

Live imaging of *Drosophila* imaginal disc development

Silvia Aldaz¹, Luis M. Escudero^{1,2}, and Matthew Freeman³

Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 0QH, United Kingdom

Communicated by A. Garcia-Bellido, Universidad Autonoma de Madrid, Madrid, Spain, June 25, 2010 (received for review March 19, 2010)

Live imaging has revolutionized the analysis of developmental biology over the last few years. The ability to track in real time the dynamic processes that occur at tissue and cellular levels gives a much clearer view of development, and allows greater temporal resolution, than is possible with fixed tissue. *Drosophila* imaginal discs are a particularly important model of many aspects of development, but their small size and location inside the larva and pupa has prevented live imaging techniques from extensively being used in their study. Here, we introduce the use of viscous culture medium to enable high resolution imaging of imaginal disc development. As a proof of principle, we have analyzed the transformation that occurs during metamorphosis of the wing imaginal disc into the mature wing and report several previously unobserved stages of this model of organogenesis. These imaging methods are especially useful to study the complex and dynamic changes that occur during morphogenesis, but we show that they can also be used to analyze other developmental and cellular events. Moreover, our viscous medium creates a platform for future adaptation of other tissue culture conditions to allow imaging of a wide range of developmental events and systems.

wing | eversion | organogenesis | epithelia

Live imaging techniques in cell and developmental biology have rapidly evolved in the past few years. The study of dynamic and complex developmental processes such as cell division, migration, and morphogenesis have particularly benefited by the ability to view living tissue by time-lapse microscopy. The nature of *Drosophila* development, together with the diversity of genetic tools available, including mutants, mitotic clone techniques, and a wide range of fluorescently marked proteins, has made this organism an excellent subject for these approaches. Live imaging of the *Drosophila* embryo has been extensively used to follow, among many other processes, dorsal closure, germ band extension, wound healing, myoblast fusion, and cell death (1–4).

In larval and pupal stages, live imaging is more difficult, but some processes, such as the migration of histoblasts in the abdomen (5, 6), have been successfully filmed. Particularly difficult to image are imaginal discs, the epithelial precursors of most adult organs, which are some of the most highly studied developmental models: they are small and buried within the opaque larval or pupal body. Reported live imaging of imaginal discs has been limited but includes analysis of rotation of the ommatidia in the eye imaginal disc (7) and analysis of epithelial cell packing, as well as glial migration in the pupal wing (8, 9). Beyond embryos and imaginal discs, a few other organ culture systems have been developed, including the migration of follicle cells in the oocyte and aspects of brain development (10, 11).

During *Drosophila* metamorphosis, extensive and complex remodeling of larval tissues sculpts the adult; specifically, the adult abdomen forms from the histoblasts and all other exoskeleton structures derive from the imaginal discs. These events provide a valuable model of organ formation, but the degree of tissue movement, and its location within the pupa, has made metamorphosis difficult to study.

Imaginal discs are formed by two contiguous epithelia, a columnar layer, the disc proper (DP), and a squamous layer, the

peripodial epithelium (PE), the latter contributing little to the final adult structure; together they form a bag-like double epithelium (Fig. 1A). During metamorphosis the imaginal discs are completely remodeled in an evagination process comprising two discrete stages: elongation, during which the columnar epithelium lengthens and changes shape; and eversion, when contraction of the PE is thought to drive the appendages through the larval epidermis (12) (reviewed in refs. 13, 14). The interaction between the PE and the larval epidermis has been described to be essential (15), as is the partial invasion of the larval walls by the stalk cells (15), but the difficulty of analyzing this dynamic process in fixed tissue has prevented a full analysis of the cellular changes that occur. Fluorescence tomography has been used to image evagination in vivo but has limited resolution (16).

We have developed an ex vivo culture system that allows direct confocal imaging of imaginal discs for up to about 24 h. It extends organ culture studies pioneered by Milner in the 1970s (17, 18) and elaborates culture conditions to allow real time and high resolution confocal microscopy of disc development. In variations of the method, we have cultured discs attached or unattached to the pupal epidermis. To validate these techniques, we here describe wing disc eversion, although they are adaptable to multiple developmental processes in all imaginal discs. Indeed, while imaging morphogenesis, we have observed processes such as the formation of sensory organs, pupal cell divisions at the dorsal/ventral boundary, patterned apoptosis, and wound healing. Comparison of live processes ex vivo with fixed tissue dissected at different stages and with previously published data (15) gives us confidence that our system reproduces normal events. Overall, this method brings together the advantages of *Drosophila* imaginal discs as a model system with the power of live imaging of development.

Results

A Method to Image Imaginal Disc Development ex Vivo. Experiments from the 1970s by Milner and colleagues (17–19) described culture medium needed to allow ex vivo evagination of imaginal discs. This media, however, is not compatible with confocal imaging of the explanted disc. The lack of attachment of the specimen to a substrate prevents positioning of the tissue; moreover, the specimen easily drifts out of focus. We have revisited these now somewhat neglected approaches but, to eliminate the problems associated with high resolution imaging of development, we have developed modifications of the media and designed simple support chambers to allow imaging of discs attached or unattached to the pupal case (Fig. 1).

