Biophysical Investigation of Cell Oscillations and
Cell Ingression in Tissue Dynamics

by

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Duke University

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Mark Kruse

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Berndt Mueller

Dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Physics
in the Graduate School of Duke University
2011
ABSTRACT
(Biological Physics)

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Abstract

Embryonic development involves a precisely orchestrated interplay between gene expression, tissue movement, and cell-shape changes. Using time-lapsed, *in vivo* confocal microscopy, we investigate the time-dependence of cell-shape changes for essentially all of the cells in the amnioserosa tissue during the early-to-late stages of dorsal closure in *Drosophila melanogaster*. Dorsal closure is a critical stage during embryogenesis, where two epidermal tissues are brought together by the force producing machinery, including that of the amnioserosa tissue. The environment these cells exist in is dominated by viscous forces, making the observable kinematics the result of active contractile force imbalances along the cell peripheries. Our image contrast is due to GFP-DE-cadherin, a relatively bright fluorescent construct that localizes at cell-cell junctions. Using custom written segmentation software we quantify cell apical areas from confocal images. By considering the kinematics of individual cells we investigate the forces produced by the amnioserosa tissue. We confirm previous observations of area pulsations or oscillations and that, within the dorsal opening, areas of peripheral amnioserosa cells are smaller than the areas of interior cells [Fernández et al., 2007, Gorfinkiel et al., 2009, Solon et al., 2009, Blanchard et al., 2010]. In addition to oscillations, we find that cells in the low-Reynolds number environment of the amnioserosa tissue exhibit ingression processes, a persistent loss of apical area resulting in the internalization of the cell. We develop an empirical model that quantifies the kinematics of the ingression processes of a substantial fraction of the
amnioserosa cells. We also account for these observations with a biophysical model that quantifies the (spatially averaged) net force from experimental data and explicitly treats the dynamics of oscillations and ingestion. Utilizing both models, we find that approximately half of the amnioserosa cells exhibit a loss of apical cross-sectional area dominated by an irreversible ingression process. For these cells, a transition is resolved from largely reversible oscillations to the onset of an ingression process. We also investigate variability in cell kinematics according to location within the dorsal opening and we find that cells ingress along each leading edge, in addition to previously observed ingestion associated with the zipping process or associated with apoptosis. We attribute cell-to-cell variability in the maximum rate of constriction during the ingression processes to be a consequence of variability in the magnitude of force produced by the cytoskeleton. Finally, we investigate invariant properties, i.e., time-independent, global properties of dorsal closure and find nearly constant rates of completion of ingestion processes as well as a constant of proportionality that relates the area of the dorsal opening to its two principle axes.
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List of Abbreviations and Symbols

Symbols

The commonly used symbols are defined here, although this list is not all inclusive. Some decoration symbols, like the $\hat{a}(t)$ refer to a process that can be applied to any function.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_i(t)$</td>
<td>Area of cell ‘i’</td>
</tr>
<tr>
<td>$a_{\text{high},i}$</td>
<td>High-frequency content of a cell ‘i’</td>
</tr>
<tr>
<td>$a_{\text{low},i}$</td>
<td>Low-frequency content of a cell ‘i’</td>
</tr>
<tr>
<td>$\tilde{f}(\omega)$</td>
<td>Fourier transform of $f(t)$</td>
</tr>
<tr>
<td>$\bar{a}(t)$</td>
<td>Box-car travelling average of $a(t)$</td>
</tr>
<tr>
<td>$\hat{a}(t)$</td>
<td>Windowed travelling average of $a(t)$</td>
</tr>
<tr>
<td>$I_i(t)$</td>
<td>Ingression function for cell ‘i’</td>
</tr>
<tr>
<td>$\alpha, \epsilon, \tau$</td>
<td>Ingression function parameters</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Quality of fit parameter</td>
</tr>
<tr>
<td>$k_{\text{eff},i}$</td>
<td>Effective elasticity for cell ‘i’</td>
</tr>
<tr>
<td>$b$</td>
<td>Coefficient of viscous drag</td>
</tr>
<tr>
<td>$d_{PS}$</td>
<td>Distance to the purse string</td>
</tr>
<tr>
<td>$d_A$</td>
<td>Distance to anterior canthus</td>
</tr>
<tr>
<td>$d_P$</td>
<td>Distance to posterior canthus</td>
</tr>
<tr>
<td>$H$</td>
<td>Height of the dorsal opening</td>
</tr>
</tbody>
</table>
Half height (H/2) of the dorsal opening

Width of the dorsal opening

Half width (W/2) of the dorsal opening

Abbreviations

Common abbreviations used throughout.

AP Anteroposterior

GFP Green Fluorescent Protein

ATP Adenosine Triphosphate

DE-Cadherin Drosophila Epidermal Cadherin

Dpp Decapentaplegic
I would like to thank my advisor, Professor Glenn Edwards for his guidance and support during my Ph.D. study. He was invaluable to my maturation in this research field. I would also like to thank Professor Dan Kiehart for his guidance and support. My knowledge of *Drosophila* and motor proteins is largely due to him. Also, Professor Stephanos Venakides for his guidance and insights into the modeling of force production as related to the problem of dorsal closure. I am honored to be the first physics graduate student at Duke to receive the Center for Theoretical and Mathematical Sciences Fellowship, and I thank them for their support. I would like to express my deep appreciation to Dr. Yusuke Toyama for his persistent help and support. Finally, I would like to thank Professor Surajit Sen, and Drs. Serdar Tulu, and Xomalin Peralta for their additional support and guidance. I am deeply indebted to Ruth Montague for preparing slides for me as well as taking care of day-to-day fly husbandry. Also, Adrienne Wells and Ginger Hunter for the times when Ruth was unavailable.

I would also like to thank my family and friends for their persistent support during my years at Duke. Specifically my parents Saul and Alice Sokolow and grandmother Dorothy Sadue, and my brothers and sister: Andrew, Justin, Jesse, Eleanor, and Gregory. Finally I’d like to thank my girlfriend and soon-to-be fiancee, Sarah Reaser, for her continual support.
Introduction

The research focus of our group is investigating tissue dynamics. Here we study the dynamics and emergent properties of amnioserosa cells in tissue. We use the model system *Drosophila* during an extremely robust and reproducible stage of development called dorsal closure. This stage occurs roughly 10 hours after fertilization when the fly is an embryo and before the nervous and musculatory systems are completed. During this stage, we observe cells in amnioserosa tissue undergoing oscillations. This system, however, exists at low-Reynolds number and is dominated by viscous forces. Under these conditions, the canonical, simple-harmonic oscillator becomes overdamped and does not support passive (non-driven) oscillations. Thus, these oscillations are driven by active contractile forces produced from within the cells and from neighboring cells, where energy for the active contractile force producing machinery is abundant, but highly regulated. The focus of this study is on the dynamics and emergent properties of amnioserosa cells and tissue.

Using time-lapsed, *in vivo* confocal microscopy we investigated the time-dependence of cell-shape changes for essentially all of the cells in the amnioserosa tissue during the early-to-late stages of closure. Image contrast is due to GFP-DE-cadherin, a relatively bright fluorescent construct that localizes at cell-cell junctions. By quantifying cell apical areas, we find that cells exhibit two general characteristics, oscillations and ingression. We have quantified the ingression process with an empirical model. Additionally we have developed a biophysical model that estimates the net forces acting...
on each cell. We also investigated variability in cell kinematics according to location within the dorsal opening and we find that cells ingress along each leading edge, in addition to ingestion associated with zipping or apoptosis. Finally, we investigated invariant properties, i.e., time-independent, global properties of dorsal closure.

Following Duke University requirements, the following specifies my contribution to the collaborative research. Day-to-day fly husbandry and slide mounting procedures were handled by the Kiehart lab. Typically slides were prepared by Ruth Montague but occasionally they were prepared by Adrienne Wells or Ginger Hunter. As an appendix, I have included a reprint that resulted from my first summer of research with the Edwards Lab (Appendix A). It represents a collaborative project with Wolfgang Wagner, Glenn Edwards, and Robert Pearlstein. I was involved in writing code to analyze signals that were measured by an interferometer, and I also aided Wolfgang Wagner in some preliminary experiments at Duke. The data sets that were used in the manuscript were collected by Wolfgang Wagner and Glenn Edwards at Vanderbilt University and brought back to be analyzed by Glenn Edwards. I contributed to parts of this analysis. In particular, determining the power-law fall-offs in the frequency spectra of the data sets (See Figure A.1). Otherwise, I performed all of the research and analyses reported here.
This represents an interdisciplinary work involving: physics and biology. As such, the average reader is likely to have a well versed background in either of these topics. To accommodate the average-physics-graduate-student reader or the average-biology-graduate-student reader, details are filled in where appropriate. This chapter begins with an overview of relevant biology. Next, it briefly covers the previous research done by our group. Finally, it concludes with an introduction to and a review of current tissue dynamics that applies specifically to our system. Additional background, specific to my research, is included in two subsequent chapters. Chapter 2 provides some basic background and terminology related to cellular force production. Chapter 3 contains a review of the principles that are relevant to \textit{in vivo} confocal microscopy. Chapter 4 reviews the projection and segmentation algorithm I developed and implemented.

1.1 \textit{Drosophila melanogaster}: A Motivated Model Organism

We study \textit{Drosophila}, the fruit-fly, as our model system to probe tissue dynamics. It is an excellent model system to investigate and has been a cornerstone in advancing
our understanding of genetics and development. Originally, it was chosen since it has many molecular, cellular, and developmental processes that can be mapped to similar processes in humans. However, a fortunate and major difference between humans and the fruit-fly is that a human (and many other vertebrates) will typically have redundancies in their genes, i.e., if a gene gets disrupted there is a second copy or similar gene that can take over. In contrast, the fruit-fly typically relies on a single gene [Alberts et al., 2008]. Another fortuitous difference is that the fruit-fly only has four pairs of chromosomes. These differences make *Drosophila* a simpler system for investigating genes and their function.

*Drosophila* also makes an excellent model system for investigating developmental processes. Similar to other multicellular organisms, the fruit-fly undergoes multiple stages of embryonic development. During the stages of early morphogenesis which take a fertilized egg to a larva, there are many stages that involve complex tissue patterning. Internal force production at the sub-cellular and cellular scale result in these embryo-scale rearrangements of tissue layers [Alberts et al., 2008]. Typically these events are coordinated and highly reproducible. Dorsal closure is one of these stages that are critical to successful development and forms the basis of our study. The enormous amounts of genetic research in *Drosophila* that has provided imaging of and modifications in the organism, along with the inherent reproducibility, geometry and symmetry of dorsal closure, make it an excellent model system for a biophysical research project on oscillations, cellular force production, and tissue patterning.

1.2 Dorsal Closure

Embryonic development is comprised of dynamic processes that link gene expression to morphogenesis [Keller et al., 2003, Wozniak and Chen, 2009]. These processes involve a precisely orchestrated interplay between gene expression, molecular force production, cell-shape changes, and tissue movement. Dorsal closure is a critical
stage during embryogenesis, where two epidermal tissues are brought together by force producing machinery in the surrounding tissues. Figure 1.1 is a confocal micrograph of a *Drosophila* embryo approximately ten hours after fertilization, an early stage of closure. The embryo is approximately 500 microns long by 150 microns wide and 100 microns deep. The dorsal opening is the region enclosed by the two intersecting bright arcs. This region is initially around 200 microns long and 120 microns wide but only 10-20 microns deep. Due to these dimensions, we make the assumption that dorsal closure occurs primarily in a plane.

The two bright arcs in Figure 1.1 are supracellular actomyosin rich cables (purse strings) that produce tension across the embryo [Kiehart et al., 2000]. At the intersections of these circular arcs are two canthi, named after their similarity in geometry to the canthi (corners) of the human eye. As dorsal closure progresses, the opposing purse strings approach one another, while a simultaneous process known as zipping occurs at each of the two canthi producing a seam between the two flanking lateral
epidermal tissues. Roughly outlined by the purse string is a central tissue called the amnioserosa. A typical amnioserosa cell is approximately 10-20 microns across and extends around 10 microns deep. The amnioserosa tissue has also been shown to produce force [Kiehart et al., 2000, Hutson et al., 2003, Toyama et al., 2008] and is comprised of a single layer of cells that undergo apoptosis (a programmed cell death) by the end of closure. The peripheral-most row of amnioserosa cells are behaviorally distinct and persists until the end stages of closure. This row is tucked underneath the leading edges of the lateral epidermis, whose cells are genetically distinct from those of the lateral epidermis [Foe and Alberts, 1983]. Throughout closure, amnioserosa cells leave the dorsal surface and are internalized through processes of ingestion. The apoptotic cells, typically in the anterior two-thirds of the dorsal opening, are one example of an ingestion process in the amnioserosa. Additionally cells are internalized at each canthus during seam formation.

Typically the entire process of dorsal closure takes 2-3 hours at 25°C to complete and is summarized by the sequence of confocal images in Figure 1.2. The cartoon in the figure highlights the same region of interest, shown sequentially from top to bottom in the time sequence of confocal images. The contrast mechanism in these confocal micrographs is due to GFP-DE-Cadherin and enhances amnioserosa cell edges and is very different from that of Figure 1.1 (GFP-moesin). It represents an enabling step in our research that is discussed in Chapter 3. The dorsal opening in Figure 1.2 is the region encompassed by, and including the brightly fluorescing cell edges. This opening maintains a robust geometry. This geometry is the basis for the setting-sun model introduced by Hutson et al. [2003] that will be discussed in detail in section 1.4.1. Further review of dorsal closure can be found in Kiehart et al. [2000], Jacinto et al. [2002], and Harden [2002].
Figure 1.2: Amnioserosa cell shapes during dorsal closure. Confocal images of the amnioserosa tissue during dorsal closure using GFP-labelled DE-cadherin where contrast is largest at amnioserosa cell boundaries. The cartoon embryo highlights the region of interest. Panels increase in time from top to bottom.

1.3 Life at Low-Reynolds Number

Dorsal closure, as well as other biological systems on the scales that typify a cell, is dominated by viscous effects, instead of the more intuitive inertial world. The tissue dynamics that we observe during closure are in the regime of low-Reynolds. Reynolds number is a dimensionless parameter that quantifies the balance of inertial effects to viscous effects [Purcell, 1977, Landau and Lifshitz, 1987]. The analogy
that is commonly introduced for this environment is that of an aircraft carrier being
towed by a tug-boat. If the aircraft carrier were immersed in a low-Reynolds number
environment (of similar magnitude to that experienced by an amnioserosa cell), and
the tug-boat stopped pulling, the aircraft carrier would come to a complete stop in
the distance of a dime. In other words, the system essentially has no memory of past
forces [Purcell, 1977, Odell et al., 1981].

Reynolds number appears in a number of fluid mechanics problems to enable
classification of similar systems, it is defined as follows [Landau and Lifshitz, 1987]:

\[ R \equiv \frac{rv\rho}{\eta} = \frac{rv}{\nu} \approx \frac{\text{inertial forces}}{\text{viscous forces}} \] (1.1)

In the context of a viscous fluid flowing past an arbitrary shaped object, ‘r’ is the
characteristic length of the object, ‘v’ its travelling velocity, ‘\(\rho\)’ the fluid density,
‘\(\eta\)’ the viscosity of the fluid, and ‘\(\nu\)’ the kinematic viscosity of the fluid. Although
the measurements of the kinematic viscosity for cell cytoplasm can vary, using the
kinematic viscosity of water when performing an order of magnitude estimation of
Reynolds number, does not affect the resulting conclusion. For water, \(\nu \approx 1 \times
10^6 \ \mu m^2/s\). The kinematics that we observe during native dorsal closure (as will be
discussed in the following section) involve velocities around \(\sim 0.01 \mu m/s\) and length
scales between \(10 - 100 \ \mu m\), resulting in a Reynolds number between \(10^{-5}\) and \(10^{-6}\).
In contrast, some laser ablation experiments in which tissues are cut, can result in
velocities \(\sim 1 \ \mu m/s\). This yields, at worst case, a Reynolds number of \(10^{-3}\). The
dynamics we observe for the amnioserosa cells (as will be discussed throughout)
have similar velocities of \(\sim 0.01 \ \mu m/s\) and length scales of \(\sim 10 \ \mu m\), resulting in a
Reynolds number of \(10^{-6}\). In all of these cases, the inertial terms are taken to be
negligible compared to the viscous terms.

Typically, inertial terms in an equation of motion are associated with second
order derivatives in time. In contrast, models for viscosity based on drag typically coincide with first order derivatives in time. Therefore, in our situation, applying the condition of low-Reynolds number to a system can be thought of as reducing the homogeneous system to first order in time. Under these conditions, the canonical, simple-harmonic oscillator becomes overdamped and does not support passive (non-driven) oscillations.

1.4 Empirical Investigation of Dorsal Closure

The following subsections review the research performed by our group in the past that is relevant to the continued research presented here.

1.4.1 Setting-Sun Model

The concept of low-Reynolds number was an essential element in the “setting-sun” model, first introduced by Hutson et al. [2003], and utilized afterward by many others in our group [Tokutake, 2003, Peralta et al., 2007, 2008, Toyama et al., 2008]. The geometry of the two purse strings in Figure 1.1 is well approximated by the intersection of two circles with similar radius-of-curvatures. These circles maintain nearly constant curvatures until the final stages of closure [Hutson et al., 2003, Peralta et al., 2007]. Figure 1.3 is a cartoon showing two intersecting circles, where the dorsal opening is given by their intersection. The height of the dorsal opening is quantified by the variable $H(t)$ which measured the maximum distance from one leading edge to another at “the symmetry point.” From this measurement it was empirically determined that the two leading edges, quantified by the purse strings, approach one another at an approximately linear rate during the mid-to-late stages of closure. This is a consequence of a near balance of the force producing tissues: the amnioserosa, the purse string, and the lateral epidermis.
Figure 1.3: Setting-sun model of the dorsal opening. The common area between two intersecting circles defines the dorsal opening in the setting sun model. Here \( r \) is the radius of the circle, \( H \) the largest purse-string-to-purse-string distance, \( W \) the distance between the two canthi, and \( h = H/2 \) and \( w = W/2 \). The angle \( \theta \) sweeps out a region from a canthus to the symmetry point of a purse string.

Hutson et al. [2003] found:

\[
\frac{dH}{dt} = -12.0 \pm 1.5 \text{nm/s} \tag{1.2}
\]

The uncertainty in the measurement of the rate of closure was later improved by Peralta et al. [2007].

Figure 1.4 is a free-body diagram of the forces acting on the purse string. Here, it was confirmed experimentally that the three tissues from Figure 1.1 produce force. The cable-like purse string is assumed to produce a tension \( T \), the amnioserosa produces a force that is assumed to act perpendicular to the mediolateral line (horizontal
Figure 1.4: Free body diagram of the forces acting along the purse string during closure. $T$ is the Tension from the purse string, while $\sigma_{AS} ds$ and $\sigma_{LE} ds$ are the forces from the amnioserosa and lateral epidermis, respectively. The symmetry points are indicated on the purse strings by the two open circles.

line connecting the two canthi), and the lateral epidermis produces a force that acts outward from the amnioserosa tissue. The exact force field produced by the amnioserosa tissue and the lateral epidermis is still an open question in research. At the symmetry point, the force from the lateral epidermis is collinear to the force from the amnioserosa tissue. Using the three tissues as applied forces we write down a vector equation for Newton’s Second Law for an infinitesimal arc length $ds$ of one of the purse strings (here boldface implies a vector):

$$\rho \dot{v} ds = \left( \frac{dT}{ds} + \sigma_{LE} + \sigma_{AS} \right) ds - b\dot{v} ds \quad (1.3)$$

Application of the low-Reynolds constraint makes $\rho \dot{v} ds$ negligible, resulting in an equation of motion:

$$b\dot{v} ds = \left( \frac{dT}{ds} + \sigma_{LE} + \sigma_{AS} \right) ds \quad (1.4)$$

Careful investigation of Equation (1.4) at the symmetry point, under the assumed geometry of the setting-sun model, reduces this vector equation to a scalar equation.
At the symmetry point, $\sigma_{LE}ds$ and $\sigma_{AS}ds$ are antiparallel and yield the following result:

$$b \frac{dh}{dt} = \sigma_{LE} - \sigma_{AS} - T\kappa \tag{1.5}$$

Here $\kappa$ is the curvature of the circular arc and $dh/dt$ is the velocity of a purse string moving towards the mediolateral line. Empirically it was determined that $dh/dt$ was constant. Thus Equation (1.5) reveals that the sum of the forces from the three tissues is constant throughout closure.

An additional perspective to describe closure is in terms of the seams that form at the canthi. Here the width of the dorsal opening reduces as closure progresses. Hutson et al. [2003] introduced an empirical rate model for zipping at the canthi, given by the following:

$$\frac{dW}{dt} = -k_z \tan(\frac{\theta_A}{2}) + \tan(\frac{\theta_B}{2}) = -\frac{k_z W}{2H} \tag{1.6}$$

Here, $k_z$ is the zipping rate constant and A/B label the two circles in the setting-sun model. This equation was subsequently shown to be a geometric property of intersecting circular arcs [Peralta et al., 2007]. Taking $H(t)$ to be linear, i.e., $H(t) = H(0) - Vt$, and integrating Equation (1.6) we obtain:

$$W(t) = W(0) \left[ \frac{H(t)}{H(0)} \right]^{k_z/2V} = W(0) \left[ 1 - \frac{Vt}{H(0)} \right]^{k_z/2V} \tag{1.7}$$

This equation gives an empirical measurement of the zipping rate constant $k_z$.

