Cell Shape Changes During *Drosophila* Germ Band Retraction: Towards Model Validation by Experimental Comparison

Xena McDonald, Monica E. Lacy, M. Shane Hutson

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BRIEF. This project seeks to experimentally validate simulated predictions of forces in a *Drosophila* embryo during germ band retraction.

ABSTRACT. Germ band retraction is a stage of *Drosophila* melanogaster development in which two epithelia, the germ band and the amnioserosa, undergo complementary movement. From their initial shapes as two interlocking "U"s, the germ band pulls around the posterior end of the embryo and the amnioserosa transforms into a smaller teardrop shape on the dorsal surface. Cell elongation is a key reporter of cellular forces in the germ band. Previous investigations have modeled the force anisotropy during retraction and used these models to predict how cell shapes should change throughout the germ band. This study focuses on experimental measurements of the predicted cell elongation in terms of aspect ratio and elongation axis orientation. Embryos with GFP-tagged cell junctions were imaged using a confocal microscope during both early and late germ band retraction. Each embryo was staged for its retraction completion percentage and analyzed for cellular aspect ratio. The goal of this study is to effectively utilize experimental cell elongation data to validate simulated data produced in previous work, contributing to knowledge of the cellular mechanics of germ band retraction.

INTRODUCTION.

The development of a *Drosophila* embryo is usually divided into individual stages, many of which have been widely studied. Germ band retraction (GBR) is an important stage of *Drosophila* development that ensures correct placement of the segments in the embryo, vital to later development. GBR involves the coordinated movement of two epithelial structures: the elongated germ band, which will become the larval epidermis; and the amnioserosa, which will later apoptose [1]. Larger-scale cell and tissue movements of this stage have been studied and largely described, but the specific cellular mechanics of the amnioserosa and germ band are still under investigation [1, 2].

At the beginning of retraction, the germ band is curled around the posterior end of the embryo, with one end covering most of the embryo's dorsal surface, and the other end covering most of the ventral surface [3]. The amnioserosa is positioned laterally on both sides of the embryo between the dorsal and ventral regions occupied by the germ band, as shown in Figure 1A. The two lateral flanks of the amnioserosa are connected by a thin bridge over the dorsal side, giving the tissues the appearance of two interlocking U-shapes. During GBR, the germ band pulls around the posterior end of the embryo while the amnioserosa shortens and moves up to the dorsal side in complementary motion. By the end of retraction, the two ends of the germ band are aligned on the ventral side and the amnioserosa forms a smaller ovoid on the dorsal side [2]. The amnioserosa and germ band remain connected and maintain epithelial integrity throughout retraction [1].

In addition to movements of the tissues as individual structures, GBR involves changes in shape at the cellular level. As the germ band retracts, it shortens along the rostrocaudal (head-tail) axis and widens in the perpendicular dorsal-ventral direction. Cells in the germ band elongate orthogonally to the rostrocaudal axis, towards the amnioserosa. Cells inside the amnioserosa undergo an opposite change in shape from extremely elongated to hexagonal and nearly isodiametric [1,2].

Previous research by Lynch *et al.* has investigated the mechanical roles of the amnioserosa and germ band during retraction, seeking to distinguish between autonomous cell elongation in the germ band and elongation due to forces exerted by the amnioserosa. Among other experiments, laser ablations were made in each segment to observe the opening of wounds made parallel and perpendicular to the rostrocaudal axis [1]. These wounds provided information on internal versus external force anisotropy and found that in most germ band segments, internal anisotropic tension dominates, while in the crook of the germ band, external anisotropic stress dominates. These experimental results were reproduced by computer simulations that predicted previously unmeasured aspects of cell shape changes in the germ band [1]. The goal of this project is to continue this research and compare the simulation's predictions to measured cell shape changes. This will help determine how much elongation is performed autonomously by the germ band during retraction and how much it is mechanically assisted by the amnioserosa, increasing knowledge of these tissues' individual roles. It will also provide a means to test the accuracy of the simulation's predictions and either validate or invalidate the underlying model. A more fluent understanding of the morphogenetic processes of a versatile model organism such as *Drosophila* may shed light on similar processes in other organisms, including that of early human development.

