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TITLE:

Imaging and Analysis of Tissue Orientation and Growth dynamics in the Developing *Drosophila* Epithelia during Pupal Stages

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KEYWORDS:

Drosophila pupa, abdomen, histoblasts, epithelia, live imaging, orientation, confocal microscopy, clonal analysis

SUMMARY:

This protocol is designed for the imaging and analysis of the dynamics of cell orientation and tissue growth in the *Drosophila* abdominal epithelia as the fruit flies undergo metamorphosis. The methodology described here can be applied to the study of different developmental stages, tissues, or subcellular structures in *Drosophila* or other model organisms.

ABSTRACT:

Within multicellular organisms, mature tissues and organs display high degrees of order in the spatial arrangements of their constituent cells. A remarkable example is sensory epithelia, where cells of the same or distinct identities are brought together via cell-cell adhesion showing highly organized planar patterns. Cells align to one another in the same direction and display equivalent polarity over large distances. This organization of the mature epithelia is established over the course of morphogenesis. To understand how the planar arrangement of the mature epithelia is achieved, it is crucial to track cell orientation and growth dynamics with high spatiotemporal fidelity during development in vivo. Robust analytical tools are also essential to identify and characterize local-to-global transitions. The *Drosophila* pupa is an ideal system to evaluate oriented cell shape changes underlying epithelial morphogenesis. The pupal developing epithelium constitutes the external surface of the immobile body, allowing long-term imaging of intact animals. The protocol described here is designed to image and analyze cell behaviors at both global and local levels in the pupal abdominal epidermis as it grows. The methodology described can be easily adapted to the imaging of cell behaviors at other developmental stages, tissues, subcellular structures, or model organisms.

INTRODUCTION:

To achieve their roles, epithelial tissues fully rely on the spatial organization of their cellular components. In most epithelia, cells are not only packed against each other to create a precise cobblestone layer but they orient themselves relative to the body axes.

The functional importance of precise tissue organization is obvious in sensory epithelia, such as the vertebrate inner ear and retina. In the first case, hair and supporting cells align in a specific axial direction to efficiently sense mechanical inputs such as sound and motion[1,2]. Similarly, photoreceptor cell spatial organization is essential for achieving optimal optical properties by the retina[3]. Spatial control of cell position and orientation is thus of particular relevance for proper physiological function.

Drosophila is a holometabolous insect that undergoes a complete transformation of its larval body structures through metamorphosis, giving rise to its adult tissues. The *Drosophila* pupa is an excellent model for the noninvasive live imaging of a variety of dynamic events, including developmental cell migration[4], cell division and growth dynamics[5], muscle contraction[6], cell death[7], wound repair[8], and cell orientation[9]. In the adult *Drosophila*, the external epithelium shows a high degree of order. This is easily observed on the arrangements of trichomes (i.e., cell protrusions originating from single epithelial cells) and sensory bristles all over the fly's body surface[10]. Indeed, trichomes are aligned in parallel rows guiding airflow[11]. The morphogenesis of the adult epithelia and the ordered arrangement of the individual cells starts during embryogenesis and culminates during pupal stages. While in embryos cell divisions, intercalations, and shape changes all decrease tissue order[12,13], this is reverted at later stages of development, especially at pupal stages, when the fly approaches maturity[9].

The immobile *Drosophila* pupa provides an ideal system to evaluate cell shape and orientation changes. The pupal abdominal epidermis presents special advantages. While the precursors of the adult head, thorax, genitalia, and appendages grow and get patterned from larval stages, the histoblasts, which are integrated into the larval epidermis, start growing and differentiating only at pupariation[14]. This feature allows the tracking of all spatiotemporal events involved in the establishment of tissue order in its entirety[9].

Histoblasts are specified during embryonic development at contralateral positions in each presumptive abdominal segment. The dorsal abdominal epidermis of the adult derives from dorsolaterally located histoblast nests present at the anterior and posterior compartments[15,16]. As histoblasts expand, replacing the larval epithelial cells (LECs), the contralateral nests fuse at the dorsal midline forming a confluent sheet[17,18,19,20].

This work describes 1) a methodology for dissection, mounting, and long-term live imaging of the *Drosophila* pupae, and 2) analytical methods to study the dynamics of cellular orientation and growth at high spatiotemporal resolution. A detailed protocol is provided, covering all the steps required from the initial pupae preparation (i.e., staging and imaging) to the extraction and quantification of directionality and orientation features. We also describe how to infer local tissue properties from the analysis of cell clones. All the steps described are minimally invasive and allow long-term live analyses. The methods described here can be easily adapted and applied to other developmental stages, tissues, or model organisms.

PROTOCOL:

NOTE: This protocol is divided into five steps: (1) staging the pupae, (2) preparing the pupae for imaging, (3) live imaging of the growing abdominal epithelia, (4) generation of genetic

mosaics, (5) data processing and analysis (including sections describing how to analyze cell orientation dynamics from cell junction outlines and growth dynamics from cell clones).

1. Staging of *Drosophila* pupae before imaging

1.1. Culture flies of the appropriate genotype on standard medium in plastic vials at 25 °C for 5 days (±12 h) after egg laying (AEL).

NOTE: Metamorphosis starts within the confinement of the third-instar larvae into the pupal case at 120 h AEL until 0 h after puparium formation (APF). This transition is easily identifiable, because larvae stop feeding and moving and the opercular region is formed (**Figure 1A**) at 0-12 h APF. The puparium is initially soft and white, but progressively hardens and tans.

1.2. Transfer white prepupae (0 h APF) to a fresh plastic vial using a moistened paintbrush. Animals can be kept at different temperatures depending on the designed experiment until the desired age.

NOTE: Pupa formation (i.e., pupation) occurs at 12 h APF, when the head and the appendages of the adult fly are totally everted (**Figure 1A**). By this time the pupal case is fully separated from the pupa, allowing its complete removal (**Figure 1A**).

2. Preparing pupae for live imaging

NOTE: After staging, the pupae are dissected and mounted as described below (see also **Figure 1**).

2.1. Remove staged pupae from the wall of the vial with a moistened paintbrush.

2.2. Glue the ventral side of each pupa on a glass slide covered with double-sided sticky tape. Gently tap on the head spiracles (i.e., opercular region) and the dorsal surface of the pupa with the tips of the forceps to assure the adhesion of the pupal case to the tape (**Figure 1A**,**B**).

NOTE: The dorsal surface should face up to facilitate the dissection of the case and the recovery of the pupa.

2.3. Begin dissection under a stereomicroscope by gently removing the operculum from the puparium with the forceps (**Figure 1C**).

2.4. Insert one tip of the forceps in a shallow angle between the pupal case and the pupa surface through the opercular opening. Tear the case from head to tail laterally in one or more swings, avoiding pinching the pupa (**Figure 1D**). Fold back the cracked pupal case to the lateral sides as you keep proceeding to the posterior end (**Figure 1E**).

NOTE: The pupal case is quite rigid and cracks easily. In case of high humidity or in particular genotypic backgrounds, the pupal case becomes softer and tearing is more difficult. In these

cases, the cracking of the pupal case can be helped by pricking its free edges with both tips of the forceps.

2.5. Remove the pupa from the opened-up pupal case by carefully inserting the forceps under the animal and gently pulling up (**Figure 1F**). The pupa will stick to the tip of the forceps (**Figure 1G**–**H**).