1.4.2 Asymmetries in the Dorsal Opening

While the time-dependent geometry of the dorsal opening is approximately symmetric, anteroposterior asymmetries in the geometry and in the zipping rate constants
have been quantified in detail [Peralta et al., 2007]. Peralta et al. [2007] extended the setting-sun model to incorporate anteroposterior asymmetries by modifying Equation (1.6) and applying it separately to each canthus. Thus there was a distinctive zipping rate constant for each canthus. During native (unperturbed) dorsal closure, they found a statistically significant difference in the two zipping rate constants, \( k_{z,\text{Anterior}} = 15.3\pm0.7 \text{ nm/s} \) and \( k_{z,\text{Posterior}} = 10.6\pm1.7 \text{ nm/s} \). Additionally, Peralta et al. [2007] quantified the geometric centroid of the dorsal opening.

\[
x_c \equiv \frac{\int x dA}{\int dA} \quad (1.8a)
\]
\[
y_c \equiv \frac{\int y dA}{\int dA} \quad (1.8b)
\]

Under the setting-sun model, \( x_c \) should fall on the symmetry axis, thus deviations of \( x_c \) from the symmetry axis quantify asymmetries in the dorsal opening. Not only did they measure an anteroposterior asymmetry in the dorsal opening, but also quantified how the centroid travels in time:

\[
\left( \frac{\Delta x_c}{\Delta t} \right)_{\text{native}} = 1.9\pm0.4 \text{ nm/s} \quad (1.9)
\]

1.4.3 Apoptotic Force

The forces that drive closure were further explored by Toyama et al. [2008] through careful investigation of \( \sigma_{AS} \). They found that the force from apoptotic cells distorted the shape of nearest neighbor and next-nearest neighboring cells. Additionally they determined that the localized force produced as part of the apoptotic process is responsible for one-half of the stress produced by amnioserosa tissue as a whole during native closure. While most amnioserosa cells undergo apoptosis (a programmed cell death) after the conclusion of dorsal closure, \( \sim10\% \) of the amnioserosa cells undergo
apoptosis during dorsal closure. Toyama et al. [2008] observed that this occurs preferentially in the anterior two thirds of the dorsal opening. By dividing the dorsal opening into thirds (anterior, central, posterior), Toyama et al. [2008] determined a ratio in the rate of apoptosis for the regions to be $5 : 4 : 1$, respectively. Finally they found that the overall stress produced by the amnioserosa tissue, the zipping rate constants, and the native rate of closure are each strongly correlated with the rate of apoptosis and are mutually correlated. This was done by using two mutant phenotypes, one in which apoptosis was completely suppressed ($AS^{-p35}$) and a second where the rate of apoptosis was enhanced ($AS^{-grim}$) [Toyama et al., 2008].

1.5 Oscillations in Cells and Tissue

Recently there has been interest in the contractile and oscillatory kinematics of amnioserosa cells. It has been observed that pulsations of apical areas [Fernández et al., 2007] accompany the constriction of amnioserosa cells during dorsal closure [Kiehart et al., 2000]. Amnioserosa cells near the two leading edges contract earlier than the more interior cells [Fernández et al., 2007, Gorfinkiel et al., 2009, Solon et al., 2009]. Amnioserosa cells exhibit pulsed oscillations at $\sim 3 - 6 \text{ mHz}$ during the earliest stages of dorsal closure (prior to the initiation of the zipping process) and have been attributed to a supracellular ratchet mechanism based on dynamic coupling of pulsed oscillations of an amnioserosa cell to a purse string of the leading edge. Here, an incremental reduction of the length of the purse string is correlated with the pulsed oscillations of the amnioserosa cells. The proposed mechanism is a molecular ratchet which catches on the contraction phase of an amnioserosa cell oscillation and resists the following expansion phase of the oscillation [Solon et al., 2009]. The rate of dorsal closure was observed to exhibit a slow phase prior to the onset of zipping, followed by a fast phase associated with the onset of the zipping process [Gorfinkiel et al., 2009]. Oscillations and contraction have been correlated with stage-dependent
accumulation of actin and myosin in the apical medial network and in the cortical ring [Blanchard et al., 2010]. The repeated assembly and disassembly of apical actomyosin networks, where assembly correlates with apical constriction, has been observed to be regulated by the PAR complex [David et al., 2010]. Pulsed oscillations and apical constriction also were observed during mesoderm invagination, and germband extension, earlier stages of *Drosophila* development. At this stage, pulsed apical constrictions were observed in ventral furrow cells and were correlated with contractions of an actin-myosin network anchored at adherens junctions [Martin et al., 2008]. These investigators raised the possibility that the force producing mechanism is based on a subcellular ratchet. Rauzi and Lecuit [2009] have commented on the similarities between the oscillations observed during dorsal closure and those observed during mesoderm invagination. With regard to germband extension, force production was attributed in part to polarized medial actomyosin flows that are oriented by DE-Cadherin and α-catenin [Rauzi et al., 2010].

With regard to single cells, shape oscillations at 20 – 100 mHz also have been observed in non-adhering fibroblast cells and attributed to a hydrodynamic instability of cortical actin as coupled to changes in calcium density that are modulated by mechanically-gated channels [Salbreux et al., 2007]. In addition, *in vitro* measurements using an optical trap have established a minimal system. More specifically, actin, myosin, ATP, and elastic loading, that exhibits spontaneous oscillations in the 1.5 – 14 Hz range [Plaçais et al., 2009].
This chapter introduces basic biological background and terminology for the key proteins that are involved in cellular force production and cell-cell adhesion. Three classes of proteins will be reviewed including those that form cytoskeleton, motor proteins, and adherens junctions. Each class is discussed to illustrate an intuitive picture of how cells produce force in tissue. We then return to the amnioserosa tissue specifically and discuss the roles of these proteins during closure.

For further background, we suggest the following three textbooks. Alberts et al. [2008] is an excellent introduction to molecular cell biology from expert biologists, Nelson et al. [2004] is an insightful introduction to biological physics emphasizing the physicist’s perspective, and Phillips et al. [2009] takes an intermediate view point.

2.1 Tissue and Cells

Professor Kiehart has always likened part of learning biology to that of learning a new language, which can present a terminology barrier for non-experts. In the interest of providing the reader with the appropriate background, we review key terminology so that the intricate interplay between proteins is not lost as an alphabet soup in the
Figure 2.1: Schematic portrayal of a subset of the proteins found within a cell and extending through the cell membrane. The Cell Interior and the Plasma Membrane are represented by light and dark grey, respectively. Contained within the Cell Interior are proteins that produce filamentous structures, labelled Cytoskeletal Proteins (yellow ellipse), and those that produce force, Force Producers (blue ellipse). The Adherens Junctions form a separate collection of proteins that result in Cell-Cell Adhesion (purple rectangle), this group of proteins is found both within the cell interior and extending through the plasma membrane to make contact with an Adherens Junction of a neighboring cell. Dashed lines represent a bond between the respective biological structures.

The key proteins relevant to cellular force production and cell adhesion are shown schematically in Figure 2.1. This listing represents a small subset of the number of proteins, nucleic acids, lipids, etc. present in the cell and is designed to map out the proteins relevant to the discussion of cellular force production. In this figure, the two grey boxes represent the cell interior and its boundary the plasma membrane, respectively. Proteins are grouped together by their functions, although it is common
Figure 2.2: Lipid molecules form part of the plasma membrane. On the left is a single phospholipid molecule where its hydrophilic head and hydrophobic tail groups have been labeled. In water these lipids form a bilayer shielding their hydrophobic tails from the water, as shown in the center panel. The right panel indicates how proteins can assemble within the lipid membrane.

In biology for proteins to serve many different purposes. The yellow ellipse groups proteins associated with the cytoskeleton that can provide mechanical structure to the cell by forming filaments within the cell interior. The blue ellipse groups the proteins relevant for force production. Adhesion proteins link the cytoskeletal proteins to the cell plasma membrane and to neighboring cells and are shown in the purple rounded rectangle.

The plasma membrane (represented in Figure 2.1) encloses the cell and separates its internal structures from its external environment. It includes many lipid molecules that individually have a hydrophilic head group and a hydrophobic tail group. The left panel of Figure 2.2 presents a cartoon of one type of lipid typical in animal cells, the phospholipid molecule. In water, collections of many of these phospholipid molecules can form a continuous sheet called a lipid bilayer (center panel) where the polar heads face the aqueous cell interior and external aqueous environment and the hydrocarbon tails form a hydrophobic membrane interior. This layer is approximately 5 nm thick and is extremely malleable. It can act like an elastic...
membrane under stretching, bending, and shear. However it is also important to note that individual lipid molecules which form the bilayer can act like a two-dimensional fluid [Phillips et al., 2009]. The picture in the center panel is a cartoon of a lipid bilayer in the absence of membrane proteins; however, this is not representative of the structure of a cell membrane, which also includes many proteins. The right panel portrays three proteins in the lipid bilayer. Here the central protein extends through the membrane and is called a transmembrane protein. The other two proteins are examples of a protein residing on the extracellular or intracellular side of the bilayer. In cell membranes, the lipid bilayer is crowded with many proteins and can have regions that are extremely structured. An *E. coli* membrane of total surface area \( \sim 6 \mu m^2 \) typically has on the order of half a million proteins, which corresponds to an average spacing between proteins of \( \sim 3 \) nm [Phillips et al., 2009]. The cell cytoplasm, which is the aqueous mixture of proteins, organelles, and vesicles (excluding the cell nucleus) internal to the plasma membrane, also is quite crowded. Approximating an *E. coli* cell to have a volume of \( 1 \mu m^3 \), the average center-to-center spacing between the millions of proteins is \( \sim 10 \) nm and the water layer between proteins is \( \sim 5 \) nm thick.

### 2.2 Cytoskeleton

The cytoskeleton is important for cellular force production and providing structure. There are many cytoskeletal proteins at play, and we choose to focus on those that form filaments. Actin is one of the important filamentous proteins found in the cell. Actin is a polarized protein, made from many actin sub-units, that can form long filaments in the cytoplasm. As summarized in Figure 2.3, each subunit assembles into a helical structure with a pseudo-repeat after 13 subunits, a linear distance of \( \sim 36 \) nm [Mehta, 2001]. Additional proteins control the initiation and termination of growth of a filament, where assembly is associated with the positive end of the
Figure 2.3: Examples of proteins that result in different connectivity of actin filaments, including: branching, bundling, and cross linking. An actin subunit is indicated by a red sphere. A: ARP complex (green) allows for actin filaments (red) to branch off at 70 degree angles. B: α-actinin (green) binds actin in parallel contractile bundles leaving room for myosin in between. C: Filamin (green) binds adjacent actin filaments allowing tangled meshworks of actin to be formed.

actin filament and disassembly with the negative end. The rates of assembly and disassembly can vary. Thus actin filaments can grow and travel as well as shorten.

Actin filaments, along with other scaffolding proteins, form more complex ordered structures that can be extraordinarily dynamic. For example, in animal cells there are proteins that complex with actin resulting in branching, parallel bundle assembly, and strengthening of crossing adjacent actin filaments (Figure 2.3). Actin filaments can branch with the help of a collection of proteins called the ARP (actin related proteins) complex. As shown in Figure 2.3A, the ARP complex (green) can bind to the minus end of an actin filament and simultaneously to the side of another actin protein making a 70 degree angle. Repeating this for many actin filaments produces a tree-like branching and a “pushing” force. In addition to branching, Figure 2.3B shows that actin can be ordered into parallel bundles in the presence of α-actinin which binds roughly perpendicular to the sides of actin filaments leaving sufficient separation between the bundles so as to allow myosin-II to enter, which will be discussed further in the next section on motor proteins. Another protein that binds actin is filamin. Panel C shows how this protein can connect adjacent actin filaments.
that cross one another. Multiple crossing of actin in the presence of filamin can result in a tangled web that can exhibit the properties of a gel [Alberts et al., 2008].

2.3 Motor Proteins

The motor protein myosin can complex with actin to produce force. As discussed earlier, the cytoskeleton is comprised of actin filaments that can form ordered structures. These structures provide the framework for myosin motors to perform their function. Here we review motor proteins in general, then we describe the motor myosin-II, the oligomers it forms (Figure 2.4) and the working model for how it produces force (Figure 2.5).

Many proteins in the cell are dedicated to acting as motors. Some of these motors are involved in muscle-like contractility while others are involved in active transport within the cell. Motor proteins of notable interest are myosins, kinesins, and dynein. Myosins complex with actin filaments while kinesins and dynein complex with microtubules (another filamentous protein found in the cytoplasm). In general, a motor protein uses repeated ATP hydrolysis to move down a filament in the cytoplasm. These filaments are polarized and specify a preferred direction of motion [Alberts et al., 2008]. The myosin head region has been identified as the force producing unit.

There are more than twenty-five classes of myosin motors [Sellers and Knight, 2007]. Of these classes, there are two major subgroups as determined by the number of myosin heads associated with “native” myosin: a single head versus having two heads. Myosin-II and myosin-V are examples of myosins with two heads. A cartoon of myosin-II is shown in Figure 2.4A. A major functional difference of the two most studied myosins are that myosin-II is related to contractile activity in both muscle and non-muscle cells [Wozniak and Chen, 2009], where as myosin-V is involved in vesicle and organelle transport [Alberts et al., 2008]. Additionally myosin-V does not self assemble into larger oligomers as does myosin-II and tends to act alone or in
Figure 2.4: Illustration of an individual myosin-II as well as an idealized myosin-II thick filament. A: Myosin-II is a motor protein that forms from two heavy chains and two copies of two light chains. Both heavy chains are indicated by a shade of green and contain the myosin head region at the N-terminus (bulbous green shape). These two heavy chains form a coiled-coil. The two light chains have been idealized by the two shades of blue for each heavy chain (four in total). B: An idealized myosin-II thick filament (not to scale). Multiple myosin-II proteins can self-assemble to form a larger thick filament. Here there are two regions of myosin heads (oriented in opposite directions) separated by a bare zone where there are no myosin heads.

Small numbers [Mehta, 2001]. Multiple myosin-II proteins can come together to form larger thick filaments (Figure 2.4B). In extremely ordered systems, such as striated muscle, these thick filaments can be made from several hundred myosins. In addition, studies in Acanthamoeba show that thick filaments comprised of 16 myosin-II proteins self assemble in the presence of 5-10 mM MgCl$_2$ [Pollard, 1982].

The combination of multiple cytoskeletal actin-rich structures, with regulated and activated myosin-II, allows for contractile force production within a cell. As discussed earlier, actin filaments are made from repeated actin sub-units. On each of these subunits there is a binding site for a myosin head. Once a myosin head finds a binding site on an actin filament it can, through ATP hydrolysis, pull on this
filament. The hydrolysis of ATP is used in a number of biological processes as one of a number of available energy currencies. While there is an abundance of ATP in a living cell, these systems are highly regulated.

Figure 2.5 shows the cycle of how ATP, myosin, and an actin filament can be coordinated to produce force. We begin the cycle starting with myosin-II in the absence of ATP, where it is attached to actin in a rigor bond (named after rigor mortis). ATP is taken up by the myosin head which causes conformational changes that result in the myosin head being released. Shortly after this step the ATP is hydrolyzed, and converted to ADP and an inorganic phosphate. This occurs simultaneously to a conformational change in the myosin which results in myosin in the cocked position. This cocked myosin head finds the next binding site on the actin filament which causes the release of the phosphate. The force generating step corresponds to a change from a weakly bound myosin head to a strongly bound state as it releases the ADP and fires the myosin. This produces a force and causes a translation of the thick filament and returns the system to the original attached state. In myosin-II, the rate limiting step involves the isomerization from ADP-\( P_i \) weakly bound to actin to ADP-\( P_i \) strongly binding state [Mehta, 2001].

The ordering of actin filaments determines some of the properties of a contractile actomyosin network. Figure 2.6 presents two alternative configurations of actin, the first are parallel bundles that exhibit some order and the second a tangled web. In both cases myosin contractility leads to force production. In the case of bundled actin filaments, the myosin act to shorten the cable length and increase its tension. In the case of the tangled web, ATP regulation can coordinate the stiffening of the entropic system. In both cases the actin filaments can be assembling and disassembling concomitant to myosin contractility.
Figure 2.5: Myosin thick filament travels toward the plus end of an actin filament through ATP hydrolysis. The actin filament, myosin thick filament, and myosin head are each labeled in the top panel. The cycle begins with the myosin head attached to the actin filament with a rigor bond. ATP is introduced into the system and binds with the myosin head. This leads to the released state where the rigor bond to the actin filament has been broken. The myosin neck region changes shape and displaces the head of the myosin into the cocked position. During this process, ATP hydrolysis occurs in the myosin head and causes ATP to be converted to ADP+Pᵢ, which both remain complexed to the myosin head. The myosin head finds the next binding site on the actin filament and forms a weak bond. After which the ejection of the phosphate results in a stronger bond of the myosin head to the actin filament and triggers the force generating power stroke. After the power stroke the ADP is loosely bound and is released from the myosin head returning it to the attached starting state, where the cycle can begin again.
**Figure 2.6:** Two examples of actomyosin networks. Upper right is an ordered array of actin filaments, here actin filaments line up in a bundle and myosin contractility results in a shortening of the cable. The lower left depicts a random assortment of actin filaments and myosin oligomers.

### 2.4 Cell-Cell Adhesion

Tissue is a collection of cells. A major difference between single-cell organisms and multicellular organisms is the existence of a group of proteins that join adjacent cells. These proteins are called cadherins. In this section, we discuss the proteins necessary for cell-cell adhesion, i.e., proteins found in the adherens junctions, and summarize how they connect to the force producing cytoskeleton.

An adherens junction links actin filaments of the cytoskeleton to the plasma membrane. Figure 2.7A shows an example of a single adherens junction. Here an actin filament from the cytoskeleton binds to anchor proteins that in turn bind to β-catenin and p120-catenin. The catenin proteins then bind to E-Cadherin which is transmembrane and extends from the cytosol to the intercellular space. E-Cadherin joins epidermal tissues by binding with the E-Cadherin of a neighboring cell. Figure
Cell-cell adhesion results from many adherens junctions. A: Illustration of a single adherens junction. Transmembrane E-cadherin is linked to actin via p120-catenin, β-catenin, and other anchor proteins. B: E-cadherin of a neighboring cell can bind the adjacent cell’s E-cadherin to produce cell-cell adhesion, where multiple adjacent adherents junctions result in cell-cell adhesion. 2.7 illustrates how multiple adherens junctions concentrate across adjacent cells. The relative strength of a single bond between adjacent cadherin proteins is small, and cell adhesion derives its strength from incorporating numerous cadherins [Alberts et al., 2008]. These adherens junctions form a belt near the apical surface of the cell. The result is a cable of actin and a belt of adherens junctions that circumnavigate the cell near its apical surface (Figure 2.8). This bundle of actin filaments form the subapical cortical ring of actin in the cells. The adherens junctions also serve as the anchoring point for the apical-medial network of actin which spans the cell.

The cellular actomyosin cytoskeleton is a dynamic structure within the overall tissue. The cytoskeleton within a cell can react to external stresses by increasing its stiffness [Mizuno et al., 2007], where experimental observations (e.g., [Franke et al., 2005, Rauzi et al., 2008, Ma et al., 2009]) and theoretical modeling (e.g., [Layton et al., 2009, Almeida et al., 2011]) suggest both elastic and contractile dynamics.
2.5 Force Production in Amnioserosa Cells

This dissertation reports an investigation of the forces produced by the amnioserosa tissue. We now qualify some of the generic, text-book description as presented above to the amnioserosa. The forces produced by individual cells begin at the subcellular level through the activation of myosin-II, which through repeated ATP hydrolysis...
pulls on actin filaments. Collectively these proteins contribute to the cytoskeletal framework that is tethered to the cell membrane by adherens junctions. One component of these adherens junctions is the transmembrane protein DE-Cadherin (the ‘D’ in ‘DE-Cadherin’ indicates it is specific to Drosophila). Thus activation of motor proteins in cytoskeletal assemblies leads to forces that are large enough to drive cell-shape change. Force production from within a cell directly affects its neighboring cell due to cell-cell adhesion.

It has been shown that myosin-II is essential to drive cell-shape changes during dorsal closure [Franke et al., 2005]. The description of the myosin filament translating along an actin filament due to ATP hydrolysis and its power stroke works well in the context of a well ordered system. In striated muscle the location of myosin and actin filaments are highly ordered forming parallel contractile assemblies. The degree of order of myosin filaments represented by the thick filament presented in Figure 2.4B has not been observed in the amnioserosa cells. However, the prevailing view is that myosin-II self assembles into smaller filaments.

Figure 2.8 presents a cartoon of the force producing structures found near the apical surface of a typical epidermal cell. Similar structures also are found in the amnioserosa tissue, where one is more ordered than the other, although the extent of ordering is an open research question. There are two principle accumulations of actin and myosin that can produce force. A cortical ring forms at cell-cell adherens junctions near the apical surface [Odell et al., 1981, Farhadifar et al., 2007] and, in addition to this ring, there is a two-dimensional apical-medial network that roughly lies in the plain of this ring [Martin et al., 2008, Solon et al., 2009, Ma et al., 2009, Rauzi et al., 2010]. Both of these structures have been shown to produce force in amnioserosa cells during dorsal closure.
This chapter is dedicated to a description of the standard techniques we utilize to investigate tissue dynamics during dorsal closure in *Drosophila*. These include a brief summary of confocal microscopy, the significance of GFP, and the preparation of a *Drosophila* embryo to be observed using a confocal microscope *in vivo*.

