustain, and pattern the oscillations. Oscillations in myosin accumulation and cellular contractility have been observed in other epithelia undergoing morphogenetic changes [29,30]. Interestingly the oscillation periods and subcellular locations of myosin accumulation differ in the different tissues, suggesting that the oscillation mechanism can be regulated tissue-specifically to achieve diverse morphogenetic outcomes.

Live imaging of collective border cell migration

At late stage 8 a small group of anterior follicle cells adjacent to the polar cells, referred to as border cells, round up in response to the cytokine Unpaired (Upd),

which the polar cells secrete. Upd activates the JAK/STAT pathway in the border cells, causing them to extend protrusions, delaminate from the epithelium and migrate in between the nurse cells. These 4–7 cells surround and carry the two non-motile polar cells from the anterior tip of the egg chamber, reaching the oocyte by stage 10 [31–33]. Genetic screens and analysis of border cell migration in fixed tissue revealed multiple signaling pathways that control distinct features of the movement. Whereas JAK/STAT signaling determines which of the follicle cells acquire the ability to move, receptor tyrosine kinases, PVR and EGFR, determine the direction of movement in response to ligands secreted by the oocyte [34–36]. The steroid hormone ecdysone by contrast controls the timing of border cell migration [32].

Analysis of border cell migration using live imaging has begun to reveal the dynamic features of their movement. For example, it was surprising to find that inhibition of both EGFR and PVR function in border cells did not suppress cell protrusion in the forward direction. On the contrary, multiple cells extend long and random protrusions in all directions, suggesting that the guidance signals may not only promote cell protrusion at the front but also inhibit protrusions in the wrong directions [15**].

A major molecular driver of protrusion in cells is the small GTPase Rac and Rac has long been known to be required for border cell migration [35,37,38]. However, both dominant-negative (DN) and constitutively active (CA) forms of Rac cause strong migration defects, indicating that Rac activity must be spatially and/or temporally regulated. Recently it has become possible to control Rac activity *in vivo* using genetically encoded and caged forms of Rac, created by Wu *et al.* [39]. These caged Rac proteins provide a novel approach to activate or inhibit Rac activity with high spatial and temporal resolution in response to flashes of blue laser light. Using this tool, we found that activating or inhibiting Rac activity in one migrating border cell causes dramatic responses of the other cells in the cluster [40**]. Activating Rac in any cell, caused the cluster to migrate in that direction. Inhibition of Rac in the leading cell caused all cells to lose their sense of direction and thus to protrude outward in all directions. In this study, the endogenous pattern of Rac activity was also monitored using a Rac FRET biosensor that was originally generated in Matsuda's lab and modified by Kardash and co-workers [41,42]. Rac activity is normally higher at the front and lower at the back of border cell clusters, and is higher in the front portion of the leading cell than in the back of the leading cell. Polarity of Rac activity is lost when guidance receptor activity is inhibited, although some uniform Rac activity persists. These findings suggest that a low level of uniform Rac activity promotes protrusion in all directions in the absence of guidance receptor activity, and that in response to asymmetric guidance receptor activation, Rac activity increases at

the front, enhancing forward directed protrusion. In addition, via an unknown mechanism enhanced Rac activity at the front inhibits protrusion in other directions of all the cells in the cluster. A key open question is by what mechanism the cells sense relative levels of Rac activity in adjacent cells.

Live imaging of transport and polarization of RNA and protein in germline cells

Initially discovered in developing oocytes, the polarized localization of mRNAs turns out to be a widely adapted mechanism to establish cell polarity in germ cells, neurons, and cells undergoing asymmetric division [43]. Localization of numerous mRNAs to distinct regions of the fly oocyte is crucial for normal patterning of the embryo following fertilization. One advantage of using egg chambers to study the subcellular localization of molecules is the large size of the germline cells. During development, the oocyte grows from a ~20 μm diameter at stage 3 to more than 100 μm at stage 10. Live imaging of RNA movement in the fly ovary was first achieved by microinjection of fluorescently labeled molecules into late stage egg chambers. An active, long-range, microtubule-dependent cytoplasmic flow termed ooplasmic streaming occurs between late stage 10 and stage 13 [44,45]. Streaming is inhibited earlier in egg chamber development by actin polymerization, kinesin and dynein motor activities and by the two proteins Capucuccino (Capu) and Spire [46,47].