Author contributions: S.A., L.M.E., and M.F. designed research; S.A. and L.M.E. performed research; S.A., L.M.E., and M.F. analyzed data; and S.A., L.M.E., and M.F. wrote the paper.

The authors declare no conflict of interest.

¹S.A. and L.M.E. contributed equally to this work.

²Present address: Hospital Virgen Del Rocío/CSIC/Universidad de Sevilla, Instituto Biomedicina Sevilla, Av. Manuel Siurot, Sevilla 41013, Spain.

³To whom correspondence should be addressed. E-mail: mf1@mrc-lmb.cam.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008623107/-DCSupplemental.

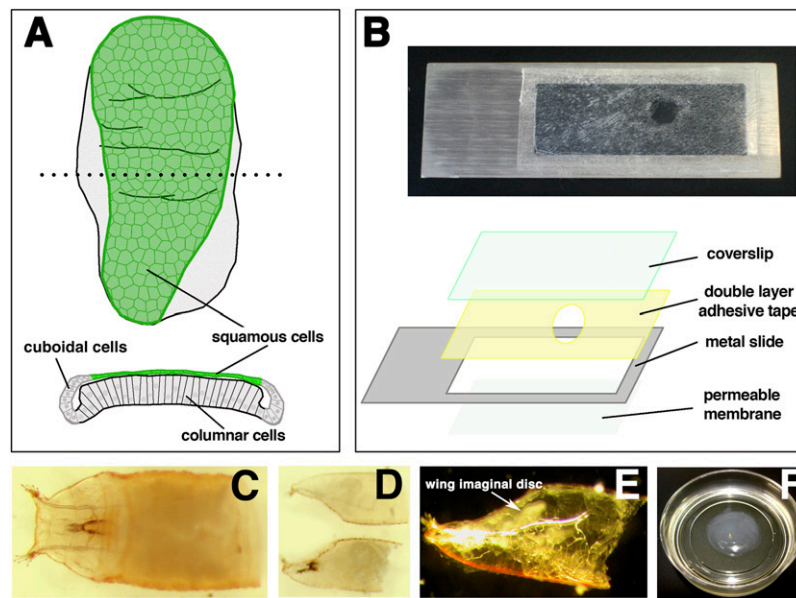


Fig. 1. Wing imaginal disc and preparation of samples. (A) A late third instar imaginal disc. (Upper) A frontal view with the squamous peripodial epithelium (PE) in green. (Lower) An axial section showing a more detailed view of the three types of cells in the imaginal disc. (B) Custom observation chamber for use with an upright microscope. (C) Anterior half of a prepupa (3–4 h APF) cut along the axial plane. (D) Same pupa bisected along the sagittal plane. (E) One of the half-pupae showing a wing imaginal disc (arrow) close to the inner side of the pupal case. (F) Culture dish containing the sample embedded in agarose and immersed in liquid medium.

In the case of explanted discs, we have modified the eversion media recipe to increase its viscosity to a point where the tissue can be held in a precise position to allow long-term imaging without drifting while leaving it sufficiently unconstrained for normal tissue remodeling not to be impeded. We experimented with several viscosity agents but found that 2.5% methyl cellulose was best. This technique allows imaging with either an inverted or upright microscope, making it easy to implement in any laboratory with access to a confocal microscope. For use with an upright microscope, we have designed a simple and cheap culture chamber that consists of a coverslip and oxygen permeable membrane, with a depth that allows morphogenetic movements but at the same time prevents discs from moving out of focus (Fig. 1B and *Experimental Procedures*).

In parallel, we developed a further modification to earlier *ex vivo* approaches. Because imaginal discs are normally attached to the larval and pupal epidermis, we also wanted to be able to image development in this more normal tissue context. For this, we dissected the pupa in culture medium but left the discs attached as normal to the pupal epidermal wall. This was done with two specific cuts: first, we removed the posterior of the pupa by cutting along the axial plane (Fig. 1C); second, we bisected the remaining anterior portion along the line of left–right symmetry (Fig. 1D). The two halves of the pupal case then contain imaginal discs that are sufficiently exposed to image (Fig. 1E). The exposed face was then placed facing the coverslip and immobilized with a drop of agarose (Fig. 1F). In this arrangement, the agarose covers the external part of the case with the discs free to move in the liquid medium; in this case, the extra viscosity of methyl cellulose is not required. The imaging of the pupal case immobilized under an agarose drop requires the use of an inverted microscope.

We studied the development of the wing imaginal disc during metamorphosis to validate these live imaging approaches. As described above, eversion remains poorly understood because the major tissue remodeling is difficult to follow in fixed tissue. We imaged unattached discs from the last 2 h of the third larval instar. It is easier to interpret the morphogenetic changes that

occur in these unattached discs because they are free of extraneous tissue (Fig. 2 and *Movies S1* and *S2*). However, because the interaction with epidermis is likely to be relevant to the final remodeling process, we validated these observations by studying similar stages of discs still attached to the pupal case (Fig. 3 and *Movies S3* and *S4*). The combination of both techniques provided information about the differential contribution to eversion of the imaginal disc itself and of the larval epidermis.