### 3.1 Confocal Microscopy

Confocal microscopy advances traditional microscopy by filtering out unwanted light from outside the imaging plane (e.g., [Bass et al., 1995, Robinson, 2001]). Our measurements of *Drosophila* during dorsal closure utilize a spinning-disc, confocal microscope. In this section, we first describe the basic principles of confocal microscopy and then briefly explain spinning-disc, confocal microscopy.

#### 3.1.1 Principle of Confocal Microscopy

Confocal microscopy filters out unwanted light by adding a pinhole after the objective. As confocal microscopy’s creator, Marvin Minsky, realized:

“An ideal microscope would examine each point of the specimen and..."
measure the amount of light scattered or absorbed by that point.” [Minsky, 1988]

His insight was achieved in practice by placing a pinhole at the focal point of the objective that selects convergent light from the imaging plane. It is perhaps best understood by walking through a simple ray tracing schematic. Figure 3.1 is a schematic illustrating the essential components for a confocal microscope. Green light from excited GFP protein (discussed in the next section) is emitted from a single point in the imaging plane and passes through the objective (ray labeled $r$). Additionally, there are other light rays from above ($r'$) and below (not shown) the imaging plane, and other scattered light rays (not shown) that can pass through the objective. In traditional microscopy all of these rays reach the detector (the “eye”), diminish contrast, and reduce resolution of the imaging plane. Minsky’s insight was to place a pinhole $P$ at the focal point of the objective before the detector so that light rays from outside of the imaging plane get filtered out ($r$ passes through the pinhole, while $r'$ is blocked). The word ‘confocal’ means having the same foci, thus confocal microscope applies the constraint that light entering the detector is convergent at the focal point of the objective lens. By sequentially illuminating points on the specimen, one can scan the entire imaging plane to reconstruct a region of interest. Additionally, (limited) subsurface imaging is made possible by a confocal microscope.

3.1.2 Spinning Disc Confocal Microscopy

Spinning disc confocal microscopy essentially takes advantage of multiple pinholes to enable realtime confocal imaging [Bass et al., 1995]. Illuminating each point of the specimen can be achieved in a number of ways. In the schematic from Figure 3.1 one can either move the specimen in a raster-scan fashion or equivalently scan the illuminating light source (laser-scanning confocal microscopy). Multiple point
Figure 3.1: Schematic of the principle components of a confocal microscope. Convergent illumination laser light from the source $S$ reaches the imaging plane in the specimen. Excited GFP protein at the plane of focus emits green light $r$. An additional ray from above the imaging plane $r'$ is presented as well. Both rays pass through dichroic mirror $D$ and reach the pinhole $P$. Rays from the plane of focus $r$ are convergent at the pinhole and pass through to the observing “eye.” Rays from out of the plane of focus $r'$ are blocked by the pinhole.

illumination on a spinning-disc confocal microscope is achieved by rotating a Nipkow disc with an array of pinholes. The distribution of pinholes on a Nipkow disc are reminiscent of a spiral galaxy. By illuminating the back of the disc, each pinhole is both a point source for illuminating the specimen and pinhole for the fluorescing light. By spinning this disc, the multiple point sources illuminate the entire imaging plane and can be collected in real time [Bass et al., 1995].

3.2 GFP Contrast Mechanisms

The light that reaches our detector is due to emission of a fluorescent protein called Green-Fluorescent Protein (GFP). Under 488 nm blue light illumination, the protein emits green light with a peak emission at 508 nm [Chalfie et al., 1994, Kiehart et al., 2006, Alberts et al., 2008]. This allows us to image the fly using light emitted by the
protein GFP instead of relying on transmitted or reflected light. GFP is a transgene from the jellyfish *Aequoria victoria* that has been expressed in the fruit fly. By adding the DNA-coding sequence for GFP to a protein of interest, it is possible to create a working form of the protein that also emits green light when illuminated with blue. This provides a source of contrast for imaging as well as gives the ability to ‘highlight’ important biological structures. It also requires that one matches a GFP imaging construct to the research question at hand.

In the past, our group has had success in quantifying the dorsal opening due to the strong fluorescence of the supracellular purse string labeled with GFP-moesin (See Figure 3.2A) [Hutson et al., 2003, Kiehart et al., 2006, Peralta et al., 2007, 2008, Toyama et al., 2008]. However, with our attention now on the shapes of the amnioserosa cells, a different contrast mechanism was needed. The expression of GFP-DE-cadherin in a *Drosophila* embryo, then results in fluorescence at epithelial cell boundaries [Oda and Tsukita, 2001]. The improved image contrast due to this transgenic fly is one of the enabling steps of this research. A comparison of confocal images from two similarly staged embryos using the GFP-moesin and GFP-DE-Cadherin contrast mechanisms is presented in Figure (3.2). The choice of GFP-DE-Cadherin to image cell-shape changes is clear by comparing the difference in contrast of the amnioserosa cells in both images.

### 3.3 Fly Mounting and Slide Preparation

Day-to-day fly farming and fly husbandry was performed by the Kiehart Lab. Typically, Ruth Montague maintained a single cage of the fly line that I was imaging and handled the day-to-day collections required for imaging. Additionally, Ruth Montague prepared slides for me to image. We investigated a fly line, GFP-DE-cadherin (driven by the ubiquitin promoter/enhancer cassette [Oda and Tsukita, 2001]), to provide image contrast of cell boundaries. The Kiehart lab used standard
methods for preparing \textit{Drosophila melanogaster} embryos for \textit{in vivo} time-resolved microscopy [Kiehart et al., 1994, 2000, 2006], which are summarized here.

The \textit{Drosophila} flies were allowed to live happy lives in a film-canister-sized cage maintained at 25$^\circ$C. At one end of the cage is a grape agar plate which serves two purposes. The first is that of a food source and the second is a substrate on which the flies can lay their eggs. After an egg is laid it takes roughly 12 hours for this fly to reach the end of dorsal closure if it is maintained at 25$^\circ$C. Typically after a number of eggs are collected, however, for convenience they were placed in a refrigerator at 18$^\circ$C which roughly doubles the time for development. Replacing the agar plate for only a short time interval ($\sim 2$ hours) during the day ensures that the eggs on that agar plate will begin dorsal closure roughly 20 hours later if they are kept at 18$^\circ$C. This means that if I want to image dorsal closure at 9am on Tuesday, Ruth was there to change agar plates on Monday between 11am and 1pm. The longer the interval of collection the more variability in staging from embryo-to-embryo [Wieschaus and Nusslein-Volhard, 1986, Kiehart et al., 2000].
3.3.1 Preparing Fly Embryos for Imaging

Time-resolved, in vivo confocal microscopy requires the marriage of a living environment for the fly embryos while still meeting imaging constraints. A full consideration of the physiological and optical constraints of Drosophila embryo imaging can be found in Kiehart et al. [1994] and the typical procedures performed in slide preparation are summarized here.

Before the flies were mounted they needed to be properly prepared so they could be imaged. Surrounding the embryo are two membranes, the inner layer is called the vitellin, the outer and thicker protective layer is the chorion. Before they are mounted to a slide, the chorion is removed by briefly placing the embryos in a diluted bleach solution, leaving the vitellin in tact. After the chorion is removed, the embryos are rinsed and arranged on an agar plate so that they can be oriented in the same direction.

As stated earlier, the flies need to survive the imaging process. The embryo relies on the yolk for food but needs an external oxygen source. Additionally, the embryos need to be stabilized and kept in an oil for imaging. To solve these issues, the flies are glued to a glass coverslip, immersed in a halocarbon oil and then sandwiched between the coverslip and a teflon membrane. Gluing the embryos helps stabilize them during imaging and, in addition, mounting the dorsal surface of the embryo to a coverslip flattens the region of the dorsal opening. This reduces the curvature of the amnioserosa tissue so that a majority of the amnioserosa falls in a single optical plane. The combination of the non-toxic halocarbon oil and the teflon membrane allows for oxygen to diffuse and reach the developing embryo, while also preventing desiccation. Additionally, the oil meets the requirements of oil-immersion objectives for a gain in image resolution. Embryos prepared and imaged in this manner survived and hatched [Kiehart et al., 1994].
Figure 3.3: Orientation of Drosophila embryos during confocal imaging. A: Perspective cartoon of the Drosophila embryo at an early stage of dorsal closure. The dorsal side of the embryo is labeled and the dorsal opening is the eye-shaped region. B: A transverse cross section of the embryo. The cartoon “eye” represents the objective of the microscope and the green horizontal lines confocal z-slices. Multiple z-slices are required to image the amnioserosa (red) due to its curvature. C: A coronal slice of the embryo created by projecting multiple z-slices into a single image. Image is a reproduction of the image from Figure 1.1 where contrast is due to GFP-moesin.

3.3.2 Time-lapsed Imaging

Figure 3.3A shows a perspective drawing of the Drosophila embryo at an early stage of dorsal closure. The cartoon in panel B represents a transverse slice along the anteroposterior axis of the embryo. The eye in the cartoon represents the microscope objective where the horizontal green lines indicate idealized planes of imaging. Here, utilizing the confocal microscope, planes are limited in thickness to roughly 1 micron and are stepped in z to interrogate deeper sections of tissue. The tissues of closure discussed in Chapter 1 are again labeled in panels B and C.

Our study focusses on the amnioserosa tissue (shown in red in Figure 3.3B)
which, due to the curvature of the embryo, requires multiple z-slices (green lines) to image. Typical data sets began at early stages of dorsal closure (before the onset of zipping) and continued for 2-4 hours at room temperature. Fluorescent images (488 nm excitation from an Argon-ion laser) were collected with a spinning-disc, confocal microscope (Yokagawa CS-10 confocal head mounted on a Zeiss Axioplan) using a 25x, 0.8 NA multi-immersion objective used with immersion oil. A complete z-scan took between 9 and 15 seconds depending on the number of slices, where each slice had a 400 ms exposure time. This produced 600-1100 three-dimensional confocal images over the time course of dorsal closure for each embryo. The process by which multiple z-slices are converted to a single image is known as projection and is discussed in Chapter 4. An example result of image projection is shown in panel C of Figure 3.3 where one can see the typical orientation for our imaging.
Our overall goal for image analysis was to segment the amnioserosa tissue with cellular resolution in a sequence of time-lapsed confocal images. In the past, we have had success segmenting the dorsal opening from the surrounding tissue utilizing fluorescence from the purse strings in GFP-moesin labelled embryos (Figure 3.2A); however, edge-detection-based segmentation of individual cells is a substantially more challenging problem due to varying contrast within and a relatively large number of segments in an image. The first step in resolving these issues was utilizing a different contrast mechanism, GFP-cadherin, that fluoresces at cell junctions (Figures 3.2B and 2.8), thus matching the contrast mechanism to the research question. While changing the contrast mechanism to GFP-DE-cadherin was a major step forward, developing a more powerful projection method and segmentation algorithm proved to be the final step in resolving the issues of quantifying confocal images into cell edges.

We developed a novel analysis method that enables us to segment each three-dimensional confocal image of the dorsal surface with cellular resolution in time. The analysis includes: a projection step to address the curvature of the dorsal surface;
a semiautomated assessment of the initial projected image that seeds subsequent
segmentation; followed by fully automated segmentation of the entire data set. The
segmentation computations were done using custom software written in C++, while
all other computations were performed utilizing custom and built-in functions in
MATLAB (The MathWorks, Natick, MA).

4.1 Mask-Projection

The first step in determining cell shapes is a Mask-Projection algorithm. The funda-
mental feature of a z-projection is to map a the three-dimensional intensity profile in
z (set of voxels with common values of x and y) to a single value for each pixel in the
xy-plane. Examples of some projection methods include: reporting the maximum,
the sum, or the average values over z. When unwanted fluorescence exists, however,
these values can be skewed, compromising the quality of the projected image. Figure 4.1A is an example of a projection where the mean value is taken over z. Here,
unwanted fluorescence from the yolk has confounded image contrast. To improve
the quality of the projected image we can exclude regions of unwanted fluorescence
before calculating the mean, obtaining the result in Figure 4.1B. Determining the
regions of unwanted fluorescence and removing them before projection is the goal of
the Mask-Projection algorithm.

4.1.1 Sources of Unwanted Fluorescence

During early stages of dorsal closure numerous z-slices are necessary to image the
entire amnioserosa where the lateral-most cells realize the curved surface of the em-
bryo as summarized in Figure 4.2. In the left-hand side of this figure, the central
dorsal surface has been slightly flattened to account for the mounting procedure (see
Chapter 3). The perspective of the microscope is characterized by the “eye.” Typi-
cally 15-20 z-scans, in 1-micron steps, were used in the experiments. For presentation
purposes, the number of z-scans has been idealized in the schematic as five confocal z-slices (left-hand side, shown in green). For mid-to-late stages of closure, the amnioserosa tissue lies almost completely in a single optical plane due in part to the mounting procedure. The left side of Figure 4.2 presents a schematic of a transverse slice of the embryo, which emphasizes how curvature of the dorsal surface as seen in this section needs to be addressed. In addition, there is curvature in an orthogonal anteroposterior direction and there can be some slight cell-to-cell variability in the z-direction (not shown in the idealized figure). We can see in the schematic that as the scanning depth increases, regions of yolk that underlie the central amnioserosa cells also will be imaged. Since endogenous yolk fluorescence degrades contrast if a traditional z-projection method is used (Figure 4.1 A), we have developed an alternative method to compensate for this effect (Figure 4.1 B).

\[\text{4.1.2 Determining the Mask}\]

In our application, we use a numerical mask to restrict the number of z-planes that contribute to the mapping. DE-Cadherin fluorescence is localized within a depth of 5 microns, located towards the apical surface of each amnioserosa cell. However, the apical cell surfaces do not lie in a single optical plane during the early stages of

\[\text{Figure 4.1: A comparison of two projection methods applied to the same data set. A: Mean-projection, reported pixels intensities represent the mean value of the z-voxel for a given xy. B: Mask-Projection, pixels of unwanted fluorescence in z are excluded before taking the mean.}\]
Figure 4.2: Mask-Projection method. Left: Schematic summarizing confocal imaging of the dorsal surface of a *Drosophila* embryo. This is a transverse section taken through the middle (along the A-P axis) of the dorsal opening (Cartoon adapted from Kiehart et al. [2000]). Right: Projection along the z-axis of three confocal images into the x-y plane (bottom image). The number of confocal slices has been idealized for presentation purposes.

closure and can exhibit other small variabilities in z as well. The computational challenge is to determine the appropriate range of z-slices to capture the DE-Cadherin fluorescence for each amnioserosa cell, while also excluding the underlying yolk fluorescence. The right-hand side of Figure 4.2 portrays an idealized view of three confocal images contributing to the overall projection, where in practice six confocal images (a subset of the 15-20 confocal images) were averaged for each amnioserosa cell. Here, the mask is represented by the magenta tint in each z-slice and the region of interest by the non-tinted pixels.

The overall masking process is the union of multiple masking steps. First we surveyed the image planes from the top down to establish a threshold for the intensity of DE-cadherin fluorescence. This is analogous to establishing first the threshold in the top image of the right column of Figure 4.2. For each value x,y in this plane that satisfies the suprathreshold condition, a value \( z_c \) was determined that locates...
this mask in z. Each mask is a numerical cylinder, typically of radius 10 microns (including fluorescent cell edges and contrasting cell interiors), where the azimuthal axis of the cylinder coincides with the z-axis of the confocal microscope. A cylinder height of six imaging planes was sufficient to capture the fluorescent edges associated with the DE-cadherin belt while excluding the yolk. Then this survey was repeated sequentially to capture peripheral DE-cadherin fluorescence corresponding to deeper z-planes, excluding from consideration all values of x,y that were successfully accounted for with the cylindrical masks in previous iterations. The union of these steps captures the curved surface of overall DE-cadherin fluorescence while excluding yolk fluorescence. This is shown schematically as the ellipse and the two elliptical rings in the three masked images, respectively, shown in the right hand column of Figure 4.2. While the intensity of the yolk fluorescence can be comparable to that of DE-cadherin fluorescence, the two are readily distinguished due to this top down analysis. At the conclusion of the overall masking process, the 3-D intensities were projected into a 2-D image as portrayed in the bottom plane on the right-hand side of Figure 4.2.

Subsequent segmentation of the projected image required a relatively smooth transition in fluorescent intensity at the tissue interface between the amnioserosa and each leading edge cells. The mask-projection method was designed to capture the fluorescent intensity from the DE-cadherin in the amnioserosa tissue but to exclude fluorescent intensity from the leading edge cells and the lateral epidermis, producing a relatively sharp transition in fluorescence intensity that could confound segmenting the outer most amnioserosa cells. Consequently, we numerically added a weak fluorescent background to the region of the projected image outside of the dorsal opening to smooth the transition at the tissue interface (see bottom image, right hand side of Figure 4.2).
**Figure 4.3**: Quantifying the curvature of the embryo due to roll-off. 

A: Cartoon of a transverse cross section of the embryo during dorsal closure. The amnioserosa (red) exhibits curvature as distance from the anteroposterior axis increases (variable \( y \)). The angle \( \psi(y) \) quantifies the roll-off angle as a function of position. 

B: Scatter plot of the relationship of length distortion to the distance from the anteroposterior axis (\( y_{from \ AP} \) is the absolute value of the position \( y \)). Length distortion is measured by considering a right triangle (inset) and is given by \( (l - l')/l \times 100\% \). Each color represents data from a different embryo.

### 4.1.3 Area Distortions from Projection

In practice, the Mask Projection method effectively excluded the endogenous fluorescence and substantially improved image contrast relative to a traditional z-projection method (see Figure 4.1). However, there were some limitations that typify projection methods. For example, the projection of a cell on a curved surface into an xy-plane can introduce slight distortions in cell shape. Due to the mounting process, discussed in Chapter 3, the central most amnioserosa cells typically constitute a plane. At the earliest stages, however, the cells found at the most extreme peripheries do not completely fall in the same plane. For these cells, length distortions are introduced when we project from an image volume to a two-dimensional image. In this section we discuss the magnitude of these effects and how they depend on the stage of closure.

Figure 4.3A shows a cartoon of the transverse cross-section of the embryo (in a
### Table 4.1: Maximum length distortions along the periphery during dorsal closure.

<table>
<thead>
<tr>
<th>Area of Dorsal Opening ($\mu m^2$)</th>
<th>Embryos Used</th>
<th>Height ($\mu m$)</th>
<th>Max($\psi$) (degrees)</th>
<th>Max % Distortion</th>
</tr>
</thead>
<tbody>
<tr>
<td>23,000</td>
<td>1.2</td>
<td>140±1</td>
<td>23.2±0.1</td>
<td>8.1±0.1</td>
</tr>
<tr>
<td>20,000</td>
<td>1.2</td>
<td>131±1</td>
<td>18.5±1.1</td>
<td>5.2±1.0</td>
</tr>
<tr>
<td>17,000</td>
<td>1-3</td>
<td>116±3</td>
<td>15.9±4.5</td>
<td>4.0±2.3</td>
</tr>
<tr>
<td>14,000</td>
<td>1-4</td>
<td>102±3</td>
<td>14.7±6.4</td>
<td>3.7±3.2</td>
</tr>
<tr>
<td>11,000</td>
<td>1-5</td>
<td>87±4</td>
<td>12.8±6.7</td>
<td>3.0±2.8</td>
</tr>
<tr>
<td>8,000</td>
<td>1-5</td>
<td>68±4</td>
<td>10.4±5.1</td>
<td>2.0±1.6</td>
</tr>
</tbody>
</table>

Columns 1 and 3 report the area and the height of the dorsal opening thereby giving an estimate on staging (see Table 5.1). Column 2 reports the number of embryos used to calculate statistics due to variability in staging. Column 4 presents the maximum roll-off angle $\psi$ measured at the symmetry point for the given area of the dorsal opening in Column 1. Column 5 is the corresponding percentage of length distortion calculated from Equation (4.2) using angles from Column 4 as closure progresses.

yz-plane). Here, the “eye” is the microscope. As the $y$-value increases we observe more roll-off in the amnioserosa tissue due to the overall curvature of the embryo. We can quantify this by measuring a roll-off angle $\psi(|y|)$ as indicated in the cartoon.

The triangle inset of Figure 4.3B summarizes the adverse effect of roll-off on projection. Given some roll-off angle $\psi$ we see the original length, given by $l$, gives a projected length of $l'$. A simple error comparison of these values will give the distortion percentage:

$$\%\text{Distortion} = \frac{l - l'}{l} \times 100\% \quad (4.1)$$

$$= (1 - \cos \psi) \times 100\% \quad (4.2)$$

Figure 4.3B shows the results of quantifying the distortion percentage as a function of $y$, the distance to the AP-axis. This data was taken at various stages of closure and at three transverse slices for each embryo. The first transverse slice was taken along the symmetry points given by the two purse strings. The other two slices were
taken 50 microns to the anterior and to the posterior of the first cross section. For lengths exceeding 40 microns of the AP-axis we can see length distortions along the y-axis fall between a $\sim 2 - 8\%$ effect.