Forrest and Gavis pioneered the use of a less invasive labeling technique to track *in vivo* movement of *nanos* RNA and found that it translocated along microtubules and then became anchored at the posterior region by an actin-dependent mechanism [48*]. This RNA labeling system takes advantage of the binding between bacteriophage MS2 coat protein (MCP) and a specific RNA sequence that forms a stem-loop structure, and was first used by Bertrand *et al.* to study mRNA localization in living yeast [49]. When MCP is tagged with fluorescent protein, and co-expressed with an RNA tagged with the MS2-binding sequence, the position of the RNA is revealed by the fluorescent signal from the MCP bound to it [50]. This mRNA labeling method was later used to localize many other important mRNAs in the oocyte including *Gurken*, *Bicoid* (*bcd*), and *Oskar* [51–53]. A more detailed review of mRNA localization in the *Drosophila* oocyte can be found in Becalska and Gavis [54].

Localized mRNAs are typically found in ribonucleoprotein (RNP) complexes, so in addition to mRNAs, many mRNA-binding proteins, including Exuperentia (Exu), Staufen (Stau), Vasa, Aubergine and Yps, also exhibit specific localizations within the oocyte. Live imaging has also been used to study the mechanisms responsible for their transport and localization. For example, fluorescently labeled Exu, an RNA binding protein required for

proper localization of *bcd* mRNA, exhibits a dynein-dependent directional movement on polarized microtubules (MTs) and travels through the ring canals that connect the nurse cells to the oocyte [55]. Imaging of Staufen (Stau), a protein associated with RNPs containing *oskar* mRNA, revealed that *oskar* mRNA is randomly transported on MTs in all directions with a weak posterior bias [53]. Interestingly, Shimada *et al.* recently discovered that the mRNA-binding protein Ypsilon Schachtel (Yps) is transported via both MT-dependent and MT-independent mechanisms and this transport is regulated by nutrient availability and insulin signaling, possibly as part of a mechanism to preserve oocytes during periods of nutrient deprivation and allowing for rapid resumption of reproduction when conditions improve [56*].

Live imaging of epithelium morphogenesis during dorsal appendage formation

Complex epithelial movements also occur during late stages of oogenesis (from stage 10B to stage 14). Live culture of late stage egg chambers was first reported by William H. Petri in 1979 [12]. The culture of late stage egg chambers may be less demanding because the egg chamber does not grow much and starts to form a vitelline membrane and thus to separate itself from the environment. The formation of the dorsal respiratory appendages during these stages had been analyzed live by Dorman *et al.* [57]. Three phases of morphogenesis were revealed, and two cell types that form the roof and floor of the structure exhibit different morphological behaviors.

Future prospects

In the past few years, live imaging of earlier stages of oogenesis has revealed patterning mechanisms that were unimaginable based on analysis of fixed tissue, such as the rotation of follicles within the basement membrane and oscillating myosin contractions. The rapid development of new genetically encoded biosensors, caged proteins and microscopy technology provides unprecedented opportunities to address biological questions using live imaging. Biosensors have been engineered to reveal changes in pH, ion concentrations, protein activities, and even the distribution of mechanical forces [58–60]. The possibilities for manipulating protein activities with high spatial and temporal resolution are also likely to expand tremendously in the near future. Recently many photoactivatable proteins have been engineered using different approaches to cage a broad spectrum of signaling molecules, including a cation channel [61], adenylyl cyclase [62], G protein-coupled receptors [63], transcription factors [64–66], and multiple protein kinases [67]. Combining these tools with innovations in whole organ cultures will allow investigators to both manipulate these pathways and monitor the immediate effects, not only on individual cells or cell types but also on the complex interactions between different cell types and ECM.

Although phototoxicity is a factor that limits long-term 3D imaging in many tissues including the ovary, advances in microscopy are likely to continue to improve our ability to see deeper, with higher resolution, and over longer periods of time. For example, light sheet microscopy or selective plane illumination microscopy (SPIM) provides a solution to image large and deep samples with greatly reduced light exposure [68]. For this technique the sample has to be suspended in a tube of transparent gel to allow imaging from multiple directions, which may require special protocol modifications. In addition, the quantity of data collected using SPIM challenges current software and hardware available in most labs. Perfusion systems may enhance survival of tissues that requires constant nutrient supply [69]. In addition, super-resolution techniques such as structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED), 4Pi, and photo-activated localization microscopy (PALM/STORM) have the potential to reveal protein dynamics at the single molecule level [70,71]. Tradeoffs for the increased resolution offered by these approaches include limitations in imaging speed, depth, and requirements for specific fluorescent probes, such as photoactivatable or photoconvertible fluorescent proteins. The depth limitation can be overcome by combining PALM/STORM with TIRF for some applications, such as imaging the basal myosin oscillations. It is very likely that with the continued improvements in culture conditions, biosensor development, and microscopy techniques, live imaging will greatly advance our understanding of the dynamic molecular, cellular and supracellular mechanisms that control *Drosophila* oogenesis.

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