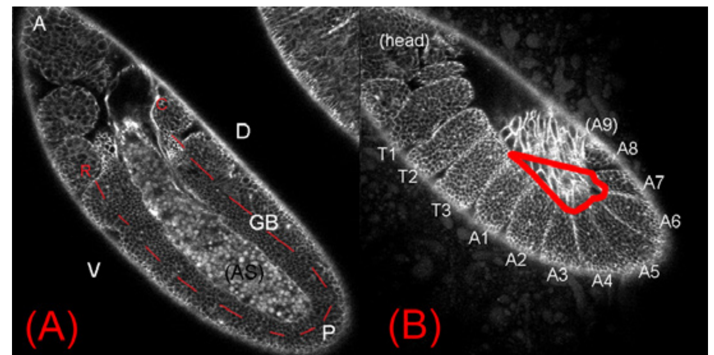

Figure 1. Anatomy of two embryos from confocal images (40x objective, zoom-1). (A) Germ band is labelled GB, and the anterior, posterior, dorsal and ventral sides are labeled A, P, D, and V respectively. AS marks the location of the amnioserosa, however the depth of this scan reveals the yolk underneath. The red dotted line between R (the rostral, or head, end) and C (the caudal, or tail, end) shows the rostrocaudal axis. (B) Twelve segments are labeled T1 through T3 and A1 through A9. A9 is not visible in this image. The red outline marks the contour traced for the contour-matching system to analyze.

MATERIALS AND METHODS.

Slide preparation procedures were similar to those performed by H.E. Lynch *et al.* [2]. The strain of *Drosophila* used was ubi-DE-Cad-GFP (*Drosophila* Genetic Resource Center, Kyoto, Japan), which expresses E-Cadherin tagged with GFP to provide a fluorescent label on cell junctions [4]. Embryos were stored at 15° C until germ band retraction, when they were dechorionated in a 50% bleach solution. They were then arranged on their sides to provide a lateral view, and mounted in halocarbon-27 oil (Sigma-Aldrich, St. Louis, Missouri), on a metal slide between an oxygen permeable membrane (YSI, Yellow Spring, Ohio) and a cover slip. The embryos were imaged using a Zeiss LSM410 laser-scanning confocal microscope (inverted) with a 40x, 1.3 NA oil-immersion objective at a scan speed of 8 seconds per frame. This scan speed provides adequate resolution while minimizing photobleaching of the GFP.

Progress through germ band retraction was determined using an ImageJ plugin and a contour-matching staging algorithm developed by our laboratory, run in Wolfram Mathematica (Wolfram Research, Champaign, Illinois) [2, 5, 6]. This method carries the advantage of being useful in post-processing scenarios where the ends of the embryo are not visible, as opposed to a method where the position of the caudal end of the germ band is measured in relationship to embryo length [2]. To achieve this, the contour of the embryo (shown in Figure 1B) was compared to the already defined standard set of contours. The standard set of contours and the set to be tested were compared using an exhaustive least squares alignment, and a chi-squared test was used to create a matrix of p-values representing alignment quality. The best-matching contour from the standard set was used to approximate the percent progression through germ band retraction. This approximation is more reliable in later stages of retraction, due to the fact that early movement shows considerably more subtle changes [2].

After scanning, images were segmented in SeedWaterSegmenter, an open-source program for image segmentation and tracking of cells over time, to provide boundary data for single cells [7]. After segmentation, data were exported and analyzed in Wolfram Mathematica using a custom algorithm for describing cell shape [1]. The algorithm determines shape by a method utilizing an area moment of inertia tensor matrix designated as **J** [1]. The matrix is first diagonalized to become **J'** to reveal the principal axes forming the best-fit ellipse that matches cell elongation [2]. The best-fit ellipse for each cell in the segment has an aspect ratio κ, calculated by the formula

$$\kappa=\sqrt{\frac{\mathbf{J}'_{11}}{\mathbf{J}'_{22}}}$$

where $\mathbf{J}'_{11}$ represents the largest diagonal entry, and $\mathbf{J}'_{22}$ the smallest. Finding the square root of terms containing **J'** allows an aspect ratio of axis lengths to be found. Testing multiple cells in the segment provided data to compare the uniformity of elongation to be seen [1].

Cell elongation was defined in terms of the aspect ratio (κ), or cell elongation along one axis, and the orientation of that axis (α) in relation to the rostrocaudal axis, so that κ and α represent magnitude and direction of elongation, respectively. A composite **J** (area moment of inertia) tensor was created from an average of **J** from multiple cells, and this was diagonalized to become **J'** for each segment. Using **J'**, composite κ and α values were found for the given segment. The composite used for the past modeling experiments was a single global composite mid-retraction κ_{cells} value, which represented all segments [1]. In this project, only one composite κ value (κ_{cells} for the entire segment, or in the comparison's case, simulated sheet) was found per segment, because there were no wounds for which to account. This is unlike the modeling study, where multiple κ values were found due to wounding. Even though the κ value (κ_{cells}) cannot be used on its own as multiple varying alignments can result in the same κ_{cells} value, they can be used to reevaluate best-fit coordinates (from the modeling study's parameter space and χ^2 distribution) in the case of a significant difference from model to experiment. While κ values were used to find the best-fit parameters for each segment, angle and aspect ratio data generated in SeedWaterSegmenter were used to create the com-parison rose plot distributions in Wolfram Mathematica. While it was intended to complete these steps for five to nine embryos, due to time constraint, segments were measured from three embryos

RESULTS.