Table 4.1 reports the stage dependence (in decrements of 3000 $\mu m^2$) for the maximum length distortion of a cell in the periphery averaged over each embryo. At early stages (largest areas) the Table shows the largest measured roll-off angles. As closure progresses, these angles decrease and the dorsal surface is well approximated by a plane. The largest distortions that occur are near the periphery and centered about the symmetry points and result in an $\sim 8\%$ distortion for the earliest confocal images. This worst case 8% is reduced to only 5% in approximately 15 minutes and
again only affects cells that are further than 40 microns from the anteroposterior axis.

An analogous analysis was performed in the orthogonal direction, assessing the distortion introduced due to roll-off into the canthus regions, the results are presented in Figure 4.4. Length distortions along the AP-axis are smaller than those discussed previously along the LR-axis. For distances within 80 microns of the symmetry line the distortions are less than one percent. The maximum distortions occur at distances larger than 80 microns from the symmetry line, near either canthus, with the worst distortion measured around 4%.

### 4.2 Segmentation

Following the Mask-Projection algorithm, the projected images were processed with a two-step segmentation method. The first step was a semiautomated, user-guided method to seed the second step, a fully-automated process for segmenting confocal images. In practice, the segmentation of a time series of images crucially depended on enumerating and locating the cell edges in the initial frame. In this initial frame, faint cell edges were enhanced by hand and then a customized seed algorithm replaced these edges with snake points on a cell-by-cell basis. The algorithm ensured consistency of snake points along common edges between neighboring cells. While user intervention for approximately two hundred cells in the initial image during the first step is tedious, providing an accurate seeding to the automated segmentation algorithm of the second step brings a huge return in the segmentation process.

Before we proceed, let us review some terminology. In our application, segmentation refers to the process by which pixels within a confocal image are assigned to biological structures within that image, i.e., assigned to amnioserosa cell edges or interiors, or excluding other biological structures such as the leading edge cells. A seed is an initial starting point for an algorithm, i.e., an educated guess. The algorithm
we implemented to segment amnioserosa cells required a seed segmentation, which was then iteratively refined to produce a final segmentation. With the exception of the initial image in a time series of confocal images, the final segmentation of the preceding image served as the seed segmentation for the subsequent image. However, the segmentation of the initial image required an independent process to create its initial guess. This computational process is what we refer to as the initial seeding process and is outlined by the green box, labeled the “Seed Segmentation of First Image,” in the flowchart of Figure 4.5. The computational process that iteratively segments the time series of confocal images is outlined in the red box in the flow chart and is labeled “Active Contour Segmentation.” In the following, we first discuss the active contour method, which in general segmented an image from a seed segmentation obtained from the previous active contour result, and then return to the special case of the first confocal image.

**4.2.1 Active Contour Segmentation**

Our active contour segmentation is enclosed by the red box in Figure 4.5 and was a modification and extension of the active contour method ‘snakes’ [Kass et al., 1988]. We extended the method to simultaneously handle multiple cells in a single confocal image and, in doing so, also introduced a hierarchical structure which kept track of nearest neighbors for the cells. Additionally, we changed the minimization procedure taken by snakes, which will be described as a point for comparison.

Snakes detects image edges by energy minimization. Different energy functions will emphasize different features of an image. The energy we calculated for a “snake” was a single loop of elastic springs over a potential landscape that corresponds to the inverse of the fluorescence intensity of a confocal image. The energy of a single snake is given by:
Figure 4.5: Flowchart of the segmentation algorithm. Steps A-G are the Seed Segmentation of the First Image of the confocal stack using a method which mimics heat flow. Step A: a threshold intensity is chosen so that most cells form closed loops of fluorescence. Steps B and C: perpendicular initial conditions are established independently so that heat can flow in Step D. Steps E-G: cells are defined by the intersections of common temperature regions from both heat flows. The periphery of these pixel sets is the seed snake for Step H. Steps H-M are the Active Contour Segmentation method for the entire stack. Step H: each snake contour has an energy that must be minimized before it becomes representative of the confocal image. We implement a Monte Carlo scheme to minimize these energies. Step I: prior steps dealt only with cells individually, here we combine the information by introducing knowledge of the tissue. Steps J and K: calculate geometrical values for each cell, determine cells that are too small (nearly completely ingressed) and are no longer tracked, and update nearest-neighbor assignments. Steps L and M: These steps set up the next iteration of the active contour segmentation.
\[ E_{\text{snake}} = \sum E_{\text{image}} + E_{\text{elastic}} \]
\[ = \sum_{i=0}^{N} k_{\text{image}} I(x_i, y_i)^{-1} + \sum_{i=0}^{N} k_{\text{elastic}} (\sqrt{(x_i - x_{i+1})^2 + (y_i - y_{i+1})^2} - l)^2 \]

Here, \( N \) is the total number of snake points that define the snake, \( x_i \) and \( y_i \) indicate the cartesian coordinates for the \( i^{th} \) snake point, and \( l \) is a rest length for the elastic springs. The ratio of the prefactors \( k_{\text{image}} \) and \( k_{\text{elastic}} \) determines the relative "importance" of the image intensity versus the spread of the snake points. Energy minimization then drives the linked snake points to seek out paths of bright fluorescence that corresponded to wells in the inverse intensity landscape, i.e., the DE-Cadherin fluorescence in the apical surface of each cell and evenly spaced snake points.

An oscillating cell can take on a larger area from one frame to the next. If this area is too large it is likely the snake ring will find the local energy minimum where all the snake points are in one location (snake collapse). It is therefore important to have a sufficiently small time step in between frames when using an active-contour method to track the movement of cell boundaries in time. In the case of an expanding cell, the snake ring may actually need to find a local minimum energy that might be larger than its current energy. In these cases the implicit Euler method implemented by the traditional snakes will likely converge on the wrong minimum (usually snake collapse). Consequently, we chose to take an alternative approach to energy minimization and implement a Monte Carlo method (see, for example, Pang [1997]) in lieu of an implicit Euler method. In our Monte Carlo scheme (Step H in Figure 4.5), a snake searches energy configurations that are both larger and smaller than its current energy, i.e., its xy snake points undergo a random walk, and accepts these configuration with a tunable probability. In practice, this flexibility, as well as
guiding the random walk of snake points, helps the ring of snake points to stay in the (correct) local minimum instead of collapsing to an erroneous absolute minimum.

As always with a Monte Carlo method, sampling is an important factor in the accuracy and the speed of convergence of the algorithm. We designed our sampling method to be well-matched to our overall goal, i.e., snaking each cell of the amnioserosa. The task of sampling in our problem was embodied in the random walks of the individual snake points that were guided by gradients of Equation (4.4), the energy function, and a hierarchial knowledge of the tissue. More specifically, for nearest-neighbor cells that are relatively small or skinny, the random walk was biased to avoid erroneously stepping from the edge of the cell of interest to a non-common edge of that neighboring cell. Additionally, for cells with relatively sharp edges or relatively small apical areas, the step size of the random walk was reduced to avoid both erroneous rounded edges or the erroneous collapse of a snake. The direction and magnitude of the step for an individual snake point was partially determined by the gradient of Equation (4.4) where choice of the random step led some snake points to step in the opposite direction of energy gradient and with some additional angular variability to this direction.

Following the Monte Carlo minimization, which was applied to every cell being snaked, the next step in the active contour method was to factor in the reality that these cells constituted amnioserosa tissue. To carry out this hierarchical step, all of the segmented cells were refined by pairwise comparison of neighboring cells. More specifically, in step I of the flowchart the common boundaries between two cells were averaged to ensure consistency, resulting in the final segmentation of this confocal image. This hierarchical step substantially improved our modified active contour method and resulted in a successful segmentation of the dorsal opening with cellular resolution.

Following the final segmentation of one confocal image, the results were saved,
cell areas were calculated, and preparations were made for the segmentation of the subsequent image (Flowchart, steps J-L). Preparations included determining nearest neighbors, calculating aspect ratios, etc., and providing the final segmentation of the current image as the seed segmentation for the subsequent image.

### 4.2.2 Seed Segmentation of the First Image

To create the seed for the first image, we developed a segmentation method that mimicked heat flow. This method is outlined in the green box in the flowchart in Figure 4.5. While this is an artificial application of heat flow in the sense that there is no applied temperature gradient across the amnioserosa tissue that results in heat flow, this abstraction resulted in an effective algorithm that generated a seed segmentation for the first confocal image. We first guide the reader through an intuitive picture of how we use heat flow to segment an image and follow it with the numerical details.

**Intuitive Description of the Seed Segmentation**

There are multiple steps to the seed segmentation algorithm. Step $A$ in the flowchart summarizes a survey of each pixel in the confocal image and, based on its intensity value, assigned it to be insulating or conducting. We chose a threshold such that bright cell boundaries were insulators and the dark centers of cells were able to conduct heat. Figure 4.6 visualizes the key steps in this algorithm, where the confocal image to be segmented is presented in the left image of the bottom row and the progression of the seed segmentation for two cells is tracked from left to right as heat flow is stepped through 1 to 50 to 350 iterations. The challenge was to pick an image intensity threshold such that the majority of the cells were enclosed by a continuous insulating boundary, i.e., a continuous loop of DE-Cadherin, without sweeping in too much fluorescence background from the cell interior. A well-imaged
cell with a continuous insulating boundary is referred to as closed cell. However, imperfections in the confocal images resulted in partially imaged cells with gaps in the DE-Cadherin loops that we interpret to be ‘thermal’ leaks between neighboring cells. We refer to such cells as open cells. To visualize these steps, consider the images in the left-most column of Figure 4.6. The survey of the confocal image (lower frame) results in the assignment of the insulating boundaries, as shown as white pixels in the middle frame (repeated in the top frame). The choice of threshold produced closed and open cells. For example, track the labeled open (red square) and closed (blue square) cells in the lower confocal image to the middle (or top) frame.

The next step in the seed segmentation was to establish two independent temperature distributions, steps B and C in the flowchart, and visualized by the top and middle images of the first column in Figure 4.6. One linear temperature distribution was along the AP axis (top image) and the other was along the LR axis (middle image). First let’s consider the AP direction. Having established a temperature for every conducting pixel (top contour plot of the left most row), we let heat flow through the conducting pixels based on the difference in temperatures for adjacent pixels, as visualized by moving from left to right through the images of the top row of Figure 4.6. We then repeated this analysis for the LR temperature difference as summarized in the middle row of Figure 4.6. Note that before heat flows, the temperature contours are straight lines, perpendicular to the direction of the temperature gradient, and present no information about cell shapes. The user tracks the iterations of heat flow until the cell edges begin to affect the temperature distribution within a cell, as can be seen in the middle and right contour plots of Figure 4.6, where previously straight contours now bend under the influence of their insulating boundaries.

It is informative to consider how this abstract heat flows in closed and open cells. In a closed system, with a non-uniform temperature distribution, heat will flow from
Figure 4.6: Seed segmentation algorithm. Left to right indicates the number of iterations of heat flow. The bottom row updates the segmentation of an open (red) and a closed (blue) cell, superimposed on a confocal image. The top row presents temperature contours in the assessment of an initial A-P temperature gradient, where contour lines are separated by 5 degrees and for presentation purposes the regions between the contour lines have been filled in with the average temperature values. The middle row presents an assessment of an initial L-R temperature gradient. False colors indicate temperature in degrees Celsius. Temperatures are considered to be common if they are within a user-defined temperature range, typically 5-15 degrees Celsius. Typical data sets imaged the entire dorsal opening, where 4-8 patches of roughly 20-50 cells were processed sequentially with the net result being the segmentation of all amnioserosa cells in the confocal image. The reference to a region of interest in the flow chart of Figure (4.5) refers to a patch of cells. This figure has been cropped for presentation purposes and therefore includes partial cells. The actual temperature distributions have been, in effect, digitized due to their presentation as a contour plot.
hot to cold regions until an equilibrium temperature is reached. During this analysis, a closed cell will be thermally isolated from its neighbors and approach its equilibrium temperature (Figure 4.6, final contour plots of the cell indicated by the blue cell). In contrast, an open cell with a small gap in its insulating boundary (Figure 4.6, red cell), however, will conduct heat along this gap with a neighboring cell as the two cells approach equilibrium. Since the gap is small, the time course to equilibrium typically is slower than what we observe in closed cells. The key is through user intervention to only let heat flow until a signature of the cell shape becomes apparent in the temperature distribution, which occurs before reaching thermal equilibrium as will be explained below. The net result is a “thermal draft” between the two open cells (Figure 4.6, red cell and its upper left neighbor).

Step $E$ in the flowchart brings together the until now independent assessments of heat flow resulting from the initial temperature distributions in the AP (upper row) and LR (middle row) assessments. As can be seen from inspection of Figure 4.6, in isolation both the AP and the LR analyses result in multiple cells having similar temperatures, e.g., the two orange cells in the middle row after 350 iterations. By combining the AP and LR assessments, we can straightforwardly segment the cells. The red and blue colors superimposed on the confocal images in the bottom row of Figure 4.6 represent the pixels corresponding to the intersection of two finite ranges of temperature (one from AP and one from LR), where the range is centered on different temperature values for each cell and each assessment. As one would expect, the intersection of the LR and AP assessments in the left most image of the bottom row is square since the boundaries of a cell have had insufficient time to influence the temperature distribution within a cell. As the number of iterations increase, these regions of intersection grow to fill the cell interior and the periphery of this region approximates the cell shape. The red cell highlights a strength of using this heat flow method. The gap in the open cell is characterized by an increase in
the density of contour lines, allowing the two open cells to be distinguished in this seed segmentation. The initial segmentation of open cells has proven to be accurate enough to be an effective seed for further analysis by our active contour method.

**Numerical Description of the Seed Segmentation**

This section describes the numerical methods we implemented to solve the time-dependent heat equation with multiple insulating boundaries. As discussed above, we take a confocal image and assign pixels as conducting or insulating based on their intensity values. We then establish two separate initial temperature distributions which we march in time to approach, but not reach, equilibrium.

The time-dependent heat equation is given by:

$$ \frac{\partial u}{\partial t} = k (\nabla^2 u) $$  \hspace{1cm} \text{(4.5)}

Where $u(x, y, t)$ is the temperature distribution and $k$ the thermal diffusivity, here $k$ is taken to be constant in space and time. This equation, in conjunction with initial conditions and boundary conditions, would then constitute a problem in heat flow (see for example Haberman [2004]). Since we are interested in a two-dimensional flow on a cartesian grid, $\nabla^2 u$ in Equation (4.5) can be rewritten to yield:

$$ \frac{\partial u}{\partial t} = k \left( \frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} \right) $$  \hspace{1cm} \text{(4.6)}

We solve this system by implementing a finite difference method. First, we discretized space to fall on a grid. Under this scheme the continuous variables $x$ and $y$ become $x_i = i\Delta x$ and $y_j = j\Delta y$. Since we work with pixels it makes sense to choose a square grid, with $\Delta x = \Delta y$. Finally we seek to solve the problem of heat flow iteratively by marching time forward along discrete intervals, so we take $t_n = n\Delta t$. Under this scheme, the function $u(x, y, t)$ becomes $u(x_i, y_j, t_n)$, or in short hand $u_{i,j}^n$.  

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Using a forward-time, centered-space difference equation, Equation (4.6) becomes:

\[
\frac{u_{i,j}^{n+1} - u_{i,j}^n}{\Delta t} = \frac{k}{(\Delta x)^2} \left[ u_{i+1,j}^n + u_{i-1,j}^n + u_{i,j+1}^n + u_{i,j-1}^n - 4u_{i,j}^n \right] \tag{4.7}
\]

While our grid spacing is determined by pixel separation, our time step \( \Delta t \) is free for us to determine. As reviewed in Haberman [2004], we can scale the time variable in this abstract temperature distribution to the diffusivity of the material, i.e., \( \Delta t = \frac{(\Delta x)^2}{4k} \). This fortuitous substitution results not only in a stable method for solving the heat flow, but also simplifies Equation (4.7). By substituting in the scaled \( \Delta t \) and simplifying, we obtain:

\[
u_{i,j}^{n+1} = \frac{1}{4} \left[ u_{i+1,j}^n + u_{i-1,j}^n + u_{i,j+1}^n + u_{i,j-1}^n \right]
\tag{4.8}
\]

We recognize Equation (4.8) as the Gauss-Seidel method, thus the forward time centered space difference equation of the time-dependent heat equation became equivalent to an iterative method for solving the steady-state heat equation. Since we merely wanted to mimic heat flow, we numerically solve the approach to equilibrium using a point successive over-relaxation method described in detail in Haberman [2004], Burden and Faires [2001].

\[
u_{i,j}^{n+1} = (1 - \omega) u_{i,j} + \omega \frac{1}{4} \left[ u_{i+1,j}^n + u_{i-1,j}^n + u_{i,j+1}^n + u_{i,j-1}^n \right]
\tag{4.9}
\]

Here \( \omega \) is the relaxation parameter which typically is around a value of 1, but can range from 0 to 2. Adjusting the relaxation parameter can speed up the approach to equilibrium, thus making the algorithm more efficient. However, in our case we needed a balance between speed but also not allowing the system to reach equilibrium. User intervention at this step was taken to allow time to increment only as long as necessary to achieve a seed segmentation of the initial confocal image.
Our approach takes the pixels of the image and assigns them to be insulators or conductors. We then incrementally scan through the grid of pixels through the variables \((i, j)\), if a pixel is surrounded by four conducting pixels we can simply apply Equation (4.9). If, however, a neighboring pixel is insulating we have to apply a special boundary condition. In our study, insulating pixels do not have a temperature assigned to them and they do not allow heat to flow through them. This means that if we are at a conducting pixel \((i, j)\) and our neighbor \((i + 1, j)\) is an insulator, there is no \(u_{i+1,j}^n\) to use in Equation (4.9) and we need to ensure there is no heat flow between pixel \((i, j)\) and \((i + 1, j)\). To solve this problem we apply Fourier’s law of heat conduction, which relates the flow of heat \(\vec{\phi}\) to the temperature gradient at these points [Haberman, 2004]:

\[
\vec{\phi} = -K_0 \nabla u
\]  \hspace{1cm} (4.10)

Here \(K_0\) is the thermal conductivity.

Ensuring no heat flow between neighboring pixels is equivalent to requiring the derivative of the temperature between the two pixels to be identically zero. For a conducting pixel \((i, j)\) and only one insulating neighbor \((i + 1, j)\), we need to address the ‘x’ component of heat flow from Equation (4.10). Using a first order difference equation for the gradient along the x-direction we obtain:

\[
\phi_x = 0 = -\frac{K_0}{\Delta x} \left( u_{i+1,j}^n - u_{i,j}^n \right)
\]  \hspace{1cm} (4.11)

Which then gives the result:

\[
u_{i+1,j}^n = u_{i,j}^n
\]  \hspace{1cm} (4.12)

This result yields a second equation to substitute in for the insulating pixel \((i + 1, j)\) when solving Equation (4.9) for pixel \((i, j)\). This approach is commonly called a ‘Ghost Cell’ method and can repeated for any number of neighboring insulating...
pixels to revise Equation (4.9). Any pixel in the image has exactly four neighbors. Thus in practice, the Ghost Cell method was applied up to a maximum of four times for a pixel with insulating neighbor pixel(s).

4.3 Results of the Segmentation Method

We applied the segmentation method to the five confocal stacks of embryos undergoing dorsal closure. We reliably segmented about 80% of the cells identified in the seed segmentation of the initial confocal image, which corresponds to greater than ~90 percent of the initial area of the dorsal opening. More specifically, the seed segmentation of five embryos identified 815 cells and 170 of those cells (about one-fifth) were not successfully tracked by the active contour segmentation during the full time course of cell ingression. The data collected from these cells could be used in some, but not all, of the analyses that follow. Figure 4.7 shows the result of our segmentation method applied to a single embryo at three different times during closure. The pink false color is the result from the segmentation method superimposed on the corresponding inverted confocal image. In most cases the difference is indistinguishable. The cell with an asterisk in panel A reveals the nature of error introduced with our segmentation method. In this specific case, a cell boundary has been erroneously smoothed by the method. Another example where we miss the full cell is indicated by the arrow in the same panel. Occasionally, a neighboring ingressing cell or other image imperfections can lead to erroneous snake points (typically distances of several microns for durations of several minutes). Less frequently, we see false edges due to strong image imperfections. Typically the cells with errors were near one or both of the leading edges. We attribute the inability to track these cells to the lack of neighboring amnioserosa cells, oblong apical cross sections, and small apical areas.

Thus in practice, about four-fifths of the cells are reliably segmented until the final stages of cell ingression when contrast between the fluorescing cell edge and the cell
Figure 4.7: Results of the segmentation method. A-C: Segmented cells are shown in pink with black borders superimposed on an inverted confocal image. Typically the difference between the confocal image and the snake points is indistinguishable. The asterisk indicates a cell with a boundary that has been erroneously smoothed during segmentation. The arrow points out a missed portion of a cell. The scale bar is 50 microns.

The subsequent analyses of the segmented images were designed to be insensitive to the limitations of the segmentation process. Consequently, in the following analyses we report the results of those 645 cells (unless otherwise specified) that were reliably segmented in five embryos.