The viscosity of the *ex vivo* medium allowed the disc to be positioned so that images can be recorded in the primary plane of the disc epithelium (Fig. 2A and *Movie S1*) or perpendicular to it (Fig. 2B and *Movie S2*). We have not analyzed in detail wing discs at earlier times because we found that the culture conditions caused premature induction of metamorphosis in young discs (*Movie S5*). The culture of attached discs can start about 3 h after pupariation, when the rigidity of the pupal case makes the dissection possible (Fig. 3).

This pair of techniques is compatible with any fluorescent labeling method. In the case described here, we have imaged development with fluorescent reporter proteins derived from publicly available protein trap constructs. A transgene with the adherens junction protein Armadillo (Arm) (also known as β -catenin) linked to GFP (Flybase) labels apical cell contours of both the columnar and squamous epithelial layers. GFP-tagged regulatory light chain of nonmuscle myosin II, Spaghetti squash (Sqh), has a stronger signal in the PE and even in the disc proper, where it accumulates apically, the cytoplasmic signal is sufficient to see the whole cell (20). We have marked nuclei with Histone2A-mRFP (Flybase). The spatial and temporal resolution achieved depends simply on the objective and the scanning speed of the microscope used. In this study we typically used a 20 \times dry or a 25 \times oil immersion objective and 20–40 min intervals between z-stacks, but greater magnification and higher temporal resolution works equally well.

Imaging Evagination of the Wing Imaginal Disc. Evagination of the imaginal wing disc takes place over about 12 h. Figs. 2 and 3 show frames from movies of, respectively, unattached and at-

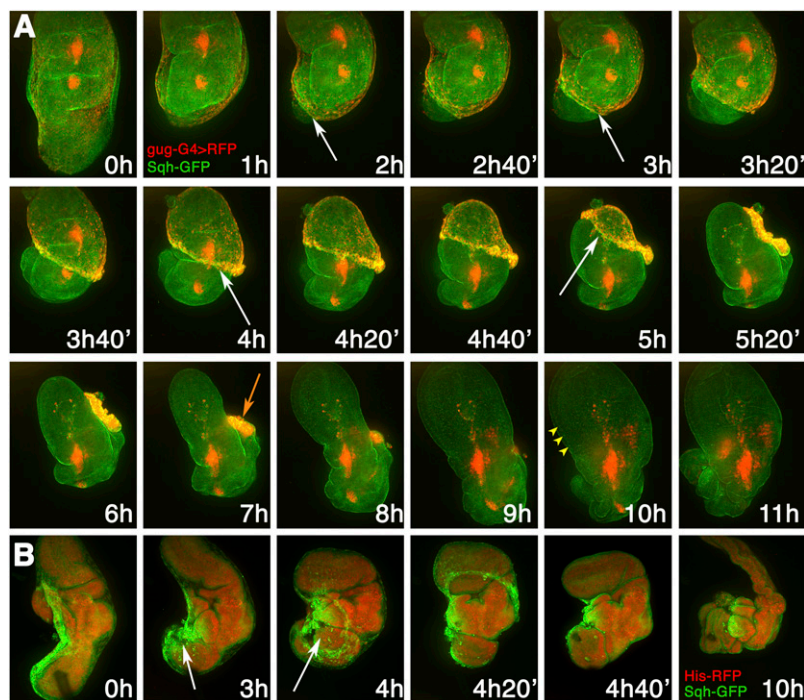


Fig. 2. Ex vivo imaginal disc eversion. (**A**) Frames from [Movie S1](#) showing a prepupal imaginal disc during the different steps of the eversion process. The disc is labeled with the *Sqh-GFP* transgene (green), and *grunge-Gal4* driving *UAS-mRFP* (red); *grunge-Gal4* is mainly expressed in peripodial cells but also in two bright spots in the region of the hinge of the columnar epithelium. This combination highlights the retraction process (white arrows mark the front of the retracting PE cells). The apoptotic bodies of the squamous cells accumulate at one side of the hinge region (orange arrow). The last images show the expansion of the wing pouch and the formation of the precursors of the wing margin sensory organs (yellow arrowheads). (**B**) Frames from [Movie S2](#) showing a prepupal imaginal disc mounted on its side showing the distribution of *Sqh-GFP* (green) and *His2A-RFP* (red). The first two frames show the folding of the whole disc after the addition of the ecdysone. This lateral view allows observation of the opening of the hole in the stalk region and the start of retraction (white arrows).

tached discs, and the processes we describe are shown diagrammatically in Fig. 4 and [Fig. S1](#). However, we encourage readers to view the movies directly: it is very difficult to illustrate dynamic 3D remodeling in still images, underscoring the need for live imaging as a primary data source in these kinds of study.