For the purposes of analysis, the germ band's twelve segments were labeled T1-T3 and A1-A9, with T1 through A2 representing the rostral (head) end of the germ band, and segments A6 through A9 representing the caudal (tail). Three embryos were imaged in both zoom-1, which shows the entire embryo as 512 by 512 pixels, and zoom-2, which scans an area one fourth of the previous size in the same number of pixels. This allows a larger view for staging of the embryo, in addition to a view providing more detail for accurate segmentation of images. For each embryo, a zoom-1 image was captured, followed by a set of zoom-2 images to capture all segments of the germ band. A second zoom-1 image was taken after the zoom-2 image group, because only zoom-1 images can be staged. Staging of the two zoom-1 images determines the timeframe (in terms of percent retraction completed) in which the zoom-2 images were taken.

Three embryos were segmented at zoom-2 and staged for completion. While the contour-matching staging algorithm provided values within the range of possibility, most of the upper confidence limits were placed above 100% completion (see Figure 2). Due to time constraints, only segments T2, T3, A1, A2 and A3 could be analyzed for angle data.

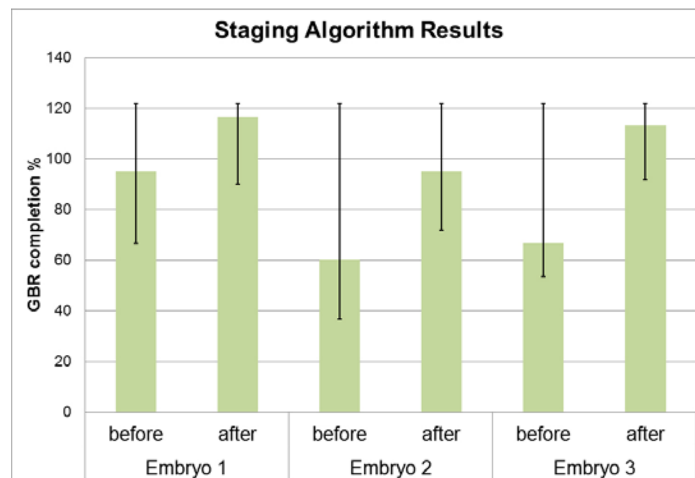

Figure 2. Staging results for each of the three embryos are shown in GBR completion percentage, both before and after zoom-2 images were captured. Error bars show the upper and lower confidence limits presented by the Mathematica algorithm. Note that confidence limits reach over 100% on every image, along with some of the results themselves showing over 100% completion.

In this project, forces applied to the germ band were investigated through descriptions of cellular elongation in each segment. As was pre-viously mentioned, aspect ratio (κ), and the orientation of the cells' major axis (α), act as the magnitude of and direction of cellular elongation, respectively. The model developed by Lynch *et al.* works with a simulated cell sheet comparable in size with a segment (~40μm wid-er to reduce boundary effects) [1]. In the case of these past experiments, the global composite mid-retraction κ_{cells} value of 1.33 was used to represent all segments [1]. For comparison with the model, a new κ_{cells} value is being developed for each remaining segment, as part of ongoing work. Most segments are still under investigation; however, a distribution of an average of three embryos for each of the segments T2, T3, A6, and A7 has been generated. Figure 3 shows these distributions as rose plots where the orientation of the cells in each segment is measured as an angle between 0 and 180 degrees. These plots show that a higher percentage of cells are elongated with an aspect ratio of roughly in between 75 and 105 degrees, where a 90-degree angle represents a position directly orthogonal to the rostral-caudal axis. Simi-lar plots for a larger average of multiple embryos for each segment will be completed.

DISCUSSION.

Although all of the intended goals have not been reached up to this point, the rose plots generated for segments T2, T3, A6, and A7 is the beginning of the comparison process. With the completion of the simulation comparison, the model created by Lynch *et al.* [1] will be either validated or improved in future research. While segment A9 was purposely omitted due to distortion and lack of visibility, the other remaining segments are still being measured. The resulting plots in Figure 3 will need to incorporate more measurements to improve on the N=3 average. In ideal conditions, the plots will represent an average of between five and nine embryos for consistency with the previous study [1]. For comparison to be completed, the same quantitative measures will be applied to both the simulated distribution images and the experimental ones made in this study. Other improvements could be made prior to plotting the distribution as well, such as cross-checking for accurate segmentation of images.