2.6. Transfer the pupa with the help of the forceps to a glass-bottomed dish and deposit it on top of a small drop of gas-permeable halocarbon oil (**Figure 1I**). Hold the pupa gently by the head to avoid any possible tissue damage.

NOTE: The drop of halocarbon oil has to be small, with a diameter approximately less than half the length of the pupa. Such amount is sufficient to adhere the pupa to the glass by capillarity and to correct the optics for oil immersion objectives.

2.7. Roll a piece of wet tissue paper at the edges of the dish to maintain humidity. Cover the dish to avoid dehydration of the pupae during imaging.

NOTE: Both female and male pupae can be employed for imaging. We recommend employing the third abdominal segments (AIII) as a reference abdominal metamere since it is almost identical in both sexes in terms of size, shape, and patterning.

3. Live imaging of growing abdominal epithelia

NOTE: An inverted laser scanning confocal microscope equipped with a 40x/1.3 NA oil immersion objective was used to image pupae at different developmental stages.

3.1. Orient the pupa over the oil drop on the glass-bottomed dishes according to the domain and the process to be evaluated (e.g. dorsolaterally for long-term live imaging of the early expansion of the dorsal nests, or dorsally to image their late expansion and tissue remodeling). See **Figure 1J**,**K** and **Figure 4**.

3.2. Transfer the glass-bottomed dish containing the mounted pupae to the microscope stage and focus on the surface of the abdominal area using the transmitted light.

NOTE: Even if this protocol is optimized for imaging on inverted microscopes, it is also possible to perform imaging on an upright microscope. In that case, the sample is placed on the microscope stage with the glass-bottom surface facing up. The halocarbon oil holds each pupa on a meniscus.

3.3. Set the acquisition parameters: 1) the number of Z-slices are usually between 20–40 to allow appropriate two-dimensional (2D) reconstruction of the abdominal epidermis; 2) step size between each slice (e.g., 1 micron); 3) time interval for recording (a 5 min interval is suitable for high fidelity analyses of cell orientation dynamics); and 4) frame resolution (e.g., 1024 x 1024).

3.4. Turn on the appropriate lasers (i.e., 488 nm and 561 nm to visualize GFP and mCherry fluorophores respectively) and adjust the laser power and gain/offset settings to visualize the marked cells. Use the lowest possible laser power (in the range 5%–20%) to minimize photobleaching and phototoxicity.

3.5. Manually set the position and the appropriate Z-stack limits for multiple pupae using the attached motorized stage and the microscope multiposition acquisition software.

4. Generation of genetic mosaics to follow behaviors of cell clones

NOTE: We employ mitotic recombination to induce genetic mosaics in the abdominal epithelium via site-specific recombination (FLP/FRT system[21,22]) (**Figure 2**).

4.1. Cross virgin females carrying a heat shock-inducible Flippase transgene (hs-FLP), a FLP recognition target (FRT) site at a specific genomic location (e.g., FRT site at position 40A at the L arm of chromosome 2), and a recognizable cellular marker (e.g., Ubi-RFP.nls or Ubi-GFP.nls) distal to the FRT site, to mutant males carrying an FRT site at the equivalent genomic location (**Figure 2A**–**C**).

NOTE: Autonomous and nonautonomous effects within or outside clones for any gene loss of function could be studied employing specific recessive alleles distal to the FRT site.

4.2. Generate FLP/FRT somatic clones in the histoblasts by heat shock treatment at the third instar larval stage of the progeny of the cross. This is performed by submerging the plastic vials containing the animals in a water bath at 37 °C for 45 min−1 h at the wandering larvae (LIII) stage.

NOTE: The sensitive period for mitotic recombination is the G2 phase of the cell cycle. Histoblasts are arrested in G2 during the whole larval development.

4.3. Score twin clones for absence (i.e., mutant cells) or enhanced (i.e., wild type twin-spot cells) levels of the fluorescent protein marker from 16 h APF onward (**Figure 2D**).

NOTE: On average, 45 min to 1 h at 37 °C renders approximately only 2−3 twin clones per region of interest (e.g., the abdominal hemisegment). Pupae showing too a high clone density (e.g., more than four twin clones per hemisegment) should be discarded from further quantitative analyses.

4.4. Upon clone identification, image living pupae at the desired stage and for the desired length of time as described in the previous section.

5. Data processing and analyses

NOTE: Data were processed using ImageJ (imagej.nih.gov/ij/).

5.1. Distinguish cell orientation dynamics from cell junction outlines.

5.1.1. Project the Z-stack slices acquired by confocal microscopy in 2D using the **Maximum Intensity Projection** (MIP) function of ImageJ.

NOTE: The number of slices per stack should be kept to a minimum to avoid the out of focus noise generated by macrophages patrolling under the epidermis.

5.1.2. Set a planar coordinate system identifying reliable tissue landmarks (e.g., A/P compartment boundaries) for analysis of each dataset (**Figure 3A**).

NOTE: Employing the same planar references for each data set will allow multiple measurements to be compared.

5.1.3. Use the **OrientationJ** plugin (bigwww.epfl.ch/orientation/) of ImageJ[23,24] on local cell edges to obtain qualitative and quantitative orientation values. The plugin renders color-coded overlays on input images based on local orientations and provides numerical values when used in quantitative mode (**Figure 3B**–**B''** and **Figure C**–**C''''**).

NOTE: The **OrientationJ** plugin is based on structure tensors, 2 x 2 matrix of eigenvalues derived from gradient and directional derivatives (See references[23,24] for a detailed description).

5.1.4. Use the **OrientationJ Distribution** option to color-code cell edge orientations relative to the set planar coordinate system (i.e., cell edge orientation maps) (**Figure 3B**). The **Distribution** option is found under the plugin menu in ImageJ. The settings to employ are: Gaussian window sigma = 1 pixel; Cubic Spline = Gradient; Minimum Coherency = 20%; Minimum Energy = 1%. Cell edge orientations are displayed as a color-coded image employing the **Color Survey** option of the plugin (Hue = orientation; Saturation = coherency; and Brightness = input image).

NOTE: Areas that contain background fluorescence do not provide any directional information and must be manually excluded from the analysis. High threshold settings reduce the pixels considered in the processed images.

5.1.5. Use the **OrientationJ Measure** option to quantify cellular orientations and directional cell-cell alignment (i.e., coherency) (**Figure 3C**). The **Measure** option is found under the plugin menu in ImageJ. Generate small adjacent non-overlapping regions of interest (ROIs) of uniform weight (64 x 64 pixels, about 20 µm x 20 µm) within the area occupied by the histoblasts (**Figure 3C**).

5.1.6. Calculate the dominant local orientation (i.e., the averaged orientation between neighboring cells–averaged cell edge orientation map) and coherency from the ROIs. The software computes the predominant orientation and local coherency within each ROI (**Figure 3C**).

NOTE: The largest and smallest eigenvalues of the structure tensor estimated by **OrientationJ** are employed to calculate coherency as the ratio between their difference and their sum.

Coherency is bounded between values of 0−1. A value of 1 indicates full alignment uniformity, while a value of 0 indicates isotropic areas with no alignment.

5.1.7. Statistically analyze the calculated orientation and coherency values from multiple images using free software packages such as PAST[25]. Axial data such as orientation values (in degrees) are appropriately described by directional statistics.