Combining the information obtained from more than 40 movies, we have divided the evagination process into two steps: folding and retraction. All of our analysis is derived from movies of discs dissected at a similar stage and which were undamaged. Before folding starts, the presumptive wing is a relatively flat bilayer epithelium (Figs. 2*A* and *B* and 4*A*); the future dorsal and ventral surfaces of the wing are adjacent to each other in the same plane, separated by the wing margin. The wing margin then moves so that the wing surfaces become apposed on opposite sides of the disc proper—the PE remains stretched over the disc proper as these movements occur, expanding to cover the entire presumptive wing (Fig. 4*B* and *C*). Once the wing margin reaches the border of the disc, the disc folds up to 90° from its initial plane (Figs. 2 and 4*A* and *C*). Although the movement of the wing margin and expansion of the PE are gradual processes, the folding happens quite fast. Fig. 4*D*, showing the attached disc, indicates how the folding brings the PE cells into close proximity with the larval epidermis (in red). The whole folding process can be seen in unattached cultured discs, but almost all of the attached discs already show some folding by the time they are dissected (Fig. 3 and 4*D*). Furthermore, attached discs only fold to about 45° from their initial plane, presumably because of constraints imposed by the associated pupal epidermis.

The second step of evagination is peripodial retraction. This step corresponds with eversion in vivo, the movement of the disc through the pupal epidermis. In unattached discs, a hole opens at

the stalk region (Fig. 2*B*) and the PE rapidly moves over the DP following a wave of retraction (Fig. 2*A* and *B* and [Movies S1](#) and [S2](#)). During this process the PE loses its epithelial morphology, and, by end of the retraction when the PE passes over the wing margin, the cells are round and separated (Fig. 2*A*); in fact, most of the squamous layer disintegrates, although some PE cells remain attached to the thorax and appear mesenchymal. The retraction is fast, being completed in 1 or 2 h (Fig. 2*A* and *B* and [Movies S1](#) and [S2](#)). After retraction, the disc expands, the folds in the columnar epithelium disappear, and both thorax and wing region expand. Particularly remarkable is the rapid expansion of the wing into an easily recognizable adult structure (Fig. 2*A* and *B*). Importantly, both the final everted discs and the intermediate steps are comparable to discs dissected from equivalent pupal stages and previous reports, suggesting that our explants resemble the process in vivo.

In the attached disc, retraction has different consequences, owing to the substrate to which the disc is anchored. The contact between disc and epidermis starts at the stalk region and, as peripodial retraction progresses, contact extends from the proximal thorax to the most distal regions, the wing blade (Figs. 3 and 4). The most distal tip of the wing never interacts with the epidermis. During the initial stages of retraction, clear separation frequently occurs between the PE and the DP (Fig. 3*B* and [Movie S4](#)). It has previously been reported that the stalk cells invade the epidermis so that the opening of the stalk creates a hole through which the disc can emerge (15). Our observations are consistent with this. We see that once the PE cells are closely apposed to the pupal epidermis, there is a subsequent contraction of the PE that corresponds to the retraction observed in movies of unattached discs (Fig. 3*B* and [Movie S4](#)). We interpret

this contraction to be responsible for driving the eversion of the disc into the space between the larval epithelium and the outer cuticle. We have observed that in the cases where, due to the mounting process, the PE and the larval walls are not in proximity to each other, the disc does not evert properly, suggesting that the interaction between them remains essential for eversion. Overall, the information coming from both culture systems, although showing some differences due to the constraints and limitations of each approach, can be integrated into a coherent interpretation of the dynamics of eversion in vivo.

During retraction, PE cells transform from epithelial to a mass of rounded cells, which appear to disintegrate. We used an apoptotic marker, *apoliner*, which moves from plasma membrane to nucleus at the onset of apoptosis (21), to confirm that, indeed, a wave of programmed cell death sweeps across the PE, eliminating most, if not all, cells (Movie S6).

Further Applications of ex Vivo Culture and Imaging. We have focused on wing disc eversion as a proof of principle for disc culturing and imaging, but the use of viscous medium is also valuable for studies of other developmental processes that occur in discs, including those that need to be followed at a cellular level with high temporal resolution. For example, using a 100 \times objective, we are able to image at 10-min intervals on a spinning disc microscope (Fig. 5A and Movies S7 and S8); in fact, the timing can be much faster if a smaller field of view and shallower z-stack is specified. To illustrate the versatility of these approaches, we have imaged a number of distinct developmental processes that we observed while studying eversion.