4.4 Quantifying Cell-Shape Changes

The segmentation process digitized cell shapes and determined their locations within the dorsal opening as a function of time providing the basis for further analyses. From the segmentation method, a cell’s periphery was represented by a set of ordered
\((x, y)\) pairs, typically containing 150 – 200 points. These \((x, y)\) pairs were used to numerically calculate the area and the centroid of the cell. The area of the cell was calculated by splitting the cell into triangles. A central point (\textit{not the centroid}) was estimated by simply taking the average value of the \((x, y)\) pairs and served as a common vertex for each of the triangles. Each triangle was then defined by sequential \((x, y)\) pairs and the common vertex. The area of an individual triangle is given by half the magnitude of the cross product of the two vectors that define its sides.

\[
\Delta_{\text{area},j} = \frac{1}{2} |\mathbf{x}_j \times \mathbf{x}_{j+1}| \\
= \frac{1}{2} |x_j||x_{j+1}| \sin(\phi_{j,j+1})
\]

(4.13a)

Here \(\mathbf{x}_j, \mathbf{x}_{j+1}\) are two neighboring \((x, y)\) pairs and \(\phi_{j,j+1}\) is the signed angle between them. These \((x, y)\) pairs were then converted to polar coordinates \((r_j, \theta_j)\) with an origin at the common vertex so that the total area of the cell was calculated from repeated application of Equation (4.13b) which iterated through the triangles.

\[
a_i \equiv a_{\text{cell}} \\
= \sum \Delta_{\text{area},j} \\
= \sum \frac{1}{2} r_j r_{j+1} \sin(\phi_{j,j+1})
\]

(4.14)

(4.15)

(4.16)

Where it is assumed the sum loops over all triangles and thus all neighboring \((r_j, \theta_j)\) pairs including the first and last point as a pair. While establishing an interior point as the common vertex was mathematically unnecessary for Equation (4.16), it numerically improved the calculation by avoiding the subtraction of otherwise similar valued \(\theta_j\)'s that could occur for an arbitrary exterior point.

The calculation of the centroid was based on Equations (1.8a) and (1.8b) but instead of being applied to the dorsal opening they were applied to individual cells.
This calculation was performed by using the trapezoidal rule for numerical integration [Burden and Faires, 2001]. Cell centroids in combination with apical areas guided studies of spatial correlations between nearest-neighbor cells and served as the foundation for investigating regional correlations within the dorsal opening. Thus giving both the area of a cell ‘i’ at a specific time, \( a_i(t) \), and its centroid, \( c_i(t) \).

4.4.1 Silent Cell-Shape Changes

Simplifying the cell-shape changes by only considering area misses shape changes that are not accompanied by an area change. Consider two idealized, sequential cell-shapes separated by a time \( \Delta t \) (Figure 4.8A and B). Calculating the area change between panels A and B we find there is no area change; however, there is a shape change that is “silent” to our area analysis. Since ultimately we attribute a cell-shape change to a spatially averaged “net” force, this indicates that our analysis of shape changes and net force is an approximation in a systematic manner. To estimate the percentage of silent area changes, we introduced two separate calculations:

\[
\Delta a = \int_0^{2\pi} \frac{r(\theta, t + \Delta t)^2}{2} - \frac{r(\theta, t)^2}{2} d\theta \quad (4.17a)
\]

\[
\delta a = \int_0^{2\pi} \left| \frac{r(\theta, t + \Delta t)^2}{2} - \frac{r(\theta, t)^2}{2} \right| d\theta \quad (4.17b)
\]

Here \( r(\theta, t) \) tracks the cell perimeter, where the \((x, y)\) pairs were converted to polar coordinates centered on the centroid at that time (this ignores cell translation). Equation (4.17a) is the change in area from one time to another time \( \Delta t \) later, i.e., the area of the blue cell in panel B less the area of the red cell in panel A. In contrast, Equation (4.17b) calculates the area that is defined by the union of the two shapes less their common intersection, i.e., the sum of the red and blue regions in panel C. The ratio of the absolute value of Equation (4.17a) to Equation (4.17b) estimates the percentage of cell shape changes that we account for:
Figure 4.8: A cartoon illustrating silent-shape changes. 

A: Cartoon of a circular cell at time $t$. B: The same cell shown at a later time $t + \Delta t$. Cell periphery is expressed as a function of $\theta$. Total area change is the difference between the red and blue regions. C: Silent-area changes, i.e., the union minus the intersection of the two concentric cell shapes. Here, the total silent-area changes is the sum of the red and blue regions.

$$R(t, \Delta t) = \left| \frac{\Delta a}{\partial a} \right|$$

(4.18)

Averaging over time (data not shown), we estimate that we are accounting for roughly half the cell-shape changes when we consider area.
Figure 5.1 summarizes several key features of the analyses used to assess the experimental images. The segmentation of three confocal images from one embryo is shown in Figure 5.1A-C. The cell edges determined by the projection and segmentation algorithms (see Chapter 4) are shown in black and have been superimposed on three inverted confocal images at 0s, 4745s, and 9490s. While there are some inaccuracies, to a large extent the segmented cell edges cannot be distinguished from the fluorescent cell edges of the inverted confocal image. Based on this segmentation, it was straightforward to calculate the cross-sectional apical area of each cell and to define cell location by its centroid, and then to track these values in time. Three illustrative cells are highlighted in red, green, and blue, where each completed ingress at about 3400s, 8100s, and 13,000s, respectively.

Figure 5.1D-H introduces methods of signal analysis as applied to the apical area for each segmented cell, which is a starting point for much of the analyses that will be described in the following chapters. Figure 5.1D presents the apical cross-sectional area, \( a_i(t) \), for the red, green and blue cells highlighted in the left column of that figure. Here \( i \) is an index that labels each amnioserosa cell in the first confocal image.
FIGURE 5.1: Cell oscillations and ingression. A-C: Inverted confocal images of dorsal closure with the results of the segmentation superimposed in a semitransparent pink with black borders. The amnioserosa cells within the dorsal opening are prominent, surrounded by two less prominent flanks of lateral epidermis. The two purse strings do not fluoresce intensely but lie near the edge of amnioserosa tissue. The two canthi are particularly evident in Panels B and C, less so in Panel A where zipping has yet to be fully engaged. Three amnioserosa cells have been highlighted in blue, green, or red for tracking purposes. The scale bar is 50 µm. D: Plots of the apical cross sectional area for the blue, green, and red cells highlighting variability in the oscillations and the ingression process. E-H: Separation of each data trace presented in panel D into its low-frequency $a_{low,i}$ (E) and high-frequency $a_{high,i}$ (F-H) contributions. Panel E also presents the fitted ingression function $I_i(t)$ (dashed curves) for each data trace.
Each of these three cells exhibits area traces that are characterized by oscillations on top of a background function. For just under half of the amnioserosa cells that we investigated, this background is shoulder shaped as exhibited by the three cells in Figure 5.1. The backgrounds are not always shoulder shaped, however, as will be considered in more detail in Chapter 6 and some additional examples will be presented in Chapter 7. As a first step in separating the oscillations from the background, the data traces in Figure 5.1D were processed with a low-pass filter or a high-pass filter. Figure 5.1E presents the low-frequency components, which isolates the background function to a large degree. Figure 5.1F-H presents the high-frequency components, which isolate the oscillations. This filtering process is represented mathematically by the expression:

\[ a_i(t) = a_{low,i} + a_{high,i} \]  

(5.1)

where \( a_i(t) \) is the area of a cell ‘i,’ \( a_{low,i}(t) \) is the low-frequency component and \( a_{high,i}(t) \) is the high-frequency component. The low-pass filter was a finite impulse response filter based on a Bohman window (301 points), implemented with MATLAB’s ‘filtfilt’ function using an optimum cutoff frequency of 1.1 mHz. The high-frequency residuals were obtained by applying the complementary high-pass filter, also based on a Bohman window. Each data trace in panel E also shows a dashed line, i.e., a generic shoulder shape that will be discussed in detail in Chapter 6. Furthermore, the area traces in panels F-H exhibit a non-trivial frequency distribution, which will be discussed in depth in Chapter 7.

5.1 Geometric and Kinematic Measures of Amnioserosa Cells

We investigated five embryos, summarized in Table 5.1, which are listed in decreasing order of the area of the dorsal opening as determined from the initial confocal image
Table 5.1: Geometric and kinematic characterization of the dorsal opening and amnioserosa cells. Column 1 identifies the embryo. Columns 2-4 report the areas of the dorsal opening and the amnioserosa cells (mean and range). The imaging time is reported in column 5, where each embryo was tracked until the end stages of closure. Columns 6 and 7 report the maximum constriction rate (mean and range) for the ingression process. Columns 8 and 9 report the linear rates and corresponding $r^2$ values for completing the ingression process. A constant of proportionality relating the area of the dorsal opening to its principle axes for the dorsal opening (see text) and the respective $r^2$ value are presented in columns 10 and 11. Typically statistics were determined from 201, 182, 144, 162 and 126 cells (totalling 815 cells) for the five embryos. However, statistics for the maximum constriction rate were restricted to a subset of the segmented cells and were determined from 166, 147, 123, 115, and 94 cells (totalling 645 cells).

(column 2). The mean area and the minimum and maximum observed cell areas, respectively, are presented in the Table under the category “Initial Area” and then the subcategory “Individual Cells.” The initial areas of amnioserosa cells typically (average of five embryos) ranged from 10 to 330 $\mu m^2$ with an average around 120 $\mu m^2$. There were concentrations of small cells near the canthi, and narrow cells along the periphery, while the majority of the large cells were concentrated in the interior of the dorsal opening (See Figure 5.1 A-C). We started imaging each of the five embryos at early-to-mid stages of dorsal closure and followed them until nearly the end of closure. Imaging for Embryos 4 and 5 began at a slightly later stage relative to Embryos 1-3. Comparing the values for the average cell sizes (column 3) reveals a stage dependence that will be discussed in detail in Chapter 6.
Figure 5.1D shows the apical area as a function of time for three illustrative cells during closure, whose low-frequency contribution $a_{low,i}(t)$ is shown in panel E. Each of these cells exhibits a relatively rapid rate of apical constriction, where inspection of the dashed lines in Figure 5.1E indicates the maximal rate occurs near 3500s, 5000s, and 8000s for the red, green, and blue cells, respectively. Table 5.1 reports calculations of the maximum constriction rates for all 645 successfully segmented amnioserosa cells. Column 6 lists the mean values, and the minimum and maximum observed values are listed in column 7. Regardless of the staging of the embryos, we find a similar distribution of maximum constriction rates. Typical values ranged from the slowest at $0.3 \mu m^2/minute$ to the most rapid at $9.5 \mu m^2/minute$, with an average of $1.6\pm0.3 \mu m^2/minute$. The calculation underlying this analysis essentially is fitting a line through the inflection point. The inflection point was determined from the dashed lines in Figure 5.1E. The method for determining the dashed lines is described in detail in Chapter 6. The duration of the linear fit was determined on a cell-by-cell basis. For example, the duration of the red cell corresponded to the difference in the times at which the cell reached 90% and 10% of its initial apical area, for the green cell the 75% and 25% values were used, and for a cell with a linear background (not shown in Figure 5.1) the total imaging time was used.

The rate at which amnioserosa cells completed the ingestion process is remarkably constant for each embryo (presented towards the right in Table 5.1). Although there is some embryo-to-embryo variability, the $r^2$ value for the linear fit of individual embryos each exceed 0.99. Completing the ingestion process was determined numerically by the segmentation algorithm, i.e., when a cell can no longer be followed due to a vanishing apical area. Thus the completion of the ingestion process is a discrete event in time for each amnioserosa cell. Given these values, it was straightforward to calculate the rate at which cells completed ingestion. On average, we found a linear loss of cells during dorsal closure. Column 8 of Table 5.1 summarizes the rate
Figure 5.2: Schematic illustrating the semimajor and minor axes of the dorsal opening. Variables \( h \) and \( w \) define the half-height and the half-width of the dorsal opening. Vertically oriented curved lines represent segment boundaries of the lateral epidermis. Compare with Figure 1.3

at which cells ingress, where we find an average value to be \( 0.8 \pm 0.2 \) cells/min.

5.2 Area Proportionality Constant

Next we considered how the area of the dorsal opening is related to the height and width of the dorsal opening, and find a linear relationship. We find the area of the dorsal opening \( A(t) \) is proportional to the product of the height \( h(t) \) (half of the maximum as calculated from leading edge to leading edge) and the width \( w(t) \) (half of the canthus-to-canthus distance), i.e., \( A(t) \propto h(t)w(t) \). Figure 5.2 is a cartoon of the dorsal opening with the two parameters \( h \) and \( w \) illustrated by arrows. The experimental value of the proportionality constant is \( 2.76 \pm 0.09 \) (average of the next to last column of Table 5.1) and can be calculated theoretically using an extension of the setting-sun model, an idealization of the geometry of the dorsal opening as introduced by Hutson et al. [2003] and discussed in Chapter 1. This calculation yields the relationship \( A = 2.70 \pm 0.05hw \), and is derived in the following.

5.2.1 Area Proportionality Constant in the Setting-Sun Model

Here we derive an equation expressing the area of the dorsal opening in terms of the canthus-to-canthus distance \( W \) and, in the perpendicular direction, the distance of the maximum opening \( H \) (compare conventions in Figure 5.2 and 1.3). This setting-
sun model is an idealization of the dorsal opening and has been shown to be a good approximation for the mid-to-late stages of closure [Hutson et al., 2003, Peralta et al., 2007, 2008]. The dorsal opening is defined by the area between two intersecting circular arcs as shown in Figure 1.3. The circles are taken to have the same radii denoted by the parameter \( r \). Within the setting-sun model, the progression of dorsal closure in time corresponds to pulling the centers of the two circles apart at a constant rate to reduce the area of the dorsal opening while maintaining the curvature \( (\kappa = 1/r) \) of the arcs. Here we define the half width, \( w \equiv \frac{W}{2} \), and half height, \( h \equiv \frac{H}{2} \), which are the semimajor and semiminor axes of the eye-shaped dorsal opening. The angle \( \theta \) is swept out from the symmetry axis to one of the canthi. For this geometry the following relationships hold:

\[
\begin{align*}
    h &= r(1 - \cos \theta) \quad (5.2a) \\
    w &= r \sin \theta \quad (5.2b)
\end{align*}
\]

It is straightforward to first calculate one-half of the dorsal opening as indicated by the shading in Figure 1.3. The area of the “piece of the pie” swept out by \( 2\theta \) is \( \theta r^2 \). Subtracting the area of the underlying triangle results in the shaded region. Multiplying by two, we get the expression for the area \( A \) of the entire dorsal opening:

\[
A = 2 \left[ \theta r^2 - \frac{1}{2}(2w)(r - h) \right] \quad (5.3)
\]

Solving Equations (5.2a) and (5.2b) for \( r \), we then make judicious substitutions into Equation (5.3). More specifically, there are two terms in the square brackets in Equation (5.3). For the first term, there are two powers of \( r \), where one is replaced by the quantity that follows from Equation (5.2a) and the other is replaced by the quantity that follows from Equation (5.2b). Completing the substitutions, we get:
\[ A = 2 \left[ \theta \left( \frac{w}{\sin \theta} \right) \left( \frac{h}{1 - \cos \theta} \right) - (w) \left( \frac{h}{1 - \cos \theta} - h \right) \right] \quad (5.4) \]

\[ = 2 \left[ \frac{\theta - \sin \theta \cos \theta}{\sin \theta(1 - \cos \theta)} \right] \quad (5.5) \]

For the values of \( \theta \) that correspond to dorsal closure (approximately \( \pi/4 \) to 0), the terms preceding \( hw \) on the right hand side of Equation (5.5) have values that range from 2.67 to 2.75. These values vary by less than two-percent of the average value 2.70, which was calculated by taking the integral of the terms in the square brackets. This two-percent variability is less than the biological variability calculated from the standard deviation of the second-to-last column in Table 5.1. Thus Equation (5.5), as applied to dorsal closure, implies that the area \( A \) is linearly related to the product \( hw \) to a very good approximation. Thus the theoretical value (2.70) for the constant of proportionality compares favorably with the experimental value 2.76 ± 0.09.

The underlying mathematics that leads to this result can be appreciated by expanding the quantity \( A/hw \) in the variable \( \theta \).

\[ \frac{A}{hw} = 2 \left[ \frac{\theta - \sin \theta \cos \theta}{\sin \theta(1 - \cos \theta)} \right] \quad (5.6) \]

\[ = \frac{8}{3} + \frac{2}{15} \theta^2 + O(\theta^4) \quad (5.7) \]

In the expansion, the term that is linear in \( \theta \) cancels, leaving a \( \frac{2}{15} \theta^2 \) as the first correction to 8/3=2.67. Typical experimental values for \( \theta \) ranged from \( \pi/4 \) to 0 during dorsal closure, thus the largest correction to 8/3 during closure is 0.08 at \( \theta = \pi/4 \).
Empirical Model for Cell Ingression

While the amnioserosa is a transient tissue and ultimately all the amnioserosa cells ingress and undergo apoptosis, there is more than one process for ingestion. For example, during the zipping process, amnioserosa cells are internalized below the seams by one ingestion process. Furthermore, during the mid-to-late stages of dorsal closure about ten percent of the amnioserosa cells are internalized via apoptosis, a second ingestion process. Here we define a geometric signature for ingestion based on the background function $a_{\text{low},i}(t)$ as shown with dashed lines in Figure 5.1E. Common characteristics of this background are the onset of a sudden decrease in cell area, followed by an interval of persistent area loss, until the segmentation algorithm can no longer track the vanishing area of the cell. To visualize the onset of an ingestion process, consider the red, green, and blue cells tracked in Figure 5.1D and analyzed in terms of $a_{\text{low},i}(t)$ in Figure 5.1E. Assessing these traces from late to early times, it is clear that all three cells complete ingestion and have an interval of persistent loss of apical area. The red cell exhibits the clearest onset of an ingestion process, preceded by an interval of a nearly horizontal plateau (Figure 5.1E). The expectation is that the transition from the plateau interval to the interval of rapid
decrease in area is mediated by as yet unknown biochemical and/or biomechanical processes. This general description of the apical cross-section of amnioserosa cells exhibiting oscillations about a shoulder-shaped function, as exemplified by the red, green, and blue cells in Figure 5.1, applies to just under half of the 815 segmented cells.

6.1 Empirical Model

A significant fraction of the segmented cells exhibited both similar and variable characteristics, as summarized by the three cells in Figure 5.1, the cell in Figure 7.2, and the two cells in Figure 7.4. We surveyed shape changes from the early-to-late stages of dorsal closure for all the successfully segmented cells from five embryos. The area of the apical cross-section for many, but not all, of the cells suggested possible common characteristics. First, there were complicated oscillations which proved difficult to assess by eye. Second, there was a prominent shoulder function; however, there was some variability in both the initial area of the shoulder and the rate of ingression. Furthermore, the amplitude of the oscillations seemed to be correlated to the shoulder. To quantitatively assess these preliminary characteristics, we applied an empirical model based on a mathematical ingression function to the $a_{low, i}(t)$ of our data sets.

Key to this analysis is the choice for the ingression function. The generic shape for the experimental data shown in Figure 5.1E is a plateau that falls off to zero, i.e., a shoulder. Thus we will need a function that falls from high to low, a parameter that specifies the value of the plateau area, and a parameter that specifies how rapidly the area falls off. We can build $I_i(t)$ from a familiar S-shaped curve by inverting and scaling its area and, in time, scaling how rapidly and when it ingresses. There are numerous S-shaped functions that could be used as the basis for $I_i(t)$, including a complementary error function, a hyperbolic tangent function, an inverse tangent
function, a logistic growth function (e.g., Spiegel and Liu [1999], McQuarrie [2003], Abramowitz and Stegun [1964]) or the Hill equation (e.g., Phillips et al. [2009]). We considered each of these functions and found that they produce essentially equivalent fits to the transition region (data not shown). While there were distinctions between the alternative S-shaped functions at times well before and well after the transition region, the differences were small relative to the low-frequency residual not captured by the analysis (see section 6.4). We have chosen to use the hyperbolic tangent function, which in the past has been interpreted as a generic switch function, as the basis for $I_i(t)$ as shown in the equation below

$$I_i(t) = \frac{\alpha_i}{2} [1 - \tanh(\epsilon_i t - \tau_i)]$$  \hspace{1cm} (6.1)

Here $\alpha_i$ scales the area of the initial plateau and the transition from the plateau to complete ingression is determined by the expression in the square brackets that specifies the time of the inflection point and the rate of the transition. More specifically, $\tau_i/\epsilon_i$ specifies the time of the inflection point. Furthermore, the value for the maximum rate of $\frac{dI_i}{dt}$ is $-\epsilon_i \alpha_i/2$. We fit Equation (6.1) to the $a_{\text{low},i}(t)$ using MATLAB’s ‘nlinfit,’ a nonlinear least squares routine. To simplify notation, we suppress the index ‘$i$’ when discussing the parameters $\alpha$, $\epsilon$, $\tau$. As will be discussed in Chapter 7 (in particular, see Figure 7.2C) an alternative analysis of the data supports the symmetry of this ingression function about it’s inflection point.