5.1.8. Through the software, calculate the mean direction and circular variance for each set of orientation distributions. The statistical significance of the difference between the distribution of the orientations between different genotypes or conditions is determined using the nonparametric Mardia-Watson-Wheeler test (W-test) for equal distributions.

5.1.9. Calculate the statistical significance of the difference in coherency applying the non-parametric Kolmogorov-Smirnov test (K-S test). Display data graphically as desired (polar plots, bar charts, box plots, etc.).

5.2. Growth dynamics from cell clones

NOTE: The following steps allow retrieval of geometrical and shape parameters for cells from 2D MIP images containing the clone(s) of interest. For comparisons between multiple clones, images must be acquired with the same settings.

5.2.1. Segment clone areas by drawing their contours with the ImageJ **Freehand Selection** tool.

5.2.2. Calculate geometrical and shape parameters by using the **Set Measurements** tool of ImageJ under the **Analyze** menu. Activate the **Area**, **Perimeter**, **Fit Ellipse**, and **Shape Descriptor** options.

NOTE: This will allow retrieval of diverse geometrical parameters including the area (sum of the pixels within the clone), the perimeter (sum of the pixel of the clone border), the aspect ratio (AR, the ratio between the major and minor axes of the best-fit Legendre ellipse inscribed into the clone border), and the angle of orientation (i.e, the angle of the major axis of the clone relative to the anteroposterior boundary).

5.2.3. Calculating non-dimensional ratios from these measurements retrieves shape parameters. These include roundness ($4 \times [\text{area}]/\pi \times [\text{major axis}]^2$), roughness (solidity - area/convex area), and circularity ($4\pi \times [\text{area}]/[\text{perimeter}]^2$).

NOTE: Each of the shape parameters represents the degree of deviation of the clones from ideal shapes such as a circle or a bounded convex hull, and they are all bounded between values of 0 and 1. Values equal to 1 indicate maximum symmetry (i.e., minimal complexity).

5.2.4. Statistically analyze geometrical and shape parameters between different genotypes or conditions using Microsoft Excel and/or PAST. Statistical significance of the difference is determined using an unpaired two-tailed Student's t-test for equal mean or the non-

parametric Kolmogorov-Smirnov K-S-test for equal distributions between conditions. Data can be graphically displayed as desired (e.g., bar charts, box plots, etc.). See **Figure 5**.

REPRESENTATIVE RESULTS:

The protocol described above covers the preparation of *Drosophila* pupae for long-term live imaging and the procedures for the analysis of cell orientation and growth dynamics of the abdominal epidermis. By applying this methodology it is possible to generate high-resolution movies of the developing pupae for periods of up to 48 h without significant photobleaching or phototoxicity. Snapshots depicting the abdominal epidermis (e.g., histoblasts and LECs) at different time points and from pupae oriented at different angles are shown in **Figure 4**. The subsequent analysis of these movies allows identification and quantification of the dynamics of local and global changes in the main geometrical and shape parameters modulated during the expansion of histoblasts and the replacement of LECs. These analyses can be performed in different scenarios and specific mutant backgrounds. They can also be employed for clones in which gene expression is altered, leading to autonomous loss or gain of function conditions. This would permit the exploration of nonautonomous responses in surrounding cells, facilitating the identification of cross talk or cell communication mechanisms (**Figure 5**). This approach has been recently employed in the identification of the Fat/Dachsous/Four jointed pathway as a key element directing and orienting cells alignment during the deploying of the planar polar pattern of the abdominal epidermis of the adult[9].

FIGURE LEGENDS:

Figure 1: Dissecting and mounting pupae for live imaging. (**A**) From left to right: dorsal view of a prepupa at 0 h APF (left) and of a pupa at 14 h APF before (middle) and after (right) the removal of the opaque pupal case. The opercular region is indicated (white arrowheads). (**B**) The essential toolkit for dissection is shown. From left to right: glass slide, double-sided tape, a pair of forceps, and a glass-bottomed dish. (**C**) Staged pupae are immobilized on double-sided sticky tape on glass slides dorsal side up. The pupal case of each pupa is opened from the opercular region with surgical forceps. (**D**–**E**) The peeling of the pupal case is gradual. The case is torn and folded to the pupal sides from the head to the abdomen. (**F**–**H**) The pupa is gently lifted from the ventral side with the tips of the forceps (**G**) and transferred to a glass-bottomed dish over a minimal drop of halocarbon oil. (**I**) The pupa is then appropriately oriented (dorsolaterally, top; dorsally, bottom). Multiple pupae can be mounted simultaneously by repeating the steps shown in panels **C**–**I**. (**J**) Image showing the outline of the cells of the dorsal histoblast nest of the AIII segment expressing the junctional marker Atpα::GFP. The pupa was oriented dorsolaterally and imaged at 14 h APF. Scale bar = 22 µm. (**K**) Image equivalent to (**J**) but from a dorsal point of view. The dynamics of tissue development can be visualized for several hours. See also **Figure 4**.

Figure 2: Generation of genetic mosaics via FLP/FRT system. (**A**) A parental heterozygous cell (pale magenta) carries a recessive mutant allele (dashed rectangle) on one chromosome arm and a gene encoding for a fluorescent marker (magenta) in the other chromosome arm (the left arm of the chromosome 2 [2L] in this example). FRT sites (orange rectangles) are engineered in both arms at the same chromosomal positions proximal to the centromere (grey ovals). (**B**) The activation of FLP recombinase by heat shock in cells paused in G2 leads to the recombination between the FRTs of sister chromatids 1-1’ and 2-2’. As a result, the region distal to the FRT sites (FRT40A on 2L) is exchanged. A magnified view is shown on the

right panel. (**C**) Upon the polar segregation of the rearranged regions (1-1' and 2-2') during mitosis, two genetically different sister cells are generated. One daughter is homozygous for the recessive allele and for the entire chromosome arm distal to the site of recombination. This cell lacks the gene that encodes for the fluorescent marker, negatively marked in white. The other daughter cell will be homozygous for the wild type arm, giving rise to a twin-spot and expressing two copies of the genes encoding for the fluorescent marker (dark magenta). For simplicity, segregations of rearranged regions to opposite poles of the mitotic cell (i.e., 1−2 and 1'−2') are not shown. These give rise to cells phenotypically indistinguishable from the parental cell (heterozygous background, pale magenta). (**D**) Image showing clones in the A compartment generated via FLP/FRT somatic recombination at the third instar larvae stage and visualized at 26 h APF. Clones of cells are marked by the absence of RFP.nls (black) and homozygous expression of RFP.nls (bright magenta, twin spots) in an otherwise heterozygous RFP.nls background (dim magenta). Equivalent events can be achieved for FRT sites located in other chromosomal locations (2R, 3L, 3R, and X). See also **Figure 5**.