Where appropriate genetically encoded markers exist, intracellular events can be tracked. We show the reorganization of intracellular myosin-II-rich fibers that occurs as cell shapes change (Fig. 5A and Movie S7), but another powerful use would be to follow signaling events in real time. An additional advantage of ex vivo culture is that it allows the simple application of exogenous markers, compounds, and drugs. For example, fluo-

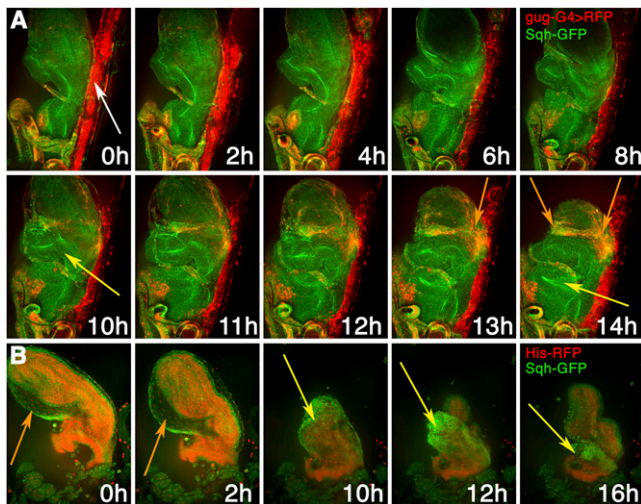


Fig. 3. Interaction between the wing disc and the pupal epidermis. (A) Frames from Movie S3 a prepupal imaginal disc attached to the pupal case. The disc expresses *Sqh-GFP* (green) and *grunge-Gal4 >UAS-RFP* (red). The red label also marks the close apposed pupal case and epidermis (white arrow). In the first frames, the PE cells are weakly stained, but when the contraction starts, intensity increases (orange arrows). At the same time the DP gets closer to the epidermis, folds, and protrudes its basal part (yellow arrows). (B) Frames from Movie S4, showing a *His2A-mRFP* (red), *Sqh-GFP* (green) wing disc. In this example, the separation between the disc proper and PE is very clear (orange arrows). The yellow arrows highlight the squamous epithelium retracting. The columnar epithelium protrudes from inside.

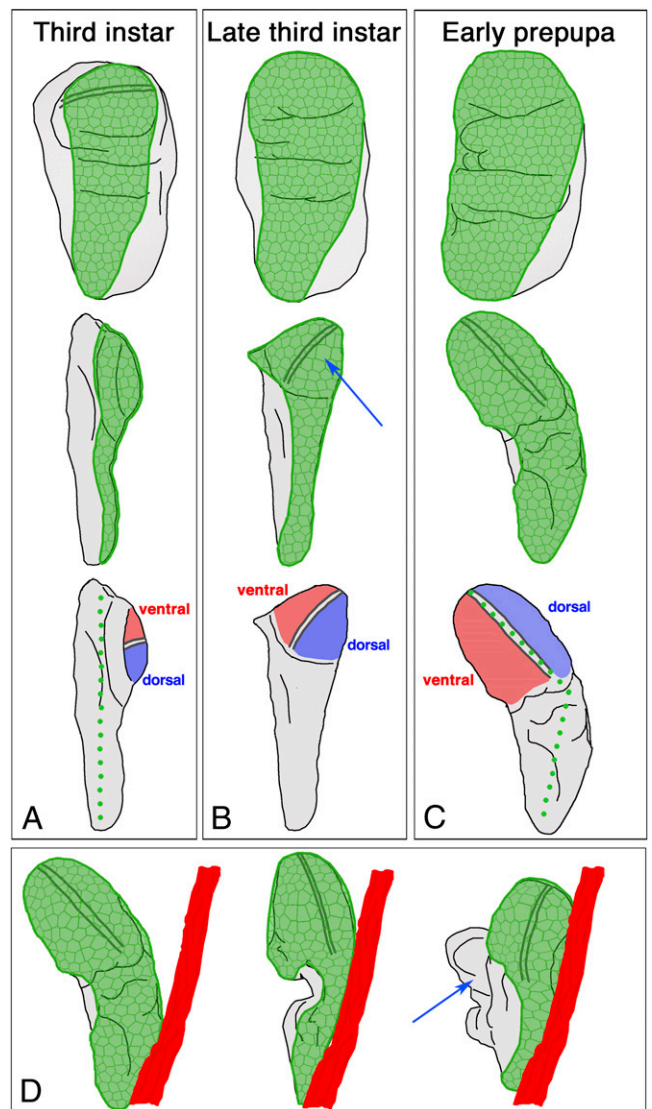


Fig. 4. Schematic representation of disc eversion. (A–C) Front view (Top in all panels) and side view (Middle in all panels) of a third instar wing disc. The squamous cells of the PE are represented in green and the columnar (and cuboidal) epithelium in gray. Note that in reality there is no sharp distinction between the squamous and columnar cells: a transition zone of cuboidal cells separates them. Here, we define the PE as the squamous cells alone. (Bottom) The image in each panel shows a side view of the disc, highlighting the dorsal and ventral compartments of the wing pouch of the imaginal disc and how they move during eversion. The green dotted line illustrates the bending between stages. (A) The third instar wing disc is flat, with ventral and dorsal compartments in the same plane. (B) By late third instar, the squamous epithelium has expanded, and the dorsal and ventral compartments of the wing pouch become apposed (arrow). (C) At the prepupal stage the disc starts to bend. The apposition of the two wing surfaces is complete. The peripodial cells cover almost the complete apical surface of the columnar epithelium. (D) Representation of the disc attached to the pupal case. The cuticle and the epidermis are represented in red. The three steps show how the disc approaches the epidermis, the PE contracts, folding the whole disc, and the basal part of the columnar epithelium protrudes (arrow).