6.2 Quality Function $\gamma$

Before discussing the application of $I_i(t)$ to the data sets, let us return to the issue of the 815 cells identified by the seed segmentation and track them through the subsequent analyses. Four-fifths of the cells (645 cells) were fully segmented, i.e., from the first confocal image through completion of a cell’s ingression process or until
the final confocal image, but the applicability of $I_i(t)$ to these cells was variable. More specifically, about one-fourth of the total cells (195 cells) were analyzed by $I_i(t)$ and produced reliable fitting parameters ($\alpha$, $\epsilon$, $\tau$; as determined by a non-linear regression) that enabled detailed comparisons between these cells. In contrast, another fourth of the cells (192 cells) had data traces that were inconsistent with the motivation that led to $I_i(t)$, i.e., these cells exhibit oscillations about a deceasing linear background but exhibited neither an area plateau nor a distinctive transition. While there are parameters that could reproduce the linear background, it is not clear that would be a meaningful application of $I_i(t)$. Finally, almost a third of the cells (258 cells) were, in a sense, in between the two previously identified classes of cells. More specifically, these cells also did not satisfy the analytical criteria to produce statistically reliable results for all three of the fitting parameters to warrant detailed comparisons in further analyses; however, to varying degrees cells in this category were consistent with the motivation for $I_i(t)$. In addition, we found that about one-fifth of the cells (170 cells) identified in the seed segmentation could not be reliably tracked by the active contour segmentation process. As mentioned in Chapter 4, these cells were small, oblong, and near one or both leading edges, i.e., a combination of biological, imaging, and numerical issues limited the extent to which these cells could be further analyzed. In particular, while it was possible to determine where and when these cells completed ingression, it was not possible to apply $I_i(t)$ to these cells. In the following, we supplement this intuitive introduction with a more quantitative classification scheme.

We developed a quantitative method to identify the cells that produce reliable fitting parameters when modelled with $I_i(t)$. To appreciate the exclusion criteria, it is instructive to consider a specific cell type. Some cells in the final class described in the previous paragraph show a clear transition but do not exhibit an extended plateau. Given the unavoidable low-frequency residual (discussed in section 6.4),
either a limited plateau or the absence of a plateau exacerbates the uncertainty in the results produced by the non-linear regression. Thus while this type of cell is consistent with the motivation for an ingression function, the application of \( I_i(t) \) to this data set results in unacceptably large uncertainties for some or all of the fitting parameters. However, calculation of centroid location, maximum rates of transition, and/or initial apical areas were reliably determined since they were insensitive to uncertainties of \( \alpha, \epsilon, \) and \( \tau \). We introduced a goodness of fit parameter, \( \gamma \), to sort the cells based on the following correlation. We found that the duration of the plateau is inversely correlated to the uncertainty in the parameters. Thus we define \( \gamma \) for each cell by:

\[
\gamma = \frac{\text{ingression duration}}{\text{observation time}}
\]  

(6.2)

where the ingression duration is the time it took a cell to constrict from 90% to 10% of its initial apical area as defined by the fitted function \( I_i(t) \). \( \gamma \) is calculated for each cell, but for notational simplicity the index has been suppressed. The observation time is the experimental duration that a cell was tracked in the active segmentation algorithm. The values of \( \gamma \) ranged from 0.1 to 8.6, with an average of 0.89 ± 0.53. \( \gamma \) can be greater than 1 when the duration of an ingression process as determined by the fitted \( I_i(t) \) exceeds the experimental observation time. We found that cells with \( \gamma \leq 2/3 \) produced reliable fitting parameters, cells with a \( \gamma > 1 \) were inconsistent with the \( I_i(t) \) analysis, and that cells with \( 2/3 < \gamma \leq 1 \) fell in between, where some cells were consistent with the \( I_i(t) \) analysis, but produced unacceptably large uncertainties in the fitting parameters and other cells were inconsistent with the analysis.

Figure 6.1 presents a false-color categorization of \( \gamma \) values for each amnioserosa cell of the five embryos. First, each embryo was assessed at its initial confocal image.
While cells with common $\gamma$ values cluster in individual embryos, we discern no overall pattern. Second, to investigate a possible stage dependence we also assessed each embryo when the area of its dorsal opening corresponded to that of Embryo 5 (see Table 5.1), the latest staged embryo (data not shown). The overall distribution of $\gamma$ values degraded for the embryos at later stages, consistent with the cells on average being at more advanced stages of ingression with reduced durations for their measured plateaus.
6.3 Summary of the Application of the Model to the Data Sets

Care was taken to track the reliability of the parameter fits on a cell-by-cell basis. We found: 1) about one-fourth of the total cells (195 cells, 23±9%) produced reliable fitting parameters (\(\alpha, \epsilon\) and \(\tau\)) to Equation (6.1) that allowed for more sophisticated analytical comparisons. Examples include the red and blue cells of Figure 5.1, the cell in Figure 7.2 and the cell in Figure 7.4B. Figure 6.2 is a histogram of the fitting parameters for all the cells with \(\gamma < 2/3\). The majority of values were contained in the ranges as follows: 50 \(\mu m^2 \leq \alpha \leq 250 \mu m^2\), 0.2 \(mHz \leq \epsilon \leq 4.0 mHz\), and 2 \(\leq \tau \leq 8\). 2) At the other extreme, a fourth of the cells (192 cells, 24 ± 6%) did not fit the generic description of oscillations about a shoulder (more appropriately characterized as oscillations about a linear background). An example is the cell shown in Figure 7.4A. 3) The remaining third of the cells (258 cells, 32 ± 4%) exhibited oscillations, but their backgrounds were neither linear, as for cells of class two, nor the shoulder as for cells of class one. These cells are more aptly described as exhibiting backgrounds that fall in between the first and second classes, with the numerical consequence that fitting these cells with \(I_i(t)\) resulted in unacceptably large uncertainties in the parameter fits. An example is the green cell in Figure 5.1. For cells in class one (\(\sim 25\%\) of the 815 total cells), \(a_{low,i}(t)\) are reliably quantified by \(I_i(t)\) that results in Equation (6.4). Cells from classes one, two and three (\(\sim 80\%\) of the 815 total cells) will be quantified by additional analyses techniques in Chapters 7 and 8 that are not based on the parameters \(\alpha, \epsilon\) and \(\tau\).

6.4 Uncertainties in the Ingression Process for Amnioserosa Cells

In practice, there were two main sources of uncertainty that contributed to the approximate nature of the application of the ingression function \(I_i(t)\) to the data sets. The first was attributable to a low-frequency residual and the second was
Figure 6.2: Values for $\alpha$, $\epsilon$, $\tau$ for cells with $\gamma < 2/3$. A: Typical distribution of $\alpha$, the plateau area, of the cells. Here each embryo is represented by a single color. B: Typical distribution of $\epsilon$ a transition rate parameter. C: Typical values for $\tau$ a dimensionless delay parameter.

summarized in our discussion of the quality factor gamma. These were the principle sources of systematic error in $I_i(t)$.

We applied frequency-domain techniques to 645 cells ($\sim$ 80% of the total cells), producing reliable results, in contrast to the more stringent reliability requirements associated with the $I_i(t)$ analyses. We took a Fourier transform of the area as a function of time, and the analysis that follows is based on the amplitude information in the frequency domain and thus the phase information has not been presented.

One key to the approximate nature of the signal analysis is the choice of the cut-
Figure 6.3: Signal analysis for a relatively fast ingressing cell. Raw data (black) in the A: time and D: Frequency domains, low-frequency signal (blue) and high-frequency signal (red) in the B: time and E: frequency domains. $I_i(t)$ (magenta) and low-frequency residual (green) in the C: time and F: frequency domains, where the low pass (blue) traces have been reproduced for comparison purposes. The insets in the right column are log-linear presentations.

off frequency that parameterizes low-pass and high-pass filters to identify the low- and high-frequency contributions. The challenge inherent in this choice of parameter is evident by comparing the blue and magenta traces in panels C and F of Figures 6.3-6.5, where the signature for a hyperbolic tangent is dominant until about 1 mHz. After reviewing the Fourier transforms for all cells, we chose a value of 1.1 mHz as the optimum cut-off frequency for all the data sets. The separation of a data set into a low-frequency contribution (blue) and a high-frequency contribution (red) is shown.
Figure 6.4: Signal analysis for a relatively slow and relatively large ingressing cell. See legend to Figure 6.3 for details.

in panel B of Figures 6.3-6.5, where the blue trace was then used to determine $I_i(t)$. However, this analysis did not fully resolve the ingression function from all of the oscillations as is explicitly revealed in panel C of Figures 6.3-6.5, where inspection of $I_i(t)$ (magenta) and $a_{\text{low},i}$ (blue) traces reveals a low-frequency residual (green trace) that is not present on the magenta trace. This contribution from the low frequency residual is inherently unresolvable and, in particular, it is not a limitation due to the choice of parameter per se as there is no value for the cut-off frequency that will fully resolve $I_i(t)$ from the oscillations. Consequently, this unresolvable low-frequency residual was dropped in the analysis, which typically introduced an error of 2-to-10 percent. Panel F of Figures 6.3-6.5 summarizes how the failure to fully resolve
Figure 6.5: Signal analysis for a relatively slow and a relatively intermediately sized, ingressing cell. See legend to Figure 6.3 for details.

the low-frequency residual (the green trace) compares with the ingression function (magenta trace) in frequency space, where we benefit greatly from its prominent, distinctive pattern as can be readily seen in the frequency domain. Thus we can make the exact assignment, followed by the approximation:

\[ a_{low,i}(t) = I_i(t) + \text{residual} \]  \hspace{1cm} (6.3)
\[ \approx I_i(t) \]  \hspace{1cm} (6.4)

In practice, this final step introduced an error of 2-to-10 percent and as such \( I_i(t) \) is a very good approximation for \( a_{low,i} \) for 195 of the cells that constitute class one.
We now present a biophysical model to investigate how the active contractile forces, passive elastic restoring forces, and the viscous drag are related to cell-area changes. To carry out this analysis, we return our attention to \( a_i(t) \) with recognition of the results of Chapters 5 and 6 that highlighted the contributions of both high-frequency oscillations \( a_{\text{high},i}(t) \) and a low-frequency contribution \( a_{\text{low},i}(t) \) that is dominated by an ingress function \( I_i(t) \).

In Chapter 2 we reviewed the key proteins present in the amnioserosa that are responsible for force production and cell-cell adhesion. The actomyosin cytoskeleton within the amnioserosa cells (subapical cortical ring and the apical-medial network) is well approximated as a two-dimensional surface in the dorsal opening. Both the subapical cortical ring and the apical-medial network are contractile and, in addition, likely have elastic properties. The subapical cortical ring likely produces a tension that acts along the boundary of a cell. The apical-medial network has been observed to assemble and disassemble, resulting in the formation of transient chords which extend across the apical cap of the cell and produce a force that acts on adherens junctions. The resultant motions are thus likely to be similar to anisotropic
longitudinal waves in the dorsal surface that are overdamped.

Contrast in our images is due to GFP-DE-Cadherin and we do not resolve individual DE-Cadherin proteins. In fact, we resolve fluorescent boundaries and have partial information about local strain. We can track displacements of this boundary but cannot resolve any flow of material within this boundary. Since we can only track resolvable displacements, we model the radial components of the net forces acting on the DE-cadherin belts, where the tangential components do not result in area changes. We return to this issue in Chapter 10. Thus we consider radial displacements of the boundary in a two dimensional model.

Our picture is that of a cell membrane that is subject to applied forces and bound by fluids on both sides. In the context of an oscillating boundary of a container of viscous fluid, Reynolds number is defined as $R = \frac{\omega a l}{\nu}$, where $\omega$ is the angular frequency of oscillation, $a$ the amplitude of oscillation, and $l$ the dimension of the oscillating body. From our data set we see oscillations of frequencies in the millihertz range, and amplitudes and body dimensions on the order of 10 microns. We therefore estimate that we have a Reynolds number of approximately $10^{-6}$, indeed justifying that inertial terms are negligible compared to our viscous terms.

Consider an infinitesimal segment of the DE-Cadherin belt and associated plasma membrane of a cell. The model we propose includes elastic forces due to the membrane and surrounding actomyosin network that we quantify with Hooke’s Law. Since we have a moving boundary with fluid on either side, we model the damping forces due to viscosity in the form of Stoke’s Law. We apply Newton’s second law to our infinitesimal segment of boundary and obtain the following equation:

$$\lambda \ddot{r}(\theta, t) ds = -kr(\theta, t) - b\dot{r}(\theta, t) + f(\theta, t)$$

(7.1)

Here $\lambda$ is a line density, $k$ is Hooke’s constant, and $b\dot{r}(\theta, t)$ the viscous drag. $f(\theta, t)$ is
the net, time-dependent radial driving force acting on the boundary that arises from forces that are both internal to the cell and from a neighboring cell. Applying the low-Reynolds number constraint and solving for $b\dot{r}(\theta, t)$, Equation (7.1) becomes:

$$b\dot{r}(\theta, t) = -kr(\theta, t) + f(\theta, t)$$  \hspace{1cm} (7.2)

Integrating this equation along the boundary we find:

$$\int b\dot{r}(\theta, t)ds = \int -kr(\theta, t)ds + \int f(\theta, t)ds$$  \hspace{1cm} (7.3)

The arc length $ds$ in polar coordinates is given by:

$$ds = r\sqrt{1 + \left(\frac{dr}{d\theta}\right)^2} d\theta$$  \hspace{1cm} (7.4)

Since most of our cell shapes are polygons (with only small variability along their edges) we assume that the derivative of the radial coordinate with respect to angle is small compared the radius. This will certainly be true when the cell is large, but will introduce error as the cell shrinks. We thus take $ds \approx rd\theta$. This results in the following integral around the periphery of the cell:

$$\int b\dot{r}rd\theta = \int -kr^2d\theta + \int f(\theta, t)rd\theta$$  \hspace{1cm} (7.5)

We exchange the order of differentiation and integration in the term on the left-hand-side, and noting that $b$ and $k$ are constants, we pull them out of the integrals:

$$\frac{b}{dt} \int \frac{1}{2} r^2 d\theta = -k \int r^2 d\theta + \int f(\theta, t)rd\theta$$  \hspace{1cm} (7.6)

We then point out that the integrals for both the damping and the elasticity will be proportional to the area of the cell and write the following definitions:
Here $b_{\text{eff}} \ddot{a}(t)$, $k_{\text{eff}}a(t)$, and $f_{\text{eff}}(t)$ are each spatially averaged, generalized forces. Making these substitutions into Equation (7.6) we arrive at the following:

$$b_{\text{eff}} \frac{d}{dt} \dot{a}(t) = -k_{\text{eff}}a(t) + f_{\text{eff}}(t)$$ (7.8)

We recognize this equation to be that of a first-order linear differential equation with an inhomogeneous driving term $f_{\text{eff}}(t)$ that is of the same mathematical form as that of a damped, driven harmonic oscillator in the limit of low-Reynolds number. We call the inhomogeneous driving term a force because it results from many subcellular forces acting along the periphery of a cell. For the remainder of this chapter we call $b_{\text{eff}} = b$, and $f_{\text{eff}} = f$.

We have measured the apical cross-sectional area $a_i(t)$ (Figure 7.1A), which integrates cell-shape changes around the periphery of a cell. A force $f_i(t)$ integrates the active forces acting on the periphery, i.e., it is a generalized force that spatially averages the intracellular and extracellular forces that act on the apical belt of adherens junctions to cause a change in cell area. Thus this spatially-averaged dynamic model is well matched to the experimentally determined kinematic values for $a_i(t)$.

We continue from our result above to write an inhomogeneous, first-order linear differential equation describing the time dependence of the areas $a_i(t)$ as shown in Equation (7.9).
\[ b \frac{da_i(t)}{dt} = f_i(t) - k_{eff,i} a_i(t) \] (7.9)

The term on the left-hand side of Equation (7.9) accounts for viscous drag. The first term on the right-hand side is a net force that accounts for all of the active contractile forces that result in area change. The second term on the right-hand side acts like a passive elastic restoring force, where \( k_{eff,i} \) is an effective Hookian force constant. This first order linear differential equation models the tissue as a viscoelastic medium, characterized by \( k_{eff,i} \) and the damping coefficient \( b \), subject to the active forces \( f_i(t) \), i.e., the driving force of this system at low Reynolds number. Here \( k_{eff,i} \) is the elastic component of the viscoelastic tissue. As will be shown below, this treatment proves adequate for investigating the frequency dependence of \( f_i(t) \) in a viscoelastic tissue at low Reynolds number.

We evaluate the Fourier transform of Equation (7.9) and obtain the following expression for the Fourier amplitudes:

\[ |\tilde{a}_i(\omega)| = \left[ \frac{1}{b \sqrt{\left( \frac{k_{eff,i}}{b} \right)^2 + \omega^2}} \right] |\tilde{f}_i(\omega)| \] (7.10)

Where the tildes denote a Fourier transform. The expression in the square brackets on the right-hand side of the equation is a transfer function that, in Fourier space, relates forces to areas. Thus the transfer function directly accounts for the viscoelastic response of the tissue through \( b, k_{eff,i}, \) and \( \omega \).

The area \( a_i(t) \) for a typical cell is shown in Figure 7.1A, with its corresponding Fourier transform \( |\tilde{a}_i(\omega)| \) in panel B. Typically, a period of an oscillation was sampled by 10-20 data points (see inset to Figure 7.1A), well above the Nyquist limit [Stearns, 2003]. Investigation of the power-law dependence of \( |\tilde{a}_i(\omega)| \) was conducted by calculating the average slope on a log-log scale. Experimentally we find
Figure 7.1: Viscoelastic analysis of cell apical area. A: Plot of $a_i(t)$ for a single cell. The inset highlights the density of the data points. B: The Fourier transform of $a_i(t)$ from Panel A, exhibiting a clear $\omega^{-1}$ dependence. Inset compares the model fit (black) using Equation (7.10) (on a log – log scale) to the data (blue). The calculated, scaled forcing function using Equation (7.15), based on the fitting parameters $k_{eff,i}/b = 5 \times 10^{-5}$mHz and $f_0/b = 0.033\mu m^2/s$. The inset summarizes the effect of varying $k_{eff,i}/b$ from 0 to $4.5 \times 10^{-4}$mHz.

the power law to be $-0.9 \pm 0.3$. Factoring an $\omega$ out of Equation (7.10) provides a theoretical framework for the observed power law:

$$|\tilde{a}_i(\omega)| = \left[ \frac{1}{\omega b \sqrt{(\frac{k_{eff,i}}{b})^2 + 1}} \right] |\tilde{f}_i(\omega)|$$  \hspace{1cm} (7.11)$$

$$\approx \left[ \frac{1}{\omega b} \right] |\tilde{f}_i(\omega)|$$  \hspace{1cm} (7.12)
Equation (7.12) predicts an $\omega^{-1}$ power law for frequencies that satisfy the condition:

$$\left(\frac{k_{eff,i}}{\omega b}\right)^2 \ll 1 \quad (7.13)$$

To estimate the parameter $k_{eff,i}/b$, we first note the approximate linearity of the Fourier transform of the cell on a log-log plot, as shown in the inset of Figure 7.1B. This feature motivated the expression $|\tilde{f}_i(\omega)| = f_{0,i} + f_{1,i}(\omega)$, i.e., identifying a frequency-independent contribution $f_{0,i}$ and a frequency-dependent term $f_{1,i}(\omega)$. Substituting this expression for $|\tilde{f}_i(\omega)|$ in Equation (7.11), we then determined the parameters $f_{0,i}/b$ and $k_{eff,i}/b$. Where we’ve assumed:

$$|\tilde{a}_i(\omega)| \approx \left[\frac{1}{\omega \sqrt{\left(\frac{k_{eff,i}}{\omega b}\right)^2 + 1}}\right] \frac{f_{0,i}}{b} \quad (7.14)$$

Fitting this equation to the 645 cells that were reliably segmented from five embryos, we find the values for $k_{eff,i}/b$ ranged from $\sim 0.01 - 0.1 \text{ mHz}$. In addition, the fitted value for $f_{0}/b$ was $0.03 \pm 0.01 \mu \text{m}^2/\text{s}$. The numerically determined values of $k_{eff,i}/b$ supports the theoretically predicted power law of $\omega^{-1}$ for frequencies larger than $1 \text{ mHz}$. Note that the transfer function is the source of the $\omega^{-1}$ dependence.

Benefitting from the numerical determination of $k_{eff,i}/b$ and $f_{0,i}/b$, we can analytically make the connection between $f_i(t)$ and the experimental data $a_i(t)$, e.g., Figure 7.1A. Scaling $f_i(t)$ by $f_{0,i}$ and solving Equation (7.9) for the scaled force we find:

$$\frac{f_i(t)}{f_{0,i}} = \frac{b}{f_{0,i}} \left(\frac{da_i(t)}{dt} + \frac{k_{eff,i}}{b} a_i(t)\right) \quad (7.15)$$

Figure 7.1C was determined using Equation (7.15). The largest uncertainty in Equation (7.15) is due to $k_{eff,i}/b$, which has its greatest effect at the turning points in
The inset expands the time interval from 90 to 210 seconds to summarize the overall uncertainty in the calculation. Uncertainties in determining values of $k_{\text{eff},i}/b$ introduce uncertainties in magnitude, but, in particular, essentially do not alter the distribution of the Fourier frequencies.