Figure 3: Illustration of cell orientation measurements. To extract information on cell orientations, first a planar coordinate system to relate cell orientation with tissue axis is set (**A**). Second, cell orientations are extracted from cell outlines (**B**). Last, cell orientations and alignments with the tissue axis are quantified (**C**). (**A**) On the left, a diagram of a lateral view of a pupa oriented according to the planar coordinate system. The red solid and the cyan dashed lines define the Cartesian plane representing the anteroposterior (A/P) boundary and the dorsal midline respectively. In the middle, an inverted image showing a dorsolateral view of the expanding histoblasts at 26 h APF outlined by the junctional marker Atpα::GFP (input image). The position of the A/P boundary is highlighted with a red line. On the right, an axial compass showing the color code applied to describe cell edge orientations (polygons) or averaged cell orientations (bars). (**B**) Display of the Plugins/**OrientationJ** menu and the **OrientationJ Distribution** window. (**B'**) Display of the **OrientationJ Distribution** window showing the settings of the parameters employed to obtain the cell edge orientation map on the right. (**B''**) Illustration of the color-coded cell outlines on idealized cells. A circle does not show any preferred color. (**C**) Display of the **OrientationJ Measure** window. (**C'**) Sequential screenshots of the **OrientationJ Measure** window showing how to measure local orientation and coherency in consecutive ROIs of uniform weight. The local orientation angle and coherency for each region are displayed as ellipsoids. (**C''**) Representation of the final results of the orientation measurement. Ellipsoids visually display the orientation (i.e., angle of the ellipsoid longest axis with respect to the A/P boundary) and coherency (i.e., ratio between the longest and the shorter axis of the ellipsoid). The numerical values of both parameters are saved in a spreadsheet for further analyses. (**C'''**) Illustration of the color-coded cell outlines and averaged cell orientation (bars) on idealized cells. A circle does not show any preferred orientation. (**C''''**) Representation of the preferred local orientation of each ROI (locally averaged orientation map). The colors highlight the orientation of each region. Anterior is to the left. Scale bar = 22 μm.

Figure 4: Long-term live imaging of growing abdominal epithelia. (**A**) Representative snapshots from long-term imaging movies from early to late phases of histoblast expansion. Top: Schematic views of a pupa oriented for dorsolateral imaging at 16 and 26 h APF. The territory occupied by the nests visible from the dorsolateral side of the pupa is highlighted in dark grey at 16 and 26 h APF. Bottom: Images showing cell outlines from both histoblasts and

LECs labeled by the ubiquitous expression of the junctional marker Atpα::GFP. The A/P boundary lies in between the two highlighted compartments (Fake colored cells in blue = anterior compartment; green = posterior compartments). (**B**) Representative snapshots from long-term imaging movies from early to late phases of nests confluence. Top: Schematic view of a pupa oriented for dorsal imaging at 32 and 48 h APF. Bottom: Cell outlines (labeled and colored as in A). Note that the Atpα::GFP marker allows delineation of the shape of individual epithelial cells over time with high resolution. Scale bar = 22 μm.

Fig. 5: Tissue properties extracted from clonal analysis. (**A**) Examples of wild type clones in the A compartment marked by the absence of RFP.nls (black) and their twin spots (bright magenta) at 26 h APF. The clones elongate along the segmental boundaries. Twin clones arrange in parallel or in tandem. Scale bar = 22 μm. (**B**) Top: Images illustrating the parameters quantified from the clone outlines. Bottom: Summary table reporting the average values for the indicated parameters for wild type animals (n = 29). (**C**) Morphology of a wild type clone at 26 h (left) and 47 h (right) APF. The clone shows complex border morphology at both stages. (**D**) Box and whisker plots for geometrical parameters at 26 h (light yellow) and 47 h APF (dark yellow). The averaged area and perimeter increase significantly in this time window. (**E**) Polar plots representing the clones orientation (bin size 18°, abundance proportional to the area). Orientation is sustained during expansion and remodeling. (**F**) Box and whisker plots for shape parameters at 26 h (light yellow) and 47 h APF (dark yellow). Roughness (solidity), roundness, and circularity barely change. The median values are shown with a red horizontal line and whiskers extend to the minimum and maximum values of the distribution. Statistics was performed with K-SM test or W-tests ($p < 0.0001$****, $p > 0.05$ not significant). Anterior is to the left, dorsal is up. Scale bar = 16 μm. Genotype is *hsflp1.22*;*FRT40A/FRT40A Ubi.RFP.nls*.

DISCUSSION:

Long-range order is an essential characteristic of most functional physiological units. During morphogenesis, order is achieved through the integration of complex instructions implemented with high temporal and spatial precision. Multiple and multilevel constrains are integrated into stereotyped tissue arrangements.

Polarity and directionality are critical to ordered spatial arrangement during development. Polarity implies symmetry breaking during development. The achievement of asymmetry is necessary for the determination of the embryonic anteroposterior (A/P) and dorsoventral (D/V) axes and adult organization[26]. Beyond this early role, local asymmetries are essential for morphological diversity at all levels. Directionality is an essential complement of asymmetry and polarity during morphogenesis. Global order is implemented by the ability of cells to sense and transmit signals locally on a cell-to-cell base with a precise sense or orientation. Single cells asymmetries harmonize cooperatively over time by orienting positions or movements within particular directions in space. Cell communication implicates secreted factors, cell-cell contacts, and mechanical inputs. Signals act on fields of cells that first locally and then globally modify their behaviors[27].

This work presents a simple way to analyze the coordinated behaviors of individual cells, including their planar orientation and growth parameters during the development of tissue order. The morphogenesis of the adult abdomen of *Drosophila* during pupation presents a series of technical advantages over other equivalent models such as germ band extension/

retraction[28] or dorsal closure[29] during fly embryogenesis, *Drosophila* wing morphogenesis ex vivo[30], ventral enclosure in *Caenorhabditis elegans*[31], or palatal fusion in mice[32], amongst others.

First, abdomen morphogenesis constitutes a process that can be followed in its entirety in vivo. Continuous live imaging of the replacement of the LECs by the histoblasts can be performed from 12 h APF, when the pupa is formed, up to the completion of adult epithelial morphogenesis. Second, it allows the analyses of a full set of cell behaviors such as cell migration, division, shape changes, delamination, and intercalation. Last, it is amenable to genetic interference and clonal analysis. Autonomous and nonautonomous effects of loss and gain of function activities can be monitored live by employing appropriate markers. However, the pupa as a model system presents some minor limitations. Due to its ovoid shape, it is not possible to perform simultaneous live imaging of lateral and dorsal events with high resolution. This issue can be overcome by performing sequential imaging in dorsolaterally and dorsally oriented pupae or better, by employing multiple angles light sheet selective plane illumination microscopy (SPIM) microscopy. Another drawback is the presence of two different cell populations of different sizes in the analyses (i.e., polyploid LECs and diploid histoblasts). This can make image segmentation complex and the two cell populations must be analyzed separately.

In the future, the long-term imaging of *Drosophila* pupae can be easily adapted to study a full range of morphogenetic phenomena including the coordination of epidermal, muscular, and neural development during metamorphosis in wild type and mutant conditions. Moreover, the algorithms and plugins in use may be employed to study the organization, patterning, and dynamics of many other tissues.

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DISCLOSURE:

The authors have no conflicts of interests.