recently labeled annexin-V (22) added to the medium provides a simple reporter of apoptosis without the need for a genetic marker (Fig. 5B and Movie S9); we have also used FM lyophilic dyes as markers. Imaginal discs are particularly intensively studied models of cell proliferation, and this is also a readily observed phenomenon in culture, both at a whole tissue level

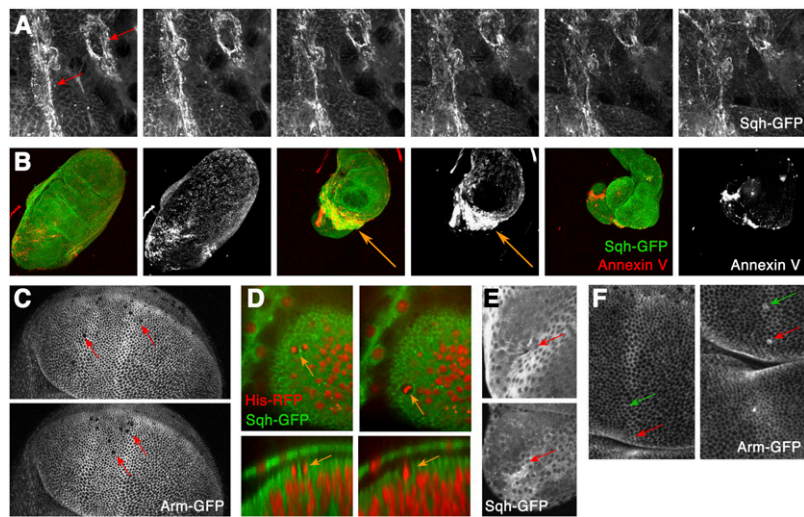


Fig. 5. Further applications of ex vivo culture. (A) Frames from [Movie S7](#) showing a wing disc labeled with Sqh-GFP. The red arrows point to the nuclei of two peripodial cells. Using a 100 \times objective, intracellular structures like myosin II fibers can be tracked. (B) Frames from [Movie S9](#) showing a prepupal imaginal disc labeled with Sqh-GFP (green) and annexin-V-Cy3 (red), which was added to the medium after dissection, as a reporter of apoptosis during retraction of the PE (indicated by arrows). (C) Two frames from [Movie S10](#) showing numerous dividing cells (several marked by arrows) in a wing disc labeled with Arm-GFP. (D) Frames from [Movie S11](#) showing a wing disc labeled with *His2A-mRFP* (red) and Sqh-GFP (green). Yellow arrows indicated dividing cells in which the different stages of mitosis can be tracked, both in the XY and XZ planes. (E) Initial (*Upper*) and final (*Lower*) frames, taken from [Movie S12](#), showing the healing of a wound (red arrow) in the notum of a wing disc labeled with Sqh-GFP. (F) Frames from [Movie S13](#) showing a wing disc labeled with Arm-GFP and the development of two wing vein sensillae in the wing pouch (green and red arrows).

(Fig. 5C and [Movie S10](#)) and, when appropriate markers are used, in individual cells (Fig. 5D and [Movie S11](#)). Wound healing is yet another process that can be imaged in ex vivo culture: Fig. 5E and [Movie S12](#) track the repair of a needle-stick injury to a wing disc, highlighting with GFP-tagged myosin II the reorganization of the actomyosin cytoskeleton. Finally, in this brief catalog to emphasize the range of developmental processes that can be imaged using these culture conditions, we have followed the patterned divisions and differentiation that leads to the development of sensory organs along the wing veins (Fig. 5F and [Movie S13](#)).

Discussion

Drosophila imaginal discs have provided great insight into mechanisms of development. Their growth and differentiation from simple epithelial sheets, comprising a small number of cells, to complex 3D organs of thousands of cells, represents a model of many general developmental processes including growth, proliferation, differentiation, morphogenesis, programmed cell death, and epithelial reorganization. The extensive genetic techniques available for their study include the use of mitotic clones, Gal4/UAS targeted gene expression, and transposon mutagenesis. Here we introduce live imaging methods that significantly extend the scope of imaginal discs as developmental models. To validate the use of live imaging to analyze morphogenesis in viscous medium, we have focused on metamorphosis as a model of epithelial remodeling and organogenesis—a process particularly suited to live imaging—but the ability to track development in real time provides a potential resource for the analysis of most processes that occur in discs.

Previous work describing metamorphosis of imaginal discs has primarily relied on observing dissected, fixed tissue. This makes it difficult to interpret the complex remodeling, does not allow high temporal resolution of the sequence of events, and introduces significant fixation artifacts (for example, the collapse onto the columnar epithelium of the adjacent squamous epithelium). Our description of disc eversion agrees in many ways with previously reported data, which, combined with our complementary data from attached and unattached discs, give us confidence that the

culture system is faithfully reproducing in vivo development. Unsurprisingly, however, it has also revealed several events that have not previously been reported, again emphasizing the extra information that is acquired by direct observation of living tissue. These include previously undescribed morphogenetic movements like the 90° folding of the disc, the subsequent rapprochement with the pupal epidermis, and the unexpected disintegration by apoptosis of most of the PE. Limited apoptosis in the stalk region has been described (15), but the coordinated death of the whole peripodial layer has previously been unreported.