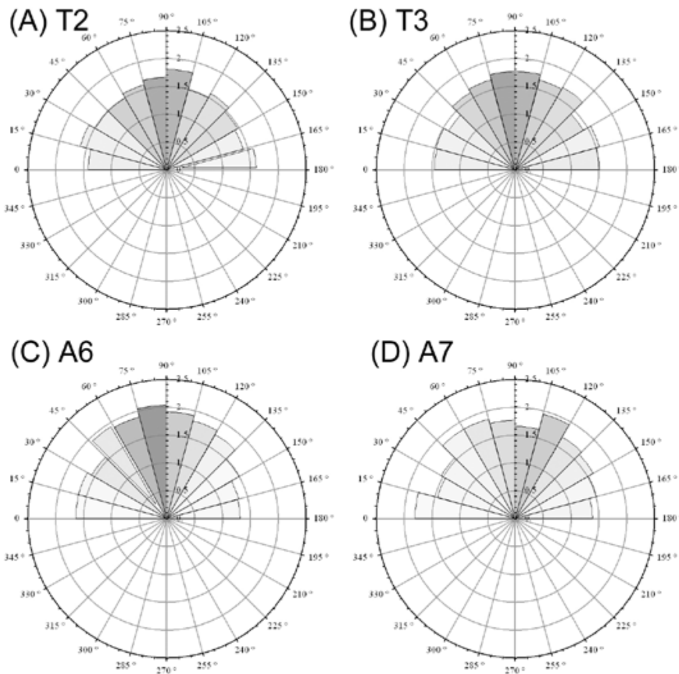

Figure 3. Preliminary rose plots generated from segments (A) T2, (B) T3, (C) A6, and (D) A7 on the three embryos staged. For each segment's plot, each wedge, or sector, represents an angle range in which the cells are oriented. The shading of the sector shows the fraction of cells aligned in the angle range, and the length of the wedge shows mean aspect ratio among cells in that sector. The darker wedges near 90 degrees show that a higher percentage of cells are elongated in a roughly orthogonal direction to the rostral-caudal axis. In the modeling study [1], the darkest shade of gray on the plot represented 35% of cells; these plots have similar shading distribution.

Even though the staging algorithm results (Figure 2) were within the range of possibility, it is preferable for the values to have a narrower, more accurate estimate of the percentage of retraction complete. While this percentage is shown to increase after the zoom-2 image captures, therefore showing time has elapsed. It is difficult to see exactly how much of retraction was missed due to the large confidence interval.

The experimental plots (Figure 3) are those generated ultimately for comparison with the modeling study, where similar plots were generated for each segment based on the simulation. Lynch *et al.* aimed to investigate and predict the balance between internal and external force anisotropy. For this purpose, they described internal edge-tension and external stress anisotropies as parameters in a four-quadrant system: external stress anisotropy acting as the x-axis, and internal edge-tension anisotropy as the y-axis [1]. Positive parameters were defined as those driving elongation towards the amnioserosa, so that in quadrants I and III, the internal edge-tension anisotropy and the external stress anisotropy act together along the axis of elongation, but in II and IV they oppose each other while acting orthogonally [1]. These parameters were the backbone for generating rose plot distributions for values of both external stress anisotropy (Δ/σ) and internal edge-tension anisotropy (polarization factor f) on unwounded segments. The data they compared with experiments for segments A2 and A5 were from late GBR and are shown with a retraction percentage. The final distributions resulting from experimental data are expected not to be significantly different from the simulated distributions, which were created from best-fit force values in this parameter space for the same unwounded segments. It is important to note that quantitative analysis has not yet been performed on the experimental plots in this study, and the process for quantifying these images through a χ^2 test is still in development.

While a significant amount of information was previously known about germ band retraction, the simulation study revealed information on the contributing internal and external influences of cell shape change [1]. Within that research, it was revealed that a cell sheet was most stable when internal and external anisotropies acted orthogonally to one another. Even though the forces found by Lynch *et al.* for each segment were best fits, the parameters were limited; there were several other good fits across the modeling study's χ^2 distribution quadrants. This explains why comparison with experimental data is key to the validation of the simulation. Assessing the underlying forces in the movement of GBR furthers the understanding of the individual roles of the germ band and amnioserosa in relationship to one another. Gaining a more complete understanding of GBR gives insight to the surrounding phases of *Drosophila* development, and will help shape future studies not only regarding the forces affecting embryonic development in *Drosophila*, but in a variety of other applications and organisms as well.

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Xena McDonald is a student at John Overton High School in Nashville, Tennessee. She participated in the School for Science and Math (SSMV) at Vanderbilt University.