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Figure 1

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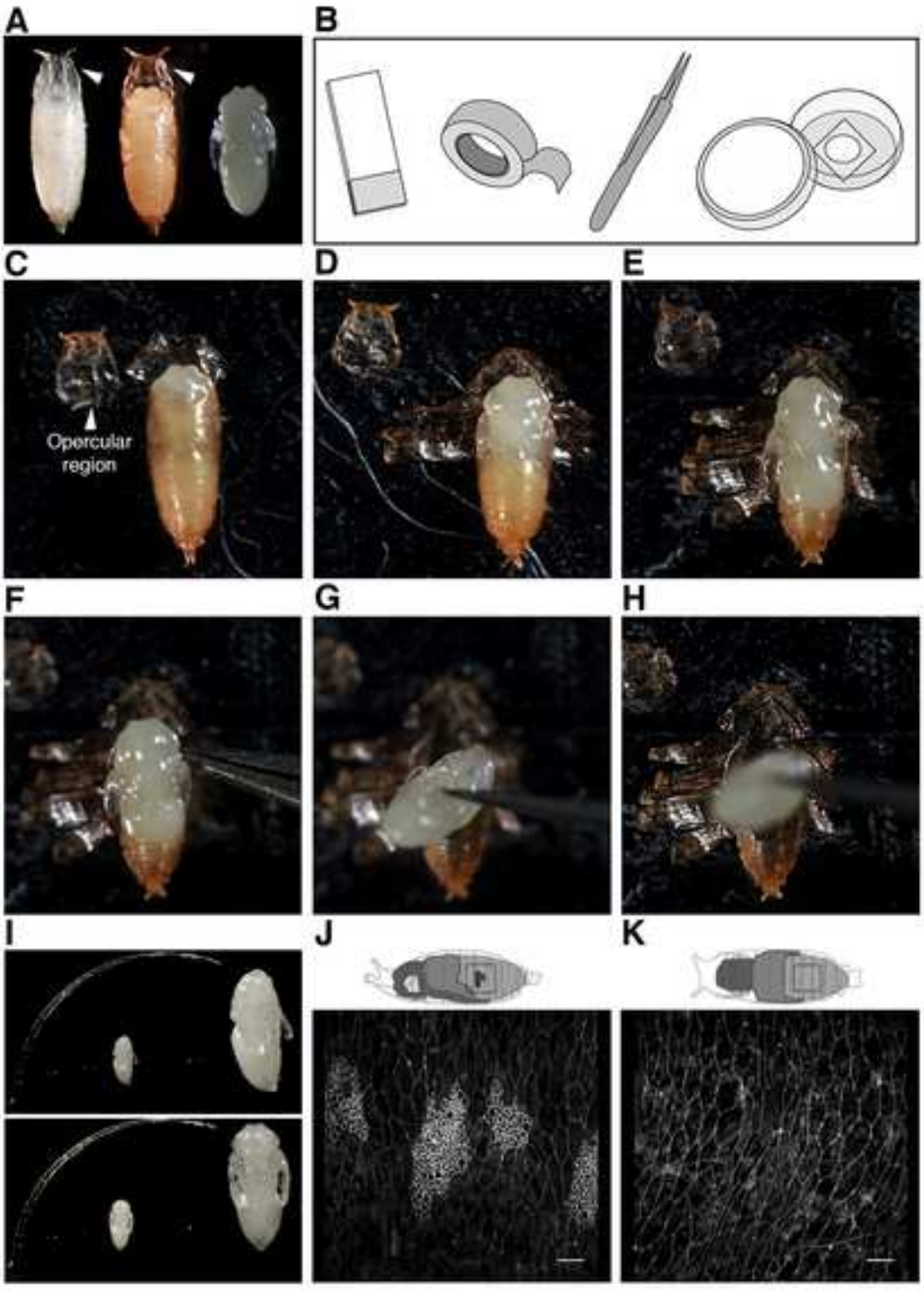