Overall, the movements we describe here suggest that the folding of the disc drives the approach of the whole disc to the pupal wall. It has previously been proposed that contraction of the PE is responsible for the forces needed during the eversion of the disc (13, 15). Our data support this and further suggest that the interaction between the PE and the pupal epidermis also participates in the mechanics of disc eversion.

The particular features of the culture methods we have developed include the ability to follow disc development over long periods (at least 24 h), viscous medium that supports discs in any orientation and prevents drifting, the use of confocal imaging to reconstruct tissue development in 3D, and temporal and spatial resolution limited only by microscope technology and the size of the tissue under observation. Another major advantage is the simplicity of the technique: it does not rely on any unusual equipment or reagents. Finally, in addition to being applicable to all imaginal discs, viscous culture media could easily be adapted to facilitate live imaging of other organ systems in *Drosophila* and other species.

Methods

Eversion Media Composition. Shields and Shang M3 insectum medium from Sigma (S3652) was supplemented with 2% FCS and 0.5% penicillin-streptomycin (15140–122; Invitrogen). Ecdysone from Sigma, 20-hydroxyecdysone H5142, was stored in a stock solution of 500 μ g/mL in 10% isopropanol at -20°C , and added at a final concentration of 0.1 μ g/mL for unattached discs and 0.5 μ g/mL for attached discs. To culture unattached discs, methyl-cellulose (M0387-100G; Sigma) was added at a concentration of 2.5% wt/vol. The methyl-cellulose was added to the media with ecdysone and stirred until dissolved, then left overnight at 4°C

to eliminate bubbles; 1 mL aliquots were stored at -20°C . The frozen media works for up to 3 mo.

Fly Stocks and Genetics. Flies were grown under standard culture techniques. The following lines were used: *sqh-GFP* (20); *arm-GFP*, *UAS-mRFP*, *His2A-mRFP1* (Flybase), *UAS-E-cad-GFP* (2), *grunge-Gal4* (23), *Ubx-Gal4^{LDN}* (24). Overexpression using the Gal4/UAS system (25) was performed at 25°C .

Annexin-V Staining. We used the annexin-V-CY3 apoptosis kit from Biovision (catalog no. K103-25). Annexin-V was added to the media at a dilution 1/200.

Dissection and Mounting. Late third instar larvae were washed in sterile PBS and disinfected in 70% ethanol for 5 min. After rinsing in PBS, they were transferred to eversion media, without methylcellulose, where the discs were carefully dissected using forceps and needles. Wounded discs were discarded. Unharmed discs were placed in an observation chamber, when being imaged on an upright microscope, or in a 35-mm culture dish with glass bottom (Fluorodish FD35-100, World Precision Instruments, Sarasota, FL), when an inverted microscope was being used. After dissection, the media was replaced with viscous media: approximately 30 μL in the observation chamber and 1 mL in culture dishes. The discs were then positioned carefully with a needle. When an observation chamber was used, it was covered with an oxygen semipermeable membrane, (catalog number 5793; YSI).

Attached discs were dissected in eversion media. The posterior tip of pupae 3 h postupariation was removed at the level of the spiracles with microdissection scissors. A second transverse cut in the medial region was then made, leaving approximately the anterior 50% of the pupa. Finally a third cut was made longitudinally, leaving two mirror-image halves, each with a wing disc attached to the pupal walls. Internal tissues obscuring the discs were removed carefully (Fig. 1 C–E). The dissected tissue was then placed in a culture dish, with the open surface facing the coverslip on the base (Fig. 1).

A drop of 2% agarose, melted in PBS and allowed to cool until just before it set, was then placed over the cuticle. Once solid, approximately 2.5 mL of fresh medium was added to the dish.

Observation Chamber Construction. The chamber was made with a double layer of double side tape (Sellotape acid free). The choice of the tape is important: some released adhesive into the medium, impairing the eversion. The two layers were perforated using a hole puncher, generating a hole of approximately 6 mm diameter. The tape was attached to a $22 \times 50\text{-mm}$ coverslip and trimmed to the same size. The coverslip was then attached to a metal slide with a cut-out panel. Finally, the chamber/hole was filled with medium and the discs placed in it and then covered with the semipermeable membrane as described.

Confocal Microscopy. We have used three different confocal systems: a Zeiss LSM510 on an upright microscope; a Zeiss LSM 710 on an inverted microscope; and a Perkin-Elmer spinning disc UltraVIEW ERS with an Orca ER CCD camera (Hamamatsu). Exact settings varied with experiment and system but were chosen to balance the need for a clear image with minimizing laser exposure to avoid tissue damage. Typically we took between 80 and 120 z sections at $1\text{-}\mu\text{m}$ interval every 20 to 50 min. When imaging the whole discs, the interval was decreased to allow a full 3D reconstruction. The images were analyzed using Adobe Photoshop CS2, Image J, and Volocity (Improvision) software.