The numerical evaluation of the derivative $da_i/dt$ in Equation (7.15) can be challenging. The inset to Figure 7.1A indicates that the data for $a_i(t)$ is not sparse and the following methods are reliable. To calculate the derivative we implemented an 11-point, time-centered, difference equation. A low-pass filter was applied before the numerical derivative was taken with a cutoff frequency of 17.5 mHz. The calculation of $|\tilde{f}_i(\omega)}/f_{0,i}$ follows from Equation (7.10).

$$\frac{|\tilde{f}_i(\omega)|}{f_{0,i}} = \left[ \frac{b}{f_{0,i}} \sqrt{\left(\frac{k_{\text{eff},i}}{b}\right)^2 + \omega^2} \right] |\hat{a}_i(\omega)|$$ (7.16)

Figure 7.2A reproduces $a_i(t)$ for the rapidly ingressing cell in Figure 7.1A, where the blue trace is the data and the black trace $\bar{a}_i(t)$ is a boxcar travelling average with a width of 1000 seconds to remove high-frequency oscillations. Figure 7.2B is the result of applying Equation (7.15) to the data of Figure 7.2A. This panel presents a good approximation of the active contractile forces that lead to cell-shape changes in a low-Reynolds environment. To isolate the forces that drive ingression from the forces that drive oscillation, the time series of $f_i(t)/f_{0,i}$ shown in Figure 7.2B has been filtered with the same boxcar travelling average that was applied to Figure 7.2A to remove high-frequency (above 1 mHz) oscillations. The result is shown in Figure 7.2C.

It is instructive to compare Figure 7.2, panels A, B and C. While initially there are relatively large amplitude and complicated oscillations in the driving force (Figure 7.2B), the filtered value for $\bar{a}_i(t)$ (Figure 7.2A, black trace) shows little net decrease...
Figure 7.2: Fourier analyses of cell oscillations. A: Plot of the area data for a single amnioserosa cell, where the blue trace is data and the black trace is filtered data (see text). B: Calculated time dependence of net applied forces acting on the cell. C: Filtered data of Panel B (see text). D: Fourier transform of the data trace for $f(t)/f_0$, where the transform was taken for the entire data trace in Panel B. The x-axis reports the frequency of the applied forces and y-axis reports the amplitude of an oscillation as a function of this frequency. E: The average of Fourier transforms for all the segmented amnioserosa cells for one embryo. F: Spectrogram of the same data trace $f(t)/f_0$, i.e., alternative Fourier analysis based on a (1300s duration) travelling window in time. For this panel, the x-axis reports time of dorsal closure, the y-axis reports the frequency of the oscillation, the amplitude is reported in the false color scale (normalized to 1 using maximum value $1.2 \times 10^{-4}$). G: The analogous sum of the spectrograms for all the segmented cells (normalized using maximum value $6.1 \times 10^{-5}$).
in area before ∼ 2800s (indicated by the vertical dashed line). From ∼ 2800 − 6500s, \( \tilde{a}_i(t) \) (Panel A, black trace) indicates a measurable net decrease. Prior to ∼ 6500s, these relatively large amplitude forces promote relatively little if any immediate loss of apical cross section. After ∼ 6500 seconds, inspection of Figure 7.2, panels A and C, indicate a qualitative change, where \( \tilde{a}_i(t) \) and \( \tilde{f}_i(t)/f_{0,i} \) now exhibit systematic decrease. Note the similarity in the functional form of \( \tilde{f}_i(t)/f_{0,i} \) during ∼ 6500−8000s in panel C to that of either the derivative of \( \tilde{a}_i(t) \) in panel A or the derivative of \( I_i(t) \) (see dashed curves, Figure 5.1E). The magnitude of the forces associated with the ingression process (Figure 7.2C, ∼ 6500 − 8000s) are small relative to the amplitudes of the forcing function that yield the oscillations (Figure 7.2B, ∼ 0 − 6500s; note the differences in the scales of the y-axes in panels B and C). Thus the high-frequency oscillating forces correlate with largely reversible changes in \( a_i(t) \), in contrast to the low-frequency forces that correlate with a coherent (systematic) decrease in apical cross section. We will return to this issue in the Discussion section.

The Fourier transform of Figure 7.2B, based on Equation (7.16), is shown in Figure 7.2D. The frequencies of the forcing function that yield the oscillations are most strongly peaked around ∼ 5 − 6 mHz in this cell. Figure 7.2E is the average of all the Fourier transforms of the segmented amnioserosa cells for this embryo and is analogous to panel D in that the transform is for the entire time series. Here we see a more classical band of frequencies for the oscillations with less broadening relative to panel D. Averaging over all the segmented cells for the five embryos, the spectrum of Fourier modes is centered at 5.7 ± 0.9 mHz. Generally there were three features exhibited in all of the amnioserosa cells in each embryo: a uniform background having a value of 1, a rise to larger amplitudes as the frequency approaches zero, and a band of frequencies between ∼ 2.4 and 9.0 mHz. The first two features are attributable to the viscoelastic nature of the tissue and the forces driving the ingression process. The band of frequencies is attributable to the high-frequency oscillations.
While the analyses that led to Figures 7.2D and E were based on the Fourier transforms of the entire time series of Figure 7.2B, we also calculated spectrograms to track changes in the Fourier transform in a travelling window in time as a cell ingressed. A spectrogram results from a sequence of Fourier transforms, each taken in a window that is stepped throughout the duration of the data set [Stearns, 2003]. While typically a Fourier transform is applied to the full time course of the data trace, analysis based on spectrograms can track the time dependence of the frequency domain as one progresses through the data trace. Mathematically we can write this as follows:

\[ S[x(t)](\omega, t_0) \equiv \mathcal{F}[x(t)W(t; t_0)] \]  
(7.17a)

\[ W(t; t_0) \equiv \begin{cases}  
W(t) & : |t - t_0| < T/2 \\
0 & : otherwise 
\end{cases} \]  
(7.17b)

Where \( W(t) \) is your favorite window function, \( t_0 \) is the center of the window and \( T \) its duration in time. To simplify the notation, here we have defined \( \mathcal{F}[f(t)] \) as \( \tilde{f}(\omega) \). The window progresses in time by stepping \( t_0 \) through the duration of the data set. More specifically, we applied a travelling window of duration \( T=1300 \) s, where \( W(t) \) is a Hann window function [Stearns, 2003]. We are interested in calculating the spectrogram of the forcing function. This can be done in two ways: the first, to use Equation (7.15) (where we calculate the numerical derivative of \( a_i(t) \)), followed by windowing and repeated Fourier transforms. The second, to recognize the following:

\[ S[f(t)](\omega, t_0) = \mathcal{F}[f(t)W(t; t_0)] \]
(7.18)

\[ = \mathcal{F}[f(t)] \ast \mathcal{F}[W(t; t_0)] \]  
(7.19)

\[ = \mathcal{F}[f(t)] \ast e^{-i\omega t_0} \mathcal{F}[W(t)] \]  
(7.20)

The second calculation recognizes that multiplication in the time domain is related to
convolution in the frequency domain and that a travelling window can be expressed as a shifted phase. This method avoids taking the Fourier transform of a numerical derivative and produced more reliable results.

Figure 7.2F is the spectrogram of the forcing function, where the frequencies are presented on the y-axis, the amplitude information represented by the false colors, and the x-axis is time. The distribution of frequencies changes as this amnioserosa cell progresses through ingression. Initially frequencies around 5 mHz are strongly peaked until around 6500 seconds, when there is a large increase in amplitudes at lower frequencies from $\sim 7000 – 8000$s. These features result directly from a decrease in the amplitude of oscillations as the ingression process initiates. Figure 7.2G is the average of all the spectrograms for the segmented amnioserosa cells for this embryo, which exhibits two notable features. First, the dominant mode ($\sim 5$ mHz) decays in amplitude steadily in time; however, the reoccurring transient features seen in the spectrograms of single cells (e.g. panel F) have, in effect, been averaged out. Second, as time progresses we see a narrowing of Fourier frequencies to near 5 mHz for this example embryo.

The corresponding analysis for a slowly ingressing cell from the same embryo is presented in Figure 7.3. Here we note that $a_{low,i}(t)$ steadily decreases for this cell. This is a direct consequence of the magnitude and sign of $\tilde{f}_i(t)/f_{0,i}$ (panel C). Both the rapidly ingressing cell (Figure 7.2) and the slowly ingressing cell (Figure 7.3) exhibit complicated oscillations. Similar to the rapidly ingressing cell, $|\tilde{f}_i(\omega)|/f_{0,i}$ (Figure 7.3D) exhibits a band of oscillation amplitudes around $\sim 5 – 7$ mHz. A notable feature that begins to emerge while considering this slowly ingressing cell is the overall trend of the oscillation amplitudes. Panel F is the corresponding spectrogram for this cell, initially ($\sim 0 – 2000$s) oscillations have large amplitudes, as time progresses and the cell reduces in apical area, the oscillation amplitudes decrease (after $\sim 5000$s, panel B and F). The correlation between the oscillation amplitudes
and the average apical area of a cell is developed further in the next section.

### 7.1 Correlation Between Oscillation Amplitude and Apical Area

In addition to investigating the active contractile forces, we searched for correlations between the high-frequency oscillations \( a_{\text{high},i}(t) \) and the low-frequency background \( a_{\text{low},i}(t) \). A feature of those amnioserosa cells that exhibit an ingression process \( I_i(t) \) is that the onset of ingression is preceded by high-frequency oscillations. Furthermore, comparing \( a_{\text{high},i}(t) \) and \( a_{\text{low},i}(t) \) in Figures 5.1E-H, 7.2A, and 7.3A raises the possibility of a correlation between the amplitude of the oscillations and the area of a cell. Figure 7.4 quantifies the relationship between the amplitude of the oscillations
and the area of a cell. Panels A and B show cells with linear ($a_{low,1}(t)$) and shoulder-shaped ($a_{low,2}(t)$) backgrounds, respectively. The corresponding high-frequency oscillations for these cells are shown in panels C ($a_{high,1}(t)$) and D ($a_{high,2}(t)$). The average value $\hat{a}_{low,i}(t)$ and the amplitude envelope $\hat{a}_{high,i}(t)$ are calculated at regular intervals and appear as diamonds in panels A and C and as open circles in panels B and D. Mathematically the method for calculating these averages was similar to performing a spectrogram, Equation (7.17), but without the Fourier transform.

$$\hat{a}_{high,i}(t_0) = \frac{1}{T} \int_{t_0-T/2}^{t_0+T/2} W(t; t_0)|a_{high,i}| \, dt$$ (7.21a)

$$\hat{a}_{low,i}(t_0) = \frac{1}{T} \int_{t_0-T/2}^{t_0+T/2} W(t; t_0)|a_{low,i}| \, dt$$ (7.21b)

Numerical integration was performed using a Composite Simpson’s Method [Burden and Faires, 2001].

Panel E assesses any correlation by plotting $\hat{a}_{low,i}(t)$ versus $\hat{a}_{high,i}(t)$ for all the segmented cells for this embryo. The entire data set exhibits a good linear correlation ($r^2 = 0.74$). There is a knee in the data near the origin; however, the data set is not robust enough for us to reliably interpret this feature. In addition, there may be a tendency for relatively large amnioserosa cells to exhibit disproportionately large deviations from linearity. Nonetheless, it is interesting that cells with widely ranging distributions of both initial areas and maximum constriction rates (whether well described by the ingression function or not) are reasonably approximated to be linearly correlated as shown in Figure 7.4E.

We also have investigated whether any quantifiable feature of the oscillations during the plateau could be correlated with the onset of a rapid decrease in apical area, finding no correlations. In particular, we investigated the distribution of the Fourier frequencies and the time dependence of the Fourier amplitudes and compared these results in some detail to those cells that are most reliably fit with the ingression
Figure 7.4: Oscillation amplitudes correlate with cell areas. Two examples cells are shown in panels A-D, a linearly ingressing cell (A,C) and a relatively rapidly ingressing cell (B,D). Data for all the segmented cells are presented in Panel E. Panel A presents the low-frequency contribution $a_{\text{low},i}$, where average values $\hat{a}_{\text{low}}$ have been superimposed in diamonds. In panel C, the averaging process identifies an envelop $\hat{a}_{\text{high}}$ of the high-frequency oscillations as shown in diamonds. Panels B and D present analogous results for a rapidly ingressing cell, where the superimposed averages appear as circles. E: Plot of envelop of the oscillation amplitude versus average cell area for all 166 cells of one embryo, indicates a good linear correlation ($r^2 = 0.74$). The two cells from panels A-D are shown in green (linearly ingressing cell) and brown (rapidly ingressing cell), respectively, in the false colored regionalization.
function $I_i(t)$. We explicitly considered the effect of the maximum constriction rates and we looked for correlations between neighboring cells. We cannot rule out more subtle correlations that may be drawn out by alternative analyses; however, our techniques should be sensitive to any robust correlation between oscillations and the onset of a rapid decrease in apical area.
Here we characterize the effect of location within the dorsal opening on cell kinematics with the aim of improving our understanding of spatial dependence within the dorsal opening for the biological processes responsible for the dynamics of amnioserosa cells. We quantify non-uniformities in the size distributions of amnioserosa cells, in the times when cells complete ingression, and in the distribution of Fourier frequencies based on a regionalization of the dorsal opening. To do so, we developed a natural coordinate system for regionalizing the dorsal opening.

8.1 Geometry of the Dorsal Opening

8.1.1 Determining the Two Leading Edges

The choice of DE-cadherin complicated the identification of the dorsal opening, which typically is defined by the two circumjacent leading edges of the lateral epidermis. DE-Cadherin provided excellent contrast for the apical edges of the amnioserosa cells in our confocal images. However, DE-Cadherin does not result in robust contrast for the two purse strings, unlike the predominant fluorescence of the two purse strings as we and others have observed when either actin or myosin has been labeled with
fluorescent constructs (Figure 3.2) [Hutson et al., 2003, Franke et al., 2005, Kiehart et al., 2006, Peralta et al., 2007, 2008, Toyama et al., 2008].

We determined the extent of the dorsal opening from the periphery of the segmented amnioserosa cells. As discussed in Chapter 4, we successfully segmented \(\sim 80\%\) of the amnioserosa cells throughout the time-lapsed, confocal images. The remaining \(\sim 20\%\) were partially analyzed when approximating the location of the purse string and typically could be used for the location where these cells completed ingression. The snake points from outermost portions of the cell boundaries were used to define the periphery of the amnioserosa tissue and thus approximated the two leading edges. The snake points were then divided into two sets according to the AP axis. At early stages of dorsal closure each set was fit with a 4th-order polynomial to approximate the two leading edges (two thick black traces, Figure 8.1A). As closure progressed, we found that a 2nd-order polynomial became sufficient to account for each leading edge. The two canthi were determined by the intersections of the two fitted polynomials. As discussed in Chapter 4, most of the cells that were not successfully segmented were near one or both leading edges, which is the major source of uncertainty in this algorithm for determining the geometry of the dorsal opening. Typically these “snake failures” were small, oblong cells with the long axis lying along the periphery (Figure 5.1A-C) and therefore, we estimate the uncertainties to be less than 3 microns for either leading edge and less than 5 microns for either canthus.

8.1.2 Cell Proximity to the Two Leading Edges

Once the two leading edges had been determined, it was straightforward to measure the distance from a cell centroid to either canthus as indicated by \(d_A\) and \(d_P\) in Figure 8.1A for two cells (red, blue). In addition, Figure 8.1A also indicates the minimum distance from the centroid to each leading edge. The minimum distance
Figure 8.1: Distances and regionalization of the dorsal opening. A: Segmented amnioserosa cells shown in grey, the two purse strings shown in thick black, and the two canthi are labelled by arrows. Two example cells (red or blue) highlight distance measurements from each cell centroid to the two canthi (\(d_A, d_P\)) and then the minimum distance to the closest leading edge (\(d_{PS}\)). B: Geometric construction of isocontours to regionalize the dorsal opening. The magenta isocontour is 35 microns from the nearest leading edge and the green isocontours are at values of -1.7, 0, and 1.7. C: Color scheme for regionalizing the dorsal opening.
to the closer leading edge is given by $d_{PS}$. In general, these distances were tracked throughout the confocal images or until a cell ingressed.

### 8.1.3 Sub-Regions of the Dorsal Opening

Measurements of cell distances from the two canthi and the two purse strings lend themselves to a natural geometry for regionalizing the dorsal opening. First, consider the two intersecting magenta traces in Figure 8.1B, which are a constant distance from the leading edges. This pair of magenta traces, and the pair of thick black traces that correspond to the two leading edges, are two isocontours (one pair corresponds to one isocontour) shown in the figure. Three additional isocontours are shown in thin black lines in Figure 8.1B. Now consider a set of green traces, each determined by a constant value for the coordinate $C \equiv \ln(d_A/d_P)$. The mathematical formula depends on the distances from the two canthi $d_A$ and $d_P$ and was borrowed from one of the two coordinates of a bipolar coordinate system [McQuarrie, 2003]. A non-zero value for $C$ corresponds to a circular arc. In contrast, a value of $C = 0$ is realized for the symmetric case $d_A = d_P$, which corresponds to the single dashed green line in Figure 8.1B. Isocontours corresponding to a total of seven values of $C$ are presented in Figure 8.1B, the three green (two thick arcs and one dashed line) and four additional isocontours (black arcs).

Five contours based on this geometrical construction were used to separate the dorsal opening into sub-regions. In practice, one isocontour was determined by taking one-fourth of the value for the maximum distance of the dorsal opening in the LR (vertical in the figure) direction, which defined the magenta isocontour in Figure 8.1B. Then the two intersections of the two magenta traces defined two values for $C$ that determined the solid green isocontours (two thick green arcs). The last isocontour was the symmetric dashed-green line. The thick black, magenta, thick green, and dashed green isocontours shown in Figure 8.1B define the colorized sub-
regions that appear in Figure 8.1C, and in the main text as Figure 7.4E, Figure 8.2A, and Figure 8.3A. The subregions of the dorsal opening were defined using the initial confocal image, false colors were assigned to all the segmented cells in the dorsal opening in this initial image, and then the color-classified cells were tracked throughout all subsequent confocal images.

8.2 Regional Kinematic Effects Within the Dorsal Opening

Figure 8.2A plots the area for each region of the dorsal opening as a function of time, where the color of each trace matches that of the region. At time zero, due to the geometric construction that leads to the regionalization, the areas of the two canthi regions are the smallest, the areas of the two central regions are the largest, and the areas of the four peripheral regions are in between. As closure progresses, there is a decrease in regional areas as the apical cross-sectional area of individual cells decreases until the cell completes the ingression process, which also decrements the number of cells in a region. While individual cells change shape in time and there is junctional remodelling when cells ingress, there is relatively little mobility of amnioserosa cells during dorsal closure. Ideally the regionalization process aimed to produce similar values for symmetric regions (dark blue and brown canthus regions; yellow and green interior regions; and cyan, blue, red, and orange peripheral regions) within the dorsal opening. In practice, however, the classification scheme is based on cell centroids, which introduces some non-ideality in the initial areas as centroids happen to fall on one side or the other of lines of regional demarcation. This effect is particularly evident in the green and yellow interior regions, which include the largest cells of the dorsal opening. Indeed as the size of the cells decreases with time, these two interior regions exhibit more comparable regional areas, starting at $\sim 5000$ s (Figure 8.2A). This suggests that categorizing the regions of cells based on the first confocal image is a reasonable analytical strategy. Based on the same regionalization
Figure 8.2: Regional analysis of dorsal closure. A: Plot of area of each of the regions (as shown in the inset) as a function of time. The eight regions (see inset) fall into three groupings: the canthi (dark blue, brown) regions, the central (yellow, green) regions, and the peripheral (cyan, red, blue, and orange) regions. B: Plots of the number of cells in each region, where an amnioserosa cell is no longer counted once it completes the ingression process. C-E: The average of the Fourier transforms of the forcing function $f(t)/b$, by region for all the segmented amnioserosa cells in one embryo, exhibiting similar groupings as seen in panel A. Regional values for $f_0/b$ in units of $\mu m^2/s$: yellow region: $0.031 \pm 0.006$, green region: $0.038 \pm 0.009$, cyan region: $0.019 \pm 0.005$, red region: $0.019 \pm 0.007$, blue region: $0.025 \pm 0.010$, orange region: $0.021 \pm 0.007$, dark blue region: $0.015 \pm 0.004$ and brown region: $0.009 \pm 0.002$

scheme, we followed the completion of the ingression process by tracking the cells in each region as shown in Figure 8.2B. There are particularly interesting results for the two interior regions. The average initial size of the amnioserosa cells in the posterior (green) interior is $\sim 270 \mu m^2$, while that of the anterior (yellow) is $\sim 200 \mu m^2$. Furthermore, completing the ingression process is substantially delayed
in the two interior regions relative to both the two canthi regions and the four peripheral regions. The traces for the two canthi regions (dark blue and brown) essentially overlap, as do the traces for the four peripheral regions (cyan, red, blue, and orange). The rates of completing the ingression process for the two canthi regions during the time interval $\sim 0 - 5000s$ compares favorably with the rates for the four peripheral regions during the time interval $\sim 7000 - 14,000s$. As for the interior regions, the rate for completing the ingression process compares favorably with that seen in the other six regions after $\sim 7500s$ for the anterior interior (yellow) region and after $\sim 12,500s$ for the posterior interior (green) region. Before $\sim 12,500s$, only one cell in the posterior interior (green) region completed ingression, in contrast to the anterior interior (yellow) region which began to lose cells to ingression at an earlier time. The rates of completing the ingression process in the anterior interior and posterior interior regions were comparable after $\sim 12,500s$, around the time when the number of cells in that region also became similar. This last comparison of the anterior interior and posterior interior regions was common for four out of the five embryos studied.