Figure 2

Figure 2_JoVE_FMEMB

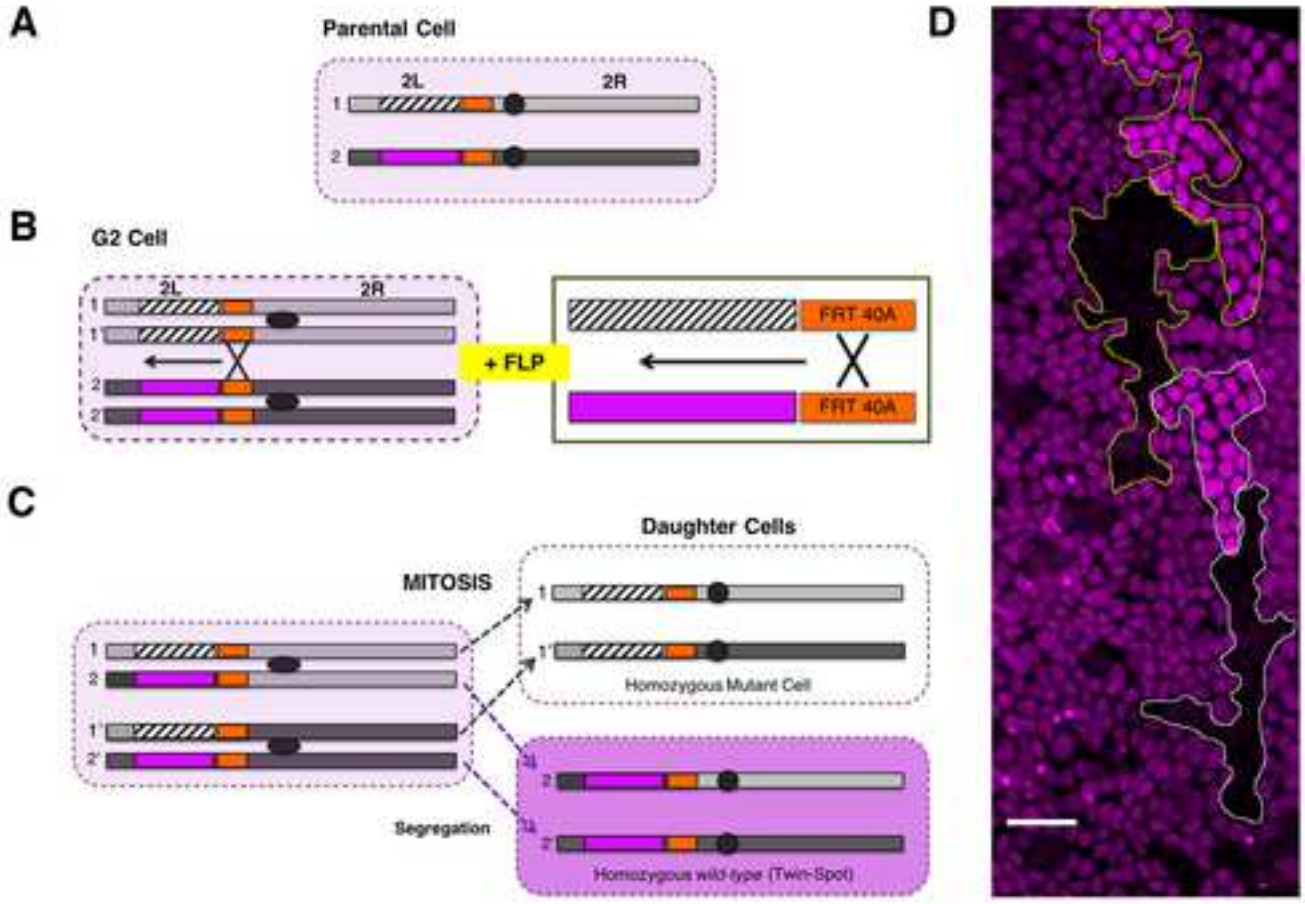


Figure 3

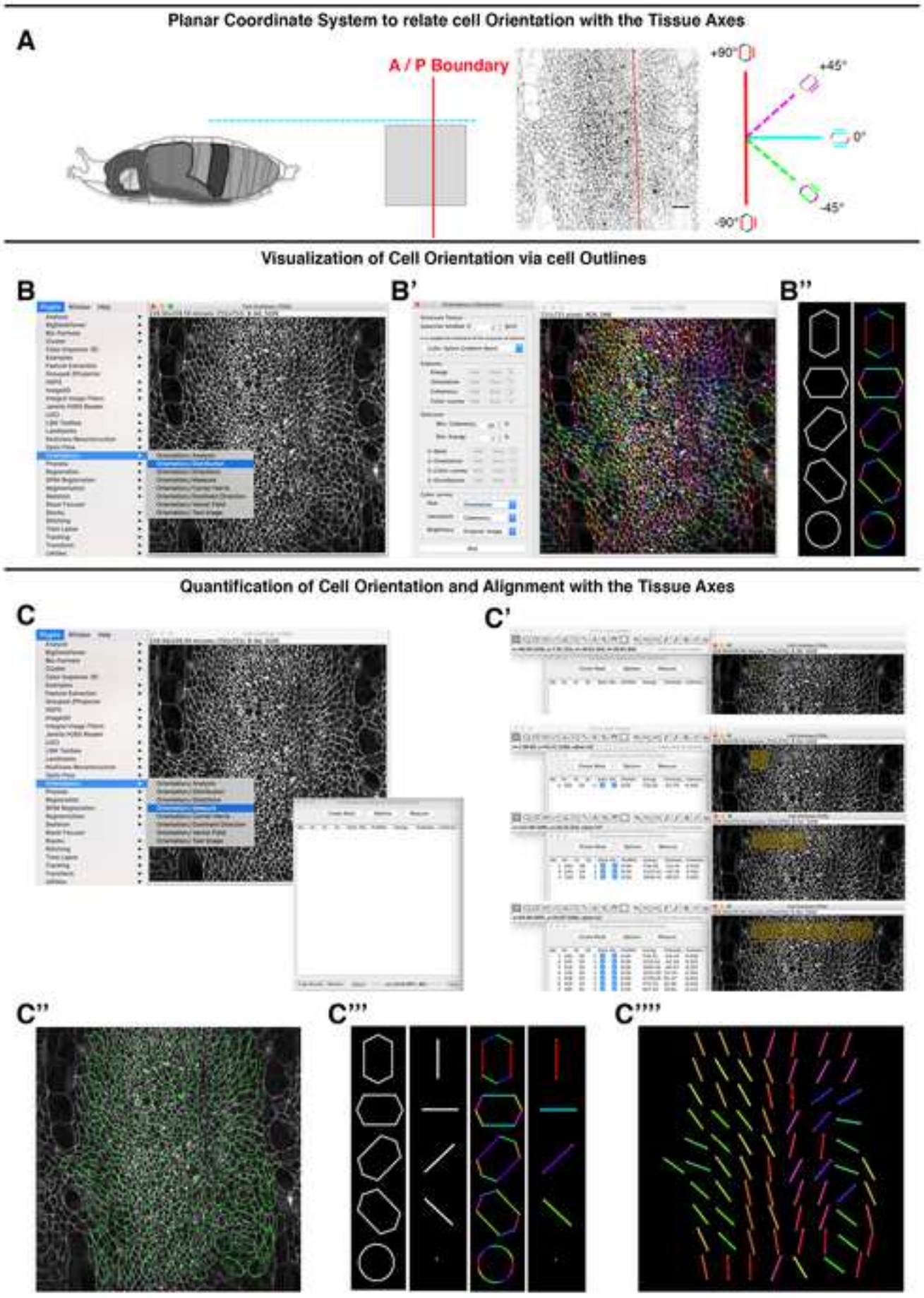


Figure 4_JoVE_FMEMB

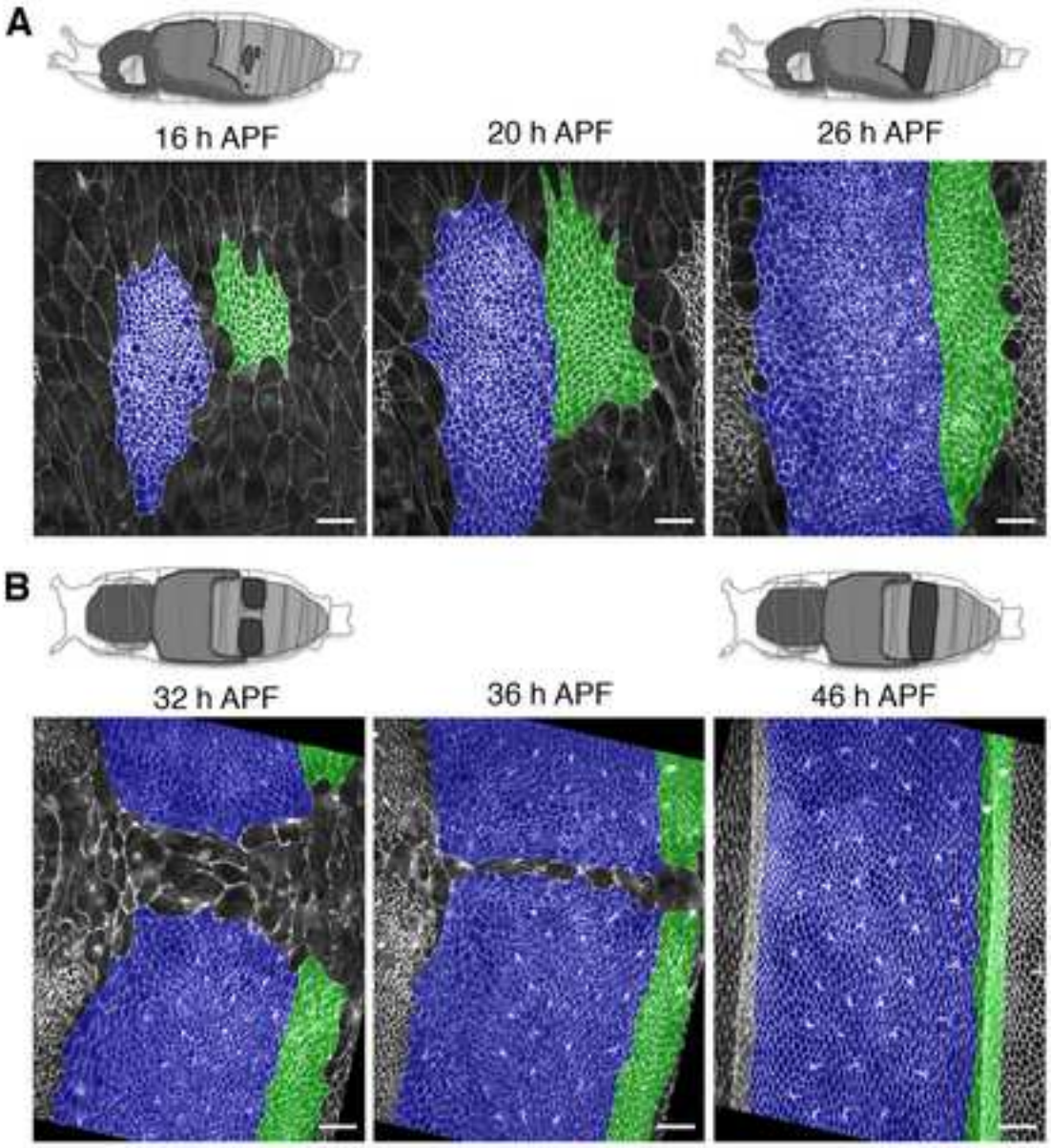
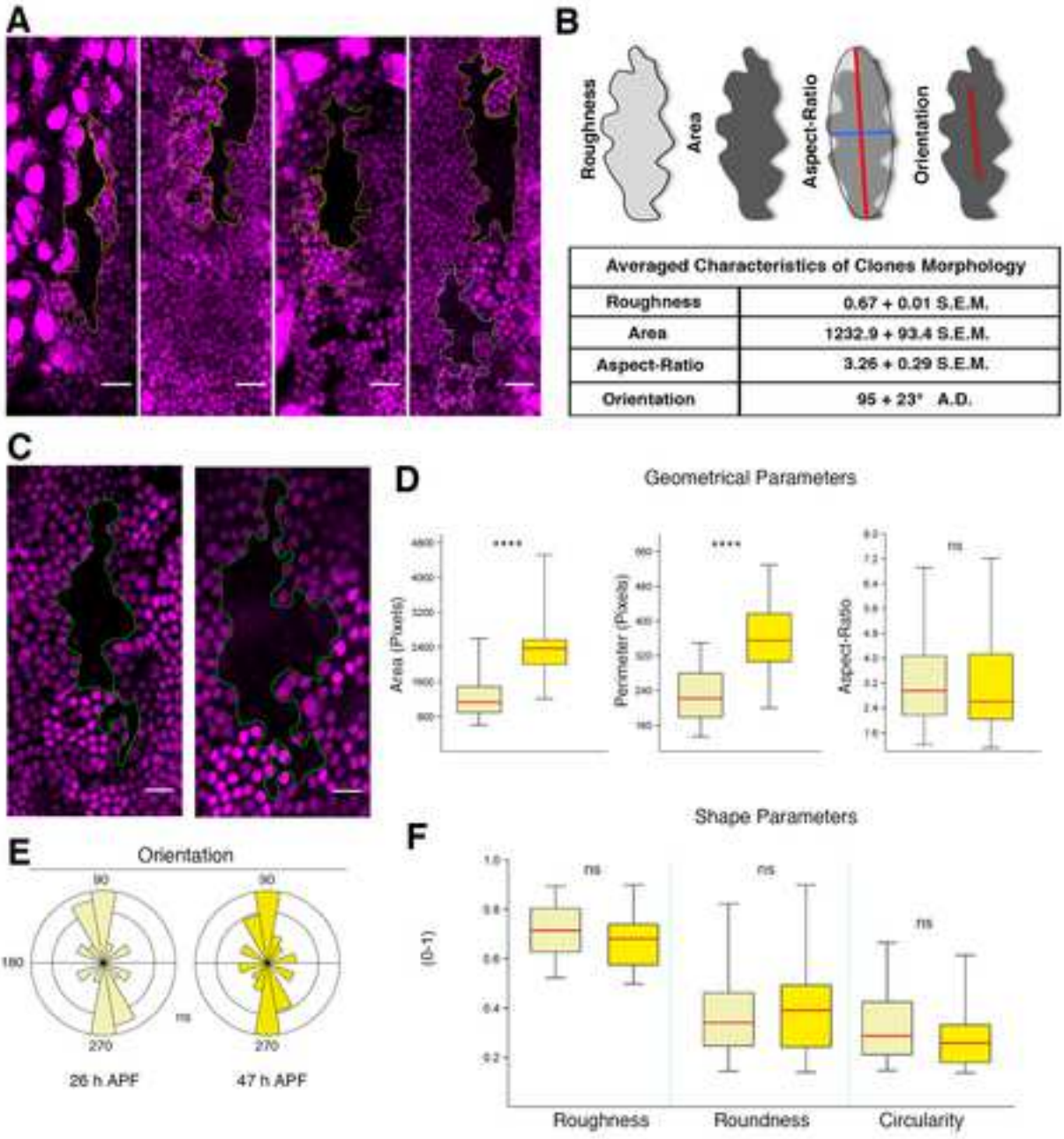


Figure 5_JoVE_FMEMB



Name of Material/Equipment	Company	Catalog Number
Analysis Software	-	ImageJ
Drosophila	*Atpa::GFP*	-
Drosophila	*hsflp1.22;FRT40A/FRT40A Ubi.RFP.nls*	-
Dumont 5 Forceps	FST	11251-20
Glass Bottom Plates	Mat Tek	P35G-0.170-14-C
Halocarbon Oil 27	Sigma-Aldrich	9002-83-9
Inverted Confocal microscope	Zeiss	LSM700
Stereomicroscope	Leica	DFC365FX

Comments/Description
Analyzing data
Strains employed for data collection
Strains employed for data collection
1.5 mm diameter for dissection
Mounting pupae for data collection
mounting pupae
Data collection
Visualization of the pupae during dissection

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Barcelona, August 4th 2019

Bing Wu, PhD.
Review Editor
JoVE

Dear Bing,
We have performed a second revision of the manuscript following your editorial advice.
I hope this time we will meet all requirements and we could proceed swiftly.
Many thanks for your understanding
All the best

Enrique Martin-Blanco
PhD.

A one by one description of what we did this second time is below

Editorial comments:

The manuscript has been modified and the updated manuscript, **60282_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We cross-checked the whole manuscript, employed the spelling and grammar tool of Word and found no mistakes.

2. Please avoid long steps/notes (more than 4 lines).

We modified all notes longer than 4 lines and adjust them to this limit.

3. For steps that are done using software, a step-wise description of software usage must be

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We described in sections 5.1.4, 5.1.5 and 5.2.2. the buttons to click under the appropriate menus.

4. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite the notes after steps 5.1.5, 5.2.2

We amended the sections 5.1.5 and 5.2.2 and we hope they do not substantially overlap with previous texts

A one by one description of what we did first is shown below

Editorial comments:

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14. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite the notes after step 5.2.2 and first three paragraphs in Discussion.
We have modified all the text as suggested.

Reviewer #1:
Manuscript Summary:
Federica Mangione and Enrique Martin-Blanco report a method for the imaging and the analysis of tissue orientation and growth dynamics in the developing Drosophila epithelia during pupal stages. Analysis of cell properties such as orientation, growth in a living organism is an essential issue to study tissue morphogenesis. This should be achieved with high spatiotemporal reliability during development. This method article provides analytical tools to address those issues. The article is really clear. The different steps are well described and illustrated and the procedures clearly lead to the described outcome. The figures are clear and well documented.
I recommend the publication of this method article in JoVE.
Major Concerns:
None.
Minor Concerns:
A better description of the junctional marker, Atpx-GFP would be helpful.
Done
In the figure 1, the labelling of figure, L, M should be modified to J, K.
L,M for J,K was modified in the figure, legend and in the text.