ACKNOWLEDGMENTS. We thank Luis F. de Navas, Simon Bullock, and Nick Barry for generously supplying stocks and support. We thank Matthew Freeman's laboratory members, Thomas Lecuit, and Enrique Martín-Blanco for helpful discussions. Natalia Azpiazu and Antonio Baonza helped us greatly with useful comments on the manuscript. This work was supported in part by Marie Curie (to S.A. and L.M.E.) and European Molecular Biology Organization (to L.M.E.) fellowships.

- Bertet C, Sulak L, Lecuit T (2004) Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. *Nature* 429:667–671.
- Oda H, Tsukita S (1999) Dynamic features of adherens junctions during *Drosophila* embryonic epithelial morphogenesis revealed by a Δ alpha-catenin-GFP fusion protein. *Dev Genes Evol* 209:218–225.
- Wood W, et al. (2002) Wound healing recapitulates morphogenesis in *Drosophila* embryos. *Nat Cell Biol* 4:907–912.
- Richardson BE, Beckett K, Baylies MK (2008) Live imaging of *Drosophila* myoblast fusion. *Methods Mol Biol* 475:263–274.
- Ninov N, Martín-Blanco E (2007) Live imaging of epidermal morphogenesis during the development of the adult abdominal epidermis of *Drosophila*. *Nat Protoc* 2: 3074–3080.
- Bischoff M, Cseresnyés Z (2009) Cell rearrangements, cell divisions and cell death in a migrating epithelial sheet in the abdomen of *Drosophila*. *Development* 136: 2403–2411.
- Escudero LM, Bischoff M, Freeman M (2007) Myosin II regulates complex cellular arrangement and epithelial architecture in *Drosophila*. *Dev Cell* 13:717–729.
- Classen AK, Anderson KI, Marois E, Eaton S (2005) Hexagonal packing of *Drosophila* wing epithelial cells by the planar cell polarity pathway. *Dev Cell* 9:805–817.
- Aigouy B, Van de Bor V, Boegli M, Giangrande A (2004) Time-lapse and cell ablation reveal the role of cell interactions in fly glia migration and proliferation. *Development* 131:5127–5138.
- Bianco A, et al. (2007) Two distinct modes of guidance signalling during collective migration of border cells. *Nature* 448:362–365.
- Ayaz D, et al. (2008) Axonal injury and regeneration in the adult brain of *Drosophila*. *J Neurosci* 28:6010–6021.
- Fristom D, Fristom JW (1975) The mechanism of evagination of imaginal discs of *Drosophila melanogaster*. 1. General considerations. *Dev Biol* 43:1–23.
- Gibson MC, Schubiger G (2001) *Drosophila* peripodial cells, more than meets the eye? *Bioessays* 23:691–697.
- Usui K, Simpson P (2000) Cellular basis of the dynamic behavior of the imaginal thoracic discs during *Drosophila* metamorphosis. *Dev Biol* 225:13–25.
- Pastor-Pareja JC, Grawe F, Martín-Blanco E, García-Bellido A (2004) Invasive cell behavior during *Drosophila* imaginal disc eversion is mediated by the JNK signaling cascade. *Dev Cell* 7:387–399.
- Vinegoni C, Pitsouli C, Razansky D, Perrimon N, Ntziachristos V (2008) In vivo imaging of *Drosophila melanogaster* pupae with mesoscopic fluorescence tomography. *Nat Methods* 5:45–47.
- Milner MJ (1977) The eversion and differentiation of *Drosophila melanogaster* leg and wing imaginal discs cultured in vitro with an optimal concentration of beta-ecdysone. *J Embryol Exp Morphol* 37:105–117.
- Milner MJ, Sang JH (1974) Relative activities of alpha-ecdysone and beta-ecdysone for the differentiation in vitro of *Drosophila melanogaster* imaginal discs. *Cell* 3:141–143.
- Milner MJ, Muir J (1987) The cell biology of *Drosophila* wing metamorphosis in vitro. *Roux Arch Dev Biol* 196:191–201.
- Royou A, Sullivan W, Karsenti R (2002) Cortical recruitment of nonmuscle myosin II in early syncytial *Drosophila* embryos: Its role in nuclear axial expansion and its regulation by Cdc2 activity. *J Cell Biol* 158:127–137.
- Bardet PL, et al. (2008) A fluorescent reporter of caspase activity for live imaging. *Proc Natl Acad Sci USA* 105:13901–13905.
- Martin SJ, et al. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182:1545–1556.
- Gibson MC, Lehman DA, Schubiger G (2002) Lumenal transmission of decapentaplegic in *Drosophila* imaginal discs. *Dev Cell* 3:451–460.
- de Navas L, Foronda D, Suzanne M, Sánchez-Herrero E (2006) A simple and efficient method to identify replacements of P-lacZ by P-Gal4 lines allows obtaining Gal4 insertions in the bithorax complex of *Drosophila*. *Mech Dev* 123:860–867.
- Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.