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Reviewer #2:
Manuscript Summary:
In this manuscript, Mangione and Martin-Blanco describe a protocol for live imaging to analyse cell behaviour during development. To illustrate this protocol, the authors use the abdomen of the Drosophila pupae during metamorphosis as model system. The protocol is strongly focused on Drosophila, although the authors state that the proposed methodology could be applied to different Drosophila developmental stages and to other model organisms.
The paper is well written and structured and constitutes a detailed description of the methodology used in their previous article (Mangione and Martin-Blanco, 2018). Therefore, I would recommend this manuscript for publication after some minor corrections.
Major Concerns:
Comments on the Figures:
Figure 1
- Panels J and K are missing or misnamed as L and M in the Figure 1, as well as in the text and in the figure legend.
L,M for J,K was modified in the figure, legend and in the text.
- In addition to the photograph shown in panel "I", it would be interesting to show the final display of the aligned pupae under the coverslip, to know how the ready-to-image sample looks like.
We think an additional figure is not necessary. The final display with multiple pupae is the same as in Figure 1I except that more than one pupa was mounted.
- I think that 6 panels (C-H) are a bit too many to show the removal of the pupal case.
Displaying 6 panels (C-H) is very useful to provide a complete overview of the dissection steps. Indeed, we are not only showing the removal of the pupal case (C-E), but also how to recover the pupa after dissection (F-H). It is essential to correctly transfer the pupa from the dissection glass to the imaging dish without damaging the animal before imaging. Damaged pupae will die during imaging.
Figure 2
The scheme of mitotic recombination in Figure 2 is confusing and the description in the figure legend is incomprehensible. I consider that this figure might not be necessary. However, if the authors believe that the scheme of the mitotic recombination process is important for the comprehension of the manuscript, I would recommend to include it as a panel in Figure 5.
We rewrote the legend of the Figure 2 to make it more accessible to the reader. Additionally, it is a key step of the protocol and to keep this schemes as a separate Figure is important to clarify the concept of mitotic recombination and to give an overview of the process to the reader (many experimentalists have issues in understanding the concept of mitotic recombination when they start performing clonal analysis).
Figure 3

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The legend of Figure 3C is not very well described. It is impossible to follow it. In addition, the photo shown at the bottom left is not described.
We rewrote the legend adding the description of the missing panels.
Figure 4
The drawing of the pupa at 26 h AFP gives the impression that the histoblasts have already fused in the dorsal midline. I would suggest the authors to leave light grey in between the growing histoblasts and the dorsal midline.
We described the figure better in the text to clarify this point. We explained why the dorso-lateral views shown in the cartoons do not let visualizing the dorsal midline (we tried to be realistic).
I would leave for the end of the figure legend the color code. I would change the sentence ….The A/P boundary lies in between the two highlighted compartments (fake colored nests in blue - Anterior - and green - Posterior).....for....."The blue color represents the A compartment cells and the green color the P compartment cells".
Done
Minor Concerns:
Comments on the text
In point 3 (line 133), it would be convenient to give the information if the imaging can be also taken with an upright microscope.
Yes it is possible to perform imaging with an upright microscope since the drop of oil ensures attachment of the pupae to the dish. We added this info in a Note after point 3.
In point 4.3: it would be interested to put a "note" indicating the expected number of clones after a 45 min/1 hour heat shock.
Done. We added a note after point 4.3: On average 2-3 clones per region of interest (hemisegment). Of course there is a certain variability; tissues with more than 4 clones should be discarded from further analyses.
There are several typing mistakes along the text.
We revisited all the text and legends

Reviewer #3:
Cell organization within tissues is often fundamental for tissue function. Therefore, characterizing the pattern of cell organization within a tissue is critical to understand hwo it works, and how its function may be disrupted in disease. In this manuscript, Mangione and Martin-Blanco describe how to image and analyze cell organization and growth in the abdominal epidermis of Drosophila pupae, a system amenable to genetic manipulations and long-term imaging.
The methods described, particularly the image analysis, will be immediately applicable to other systems, and thus of interest to a broad readership. The description of mitotic clones using the FLP/FRT system is really nice and clear, and I am sure it will be referenced by many.
Two aspects in which I think the manuscript can be improved are (1) better definition and justification of some of the image analysis parameters selected; and (2) better description of

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the statistical methods to be used analyzing the data. I proposd to address the following points before further considering the manuscript:
MAJOR:
1. A number of technical terms, particularly anatomical and image analysis-related, could use a definition. This would help reach a wider audience. Perhaps including a box with brief definitions to some of these terms will help the unfamiliar reader. A few examples: holometabolous, operculum, metamere, coherency, hue, saturation, guassian window, cubic spline, etc.
Including definitions could have been an option. We think this would have increase the length of the manuscript and alternatively, we tried to simplify the terminology when possible. Most of these terms are well known and fully defined elsewhere.
2. Lines 91-92: the onset of metamorphosis in larvae is described as "easily identifiable as larvae stop moving and evert the spiracles". The authors should show images of animals before and after spiracle eversion to better illustrate how to identify the onset of metamorphosis.
We rephrased this sentence to avoid confusion. Pupal development is well described in multiple reviews (e.g. Reference 14) and we think it is not necessary to add an extra figure.
3. The authors should provide a paragraph briefly describing how the different algorithms in OrientationJ work. I understand that they did not develop the algorithms, but at the same time, they should provide some background for readers to understand what they are doing if they apply these methods.
The properties of the algorithm are fully described by its authors very clearly bot in the references (References 23 and 24) and in the web page provided.
4. Lines 191 and 204: what is the mathematical definition of coherency? The parameters used in OrientationJ should be defined similar to the measurements of growth dynamics in clones (see line 223).
We added the mathematical definition of coherency in the text and provided definitions for growth parameters.
5. The authors provide sample parameter values for OrientationJ, but it would be useful to show how changes in the values of these parameters affect the results of the analysis. Adding an additional figure demonstrating an example of the effects of changes of key parameters would greatly increase the value of the manuscript.
How different parameters might affect the analysis is something that relate to how the algorithm is made. In the Fiji manual all parameters are well described. We present the values employed in our case and they should be tested by trial and error for other model systems.
6. Line 207: the section on statistical analysis is weak. What are appropriate statistical tests to compare the outputs of OrientationJ? Citing specific tests -maybe those in the legend of Figure 5?- and justifying their use, would also allow the readers to use alternative software tools. Related to this, only mentioning Excel as the tool to conduct statistical testing does not seem appropriate, particularly when there exist specialized statistical packages such as SAS or SPSS, and even better, free statistical software tools, including R/RStudio or Octave. I

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suggest that the authors at least include references to some of these other tools, particularly the open access ones.

We used both the PAST software (free software tool) (new Reference 25 and Excel to perform statistics.

MINOR:

1. Line 55: the authors argue that during embryonic development, the Drosophila epidermis becomes disordered, and this process is reverted at larval stages. While it is true that all the cell behaviours mentioned in this sentence contribute to tissue disorder, it must also be pointed out that epidermal order increases during the second half of embryonic development, as evidenced by the regular pattern of larval denticle precursors produced by cells in the embryonic epidermis. The authors may want to point out that the increase in epidermal order begins during the final stages of embryonic development.

We modified the text accordingly

2. The authors should clarify if OrientationJ requires segmentation of junctions or cells? If so, how should this be accomplished?

OrientationJ does not require segmentation of cell junction (of course it will work with segmented images as well). Segmentation can be done in several ways, including ImageJ watershed algorithms. Of course, the quality of the segmentation output strongly will depend on the original input image.

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