Finally, we carried out a region-by-region Fourier analysis as shown in Figure 8.2C-E. We calculated the Fourier transform $|\hat{f}(\omega)|/f_0$ using Equation (7.16) for the area as a function of time, $a_i(t)$, for each cell and then averaged the results region by region, i.e., we added the Fourier transforms for cells in the same color category and then divided by the number of cells in that category at the initial time. Panel C-E shows that, when comparing the two interior regions (panel C) to the four peripheral regions (panel D), the distribution of relatively large forces at lower frequencies shift to higher frequencies within the overall band and decrease in amplitude. In addition, the smallest cells are in the two canthus regions (panel A), which have the smallest average Fourier amplitudes (panel E) and the smallest values for $f_0/b$ (listed in the legend to Figure 8.2). Thus we find that the average Fourier amplitude scales with
8.3 Where Amnioserosa Cells Delaminate in the Dorsal Opening

We investigated the locations within the dorsal opening where amnioserosa cells completed the ingression process. Since the time and location for completing ingression are readily determined by the segmentation algorithm, this analysis was based on all of the 815 cells from five embryos. Figure 8.3A-C presents a false color representation based on the regionalization geometry of three confocal images from one embryo at 0s, 4853s, and 9705s. The color of each cell was determined by the region that its centroid resides in for the initial confocal image and did not change for subsequent confocal images. There is a notable loss of cells at the canthi when comparing panel A and B, consistent with the current view that amnioserosa cells can ingress as part of the zipping process. Extending the comparison to panel C reveals that in addition to the complete ingression of all the cells near the two canthi regions, there is a substantial reduction in the number of cells near the periphery and away from the canthi (blue, cyan, red, and orange regions). In contrast, there is markedly less ingression during closure within the interior regions (yellow and green).

To quantify these qualitative observations, we assessed the proximity of a cell completing the ingression process to either purse string (Figure 8.3D) and compared it to the proximity to either canthus (Figure 8.3E). Figure 8.3D is a histogram of the distance to the nearest purse string \(d_{PS}\) for each such cell, the solid black line represents the average over the five embryos. The y-axis presents the normalized fraction of cells completing the ingression process for each embryo and the x-axis is the distance to the nearest purse string. This histogram shows a large clustering of cells completing ingression within 10 microns of the purse string. The cumulative distribution is shown as an inset, where the two dashed lines correspond to a distance of 10 microns and 90%. We find that on average more than 90% of the cells complete
Figure 8.3: Amnioserosa cells also complete ingression along the two leading edges. 
A-C: False-colored regionalization of three inverted confocal images. Color coding of each cell determined during the first frame and maintained throughout dorsal closure. Inspection of the three images indicates that cells in the periphery (brown, blue, cyan, dark blue, red, and orange regions) preferentially complete ingression before interior cells (yellow and green regions). The scale bar is 50 µm. D-E: Quantification of the location of each cell at the conclusion of an ingression process. Panel D presents a histogram of the fraction of cells that completed ingression as a function of distance from the purse string ($d_{PS}$), where the inset integrates these results to show the fraction of cells that have closed within a given distance from the purse string. As guides to the eye, the vertical dashed line indicates the 10 micron value and the horizontal dashed line indicates the 90% value. Panel E then assesses those cells that are located within 10 microns of either purse string and calculates the distance from each of those cells to the nearest canthus ($d_C$ is the minimum of $d_A$ and $d_P$). The inset again integrates these results, where the dashed line indicates the 90% value: the total fraction tends to $\sim 90\%$ since about 10% of the cells ingress in the interior regions. These two panels demonstrate that less than half of the amnioserosa cells close near either canthus as part of the zipping process and, in contrast, more than half of the amnioserosa cells ingress near either purse string, but not within either canthus region.
ingression within 10 microns of a purse string. This region includes both the canthi and the periphery of the dorsal opening. The remaining 10 percent of the cells completed the ingression process within the interior regions.

We then analyzed these 90% of the cells that complete the ingression process in the peripheries (as identified in Figure 8.3D) to distinguish whether the location where a cell completes ingression is near to or far from the two canthi. Figure 8.3E is a histogram of the distance to the nearest canthus (either \( d_A \) or \( d_P \)) when ingression is completed. The cumulative distribution is shown as an inset, where the horizontal line corresponds to 90%. These results indicate that more than half of these cells are located at distances larger than 30 microns from the nearest canthus. For values smaller than 30 microns, the fraction increases. Thus a significant amount of the cells complete ingression near a purse string but away from a canthus. These results should be compared to those of the previous section and Figure 8.2B which quantified the delay in completing the ingression process on a region by region basis. Jointly, these observations suggest the interior cells are gaining proximity to either leading edge as dorsal closure progresses and raises that possibility that the leading edge may signal the ingression process in amnioserosa cells. We will return to this issue in the Discussion section.
Discussion

We have observed the apical area of amnioserosa cells in tissue for five embryos, where confocal imaging commenced during early-to-mid stages of closure and continued to near completion. We confirm previous observations of area pulsations or oscillations and that, within the dorsal opening, areas of peripheral amnioserosa cells, are smaller than the areas of interior cells [Fernández et al., 2007, Gorfinkel et al., 2009, Solon et al., 2009, Blanchard et al., 2010]. We have quantified the kinematics of a substantial fraction of the amnioserosa cells and developed a model to advance our understanding of the dynamics. An enabling step of our research was the segmentation of apical cross sections, quantifying area changes throughout closure. We find that the apical areas exhibit two common kinetic features: complicated oscillations and ingression. We attribute both types of area changes, which occur in the low-Reynolds number environment of amnioserosa tissue, to be a consequence of time-dependent imbalances in the net force acting on the cell peripheries. We account for these observations with a biophysical model that quantifies the (spatially averaged) net force from experimental data and explicitly treats the dynamics of oscillations and ingression. We find that approximately half of the amnioserosa cells
exhibit a loss of apical cross-sectional area dominated by an irreversible ingression process. For these cells, a transition is resolved from largely reversible oscillations to the onset of an ingression process. We attribute cell-to-cell variability in the maximum rate of constriction during the ingression processes to be a consequence of variability in the magnitude of force produced by the cytoskeleton.

A central feature of our time-dependent analyses was to convert changes in two-dimensional cell shape into the area traces $a_i(t)$, e.g., Figure 5.1. While the apical cross section of a cell in amnioserosa tissue can be described as cell-cell edges and multicellular junctions, a full two-dimensional treatment of cell shape changes would require subcellular knowledge of stresses and strains. The analyses reported here are well matched to the optical resolution and image contrast of the experimental measurements and the inaccuracies inherent in the numerical methods. Summarizing a cell shape as an area allows us to connect to a biophysical model that treats a spatially-averaged, time-dependent net force $f_i(t)$. A feature of our model is any time dependence in elasticity (or equivalently compliance) will be accounted for in $f_i(t)$, while spatial variability is treated in the spatial averaging process implicit in $f_i(t)$. In addition, previously it has been shown that treating $b$ as constant is a very good approximation within the dynamic range of our observations (supporting online text, Section II, in Toyama et al. [2008]). Since $f_i(t)$ is a net force, time-dependent changes in $f_i(t)$ are a consequence of changes in intracellular and/or extracellular forces. Our approach is sensitive to forces that result in contractions and dilations, but is insensitive to forces that are not accompanied by area changes. There are “silent forces” that change a cell shape without changing the area of the cell in addition to the forces $f_i(t)$ reported here. We have quantified cell-shape changes and estimate that half of them correspond to area changes (See Section 4.4.1). Thus the forcing function $f_i(t)$ does summarize those net stresses acting along a cell’s apical belt of adherens junctions, where the sum of the associated strains is the area change.
9.1 Oscillations in Apical Cross Sectional Area and Force at Low-Reynolds Number

Our initial interest was motivated by curiosity as to how cells in the low-Reynolds environment of tissue exhibit intrinsic oscillations. Amnioserosa cells exhibit both complicated oscillations in time as quantified in Figure 5.1 and distinctive frequencies of oscillations as quantified in Figures 6.3-6.5. Thus we concentrated on the active nature of amnioserosa cell dynamics, since passive cell oscillations are substantially overdamped in a low-Reynolds number environment. This is reflected in our biophysical model described in Chapter 7 and summarized by Equation (7.9). While we cannot rule out other models that may account for these oscillations, our choice of modelling is based on the simplest phenomenological model that includes passive viscoelasticity of the tissue and other time-dependent forces, including the active driving forces of the cytoskeleton. This model allowed us to approximate, from experimental data, the forces that drive cell area changes. The time dependence of \( f_i(t) \) is shown in Figure 7.2B and the frequency of \( |\tilde{f}_i(\omega)| \) is shown in Figures 7.2D-G and 8.2C-E.

A key feature of our biophysical model is the low-frequency components of forces are more effective at driving cell area changes then high-frequency components. This can be seen by inspection of the transfer function (square brackets) in Equation (7.10). Since \( k_{eff,i}/b \) ranged from \( \sim 0.01 \) to \( 0.1 \) \( mHz \), \( (k_{eff,i}/b\omega)^2 \ll 1 \) for frequencies above \( 1 \) \( mHz \) and therefore \( |\tilde{a}_i(\omega)| \) is well approximated as being proportional to \( |\tilde{f}_i(\omega)|/\omega \) (Equation (7.12)). The physics underlying this \( \omega^{-1} \) dependence is directly attributable to the viscous drag term and thus is likely to be a general characteristic of tissue dynamics and a model independent result. We will return to the issue of the \( \omega^{-1} \) dependence in section 9.2.

A key consequence of estimating \( |\tilde{f}_i(\omega)| \) is the band of relatively high-frequency
oscillations evident in Figure 7.2E. The band is centered at 5.7 mHz, extending from \( \sim 2.4 - 9.0 \) mHz, and highlights the frequency dependence of the forces that, when multiplied by the transfer function, drive the oscillations in area. Our understanding of the mechanism(s) for generating these forces is incomplete, where any consideration must address the contributions of both intracellular and intercellular force generation within a cell and between cells that contribute to the net force \( f_i(t) \). Previously, pulsed oscillations in the millihertz range have been correlated with assembly/disassembly cycles of actin and myosin in the apical medial network [Martin et al., 2008, Blanchard et al., 2010] and are thought to be regulated by the PAR complex [David et al., 2010]. In addition, free calcium can elicit contractions in isolated cytoplasm from single amoeba [Taylor et al., 1973] and it has been proposed that an increase in free calcium concentration can sever actin filaments in the cytoskeleton [Salbreux et al., 2007]. Two of the outstanding questions include: i) what intercellular feedback mechanism accounts for oscillations in the first place given the contractile nature of the actomyosin cytoskeleton in a cell and in each of the neighboring cells, and ii) what aspect of the molecular mechanism leads to the observed band of frequencies for these oscillations. It has been suggested that load dependent detachment rates in the actin-myosin network may be a possible feedback mechanism [Plaçais et al., 2009].

9.2 Cell Ingression

A key feature of our investigation is that about one fourth (195) of the 815 imaged amnioserosa cells are well described by the ingression function \( I_i(t) \) (Equation (6.1)), while another third of the cells are consistent with this description. This shoulder-shaped function likely describes a range of biological processes that result in ingression. These include the ingression of amnioserosa cells near either canthus during the zipping process, the ingression of cells in the peripheral regions of the
dorsal opening, and the ingression of apoptotic cells that preferentially occurs in the anterior two thirds of the dorsal opening. We find a distribution of values for the parameters ($\alpha$, $\epsilon$, $\tau$) that characterize the ingression process, where the maximum constriction rates varied from 0.2 to 13.6 $\mu m^2/min$. An extant question is the degree to which this variability is due to one biological mechanism for force production subject to varying intensities of regulation as compared to enlisting additional cooperative mechanisms, with the net result possibly being a family of ingression processes based on the contractile properties of actin and myosin.

What remains unclear are the mechanism(s) that is responsible for the onset of ingression. At the molecular level, actomyosin complexes are involved in force production for both oscillation and ingression. However, the systematic/coherent ingression process is qualitatively and quantitatively different from the oscillations. While the oscillations lie at relatively high-frequencies ($\sim 2.4$ to $9.0 mHz$), the ingression process is dominated by an order-of-magnitude lower, sub-millihertz frequencies.

An intriguing result, presented in Chapter 7, is the amplitude of forces that drive high-frequency oscillations (Figure 7.2B) in the millihertz range exceeds, by a factor of $\sim 5$, the magnitudes of the forces that drive ingression (Figure 7.2C). To comment further on this observation, we now return to the $\omega^{-1}$ dependence of the transfer function that relates force to area changes as described in the previous section. In essence, biology is benefiting from ten-times more effective coupling between force production and area changes in the ingression process relative to the oscillations due to the $\omega^{-1}$ dependence.

It seems a mechanistic understanding of oscillations, the onset of ingression, and the variable rates of ingression will involve a more complete understanding of actomyosin complexes and their regulation. For example, does the time course of force production resulting in oscillations and then coherent ingression correspond to expanding subcellular regions of persistent force production across the apical surface.
of a cell? Previously it was observed that the average fluorescent levels of myosin progressively increase (both in the apical medial network and along cell junctions) as dorsal closure enters a fast phase [Blanchard et al., 2010]. The intracellular and intercellular (feedback) mechanisms that produce this regulation are poorly understood.

9.3 Regional Dynamics and Regulation

We have observed systematic differences in the area and dynamics of amnioserosa cells as a function of location within the dorsal opening. Our initial confocal image for each embryo found that the largest cells were in the two interior regions (Figure 8.2) and that cell size typically decreased with proximity to either purse string (or canthi, Figure 8.3), consistent with previous reports [Gorfinkiel et al., 2009, Solon et al., 2009]. It may be the case that the smaller cells in the canthi and peripheral regions have progressed into an ingression process prior to our imaging, with adverse consequences for the numerical implementation of any analysis based on an ingression function $I_i(t)$. Figure 8.3D and E demonstrates that cells preferentially complete ingression near either purse string in addition to completing ingression near the two canthi. This correlation suggests the possibility that each leading edge is involved in the regulation of cell ingression, whether by chemical and/or mechanical signalling processes [Peralta et al., 2007, Salbreux et al., 2007, Gorfinkiel et al., 2009]. Previously it has been reported that Dpp (a member of the JNK signaling cascade, which is activated in leading edge cells) can regulate cell-shape changes in both the lateral epidermis and in the amnioserosa [Fernández et al., 2007]. While this apparent regulation promotes the completion of ingression, as seen in Figure 8.3A-C, some cells do persist in the peripheral regions. This raises the possibility that proximity to the leading edge is not the only contributing factor, i.e., there may be variability in the response of amnioserosa cells to the signal(s) that regulate the
onset of ingestion.

The dorsal opening steadily progresses towards closure despite the variability in the details of cellular dynamics that we have described. While cells can transition into a rapid loss of apical cross-sectional area, the amnioserosa stress is relatively homogeneous along each purse string of the two leading edges [Hutson et al., 2003, Peralta et al., 2007, Toyama et al., 2008, Ma et al., 2009]. Several parameters that characterize the overall geometry of the dorsal opening are nearly invariant as closure progresses, including the area proportionality constant (Table 5.1 and Chapter 5.2), and as reported previously $dH/dt$ and the curvature of the two leading edges [Hutson et al., 2003, Peralta et al., 2007]. We propose that the emergence of these invariant parameters may be due to the distribution of rates of ingestion. While Figure 8.2B indicates that there is a regional dependence to the cellular rate of completing ingression, when these rates are summed over all of the regions it results in a constant rate of completing ingestion from mid-to-late stages of dorsal closure (Table 5.1). This implies the inhomogeneities in cellular dynamics are connected to the homogeneity of tissue dynamics via the invariant ingression rate, where causality remains unclear. As a consequence, a temporal averaging of a spatial distribution of local cellular forces produces nearly uniform spatial and temporal tissue stress. These observations are consistent with a previous observation, where a cellular rate of ingestion has been implicated in the upregulation of the stress of the amnioserosa in response to laser perturbation via a cellular apoptotic force [Toyama et al., 2008]. The global regulation of the cellular ingestion rates is an open research question.
Future Directions

There are a number of future directions that directly follow from the research presented in earlier chapters. It would be interesting to perturb the ingression process and the oscillations through the use of mutant phenotypes. These include mutants that affect Dpp signalling (tkv, put, shn), the apoptotic process (AS – p35, AS – grim), and myosin-II contractility (zip/MyoII). In addition, it would be advantageous to have more detailed knowledge of the force field to characterize the stresses and strains at the subcellular level. The aim would be to more fully describe the time-dependent molecular mechanisms for force production and their regulation, including the biological significance associated with the emergence of a band of oscillation frequencies extending from $\sim 2.4 - 9.0 \text{ mHz}$. Here we briefly discuss some follow-up experiments and form some hypotheses.

10.1 Perturbation of the Forcing Function and Ingression Processes with Mutant Embryos

Mutant phenotypes of *Drosophila* enable the investigation of dorsal closure in embryos that selectively lack a gene product. Fernández et al. [2007], Franke et al.
[2005], and Toyama et al. [2008] are three studies that have established the importance of a number of specific mutants to dorsal closure. To extend these studies, we would characterize these mutants and compare them to our analysis of the wild-type fly. First we would explore the role of the Dpp signalling pathway on the dynamics of the ingestion function using the mutants tkv, put, and shn. Second, using AS – p35 and AS – grim, we would explore the effect of apoptosis on oscillations and ingestion. Third, we would investigate the role of myosin-II (or rather the lack of) on oscillations and ingestion using zip/MyoII. Each of these experiments would require the mutant fly line be crossed with a GFP-DE-Cadherin fly line so that amnioserosa cell boundaries could be tracked in conjunction with the mutant phenotype.

Decapentaplegic (Dpp) is a morphogen in Drosophila that is necessary during dorsal closure. There are many mutants that exhibit a disrupted signalling pathway for Dpp, but three mutant phenotypes in particular reach the extended germ band stage and fail during closure. These three mutants, identified in Fernández et al. [2007], include: the thick veins mutant (tkv), punt mutant (put) and the Schnurri (shn). Each of these mutant phenotypes develop normally during embryogenesis but ultimately exhibit defects during germ band retraction or during dorsal closure. Fernández et al. [2007] state that these mutants exhibit oscillations but do not reduce in apical surface area. In the same study, Fernández et al. [2007] allow for a rescue of Dpp signalling in the amnioserosa cells of the tkv mutant and observes restored apical constriction. While providing a qualitative account, they do not quantify the oscillations nor the equivalent of an ingestion function of the amnioserosa cells in these mutants. Their results strongly suggest that Dpp is necessary in the signalling process and/or the cytoskeletal components for the ingestion function. Using similar experiments we could quantify the role of disrupting Dpp in the ingestion function by using the tkv mutant and the perform the corresponding rescue experiment. If
Dpp is necessary for the onset of the ingression process, we would expect to see oscillations on an extended plateau region of the ingression function followed by no net loss of apical area. After rescuing Dpp, we would expect cells initiate their respective ingression processes. Although the tkv mutant does not exhibit reductions of apical areas, it does, along with put and shn, exhibit oscillations. Therefore put and shn are great candidates for studying the roles of the active contractile force in the absence of ingression. Presently we find that oscillation amplitude is correlated to the area of a cell. By inhibiting ingression, we would expect to see the oscillation amplitudes to continue to be large and, in contrast to wild-type, continue to oscillate until failure.

Another research direction would perturb the apoptotic process to investigate the oscillations and the onset of ingression. The apoptotic process is one of at least three ways that a cell can ingress. Previously, Toyama et al. [2008] explored the role of the anti-apoptotic caspace suppressor AS−p35 and pro-apoptotic AS−grim. Where these mutants decrease (increase) the rate of apoptosis, and that this decreased (increased) rate is correlated to the rate of closure. In four out of the five embryos that were studied here, we found an asymmetry in the onset of completing the ingression process between the anterior interior cells from the posterior interior cells. In these cases, the anterior region initially had more cells and began to lose cells to ingression processes earlier than the posterior region. Toyama et al. [2008] determined that apoptosis occurs preferentially in the anterior two thirds of the dorsal opening and that the mutants AS−p35 and AS−grim change the rate at which cells complete ingression via the apoptotic process. Both AS−p35 and AS−grim also promise to provide useful information about the role oscillations play in the ingression process. Toyama et al. [2008] present time sequences of dorsal closure and quantified $H(t)$ and $W(t)$. The rates of change for $H(t)$ and $W(t)$ both correlate with the rate of apoptosis, which might suggest the area constant of proportionality is maintained.
Since this is a time-independent quantity, and the rate of apoptosis is inherently time-dependent, it is likely the manifested changes will occur in the distributions of $\alpha$, $\epsilon$, and $\tau$. Increased rates of apoptosis could result in a shift to larger constriction rates $\alpha\epsilon/2$, as well as a shift to earlier times of ingression controlled by the combination $\tau/\epsilon$.

The last experiment that would involve a mutant phenotype would follow the research done by Franke et al. [2005]. They showed that myosin-II contractility was necessary to drive cell-shape changes in epithelial cells during dorsal closure. Using a transgenic mosaic in the mutant $zip/MyoII$, they imaged individual amnioserosa cells with no myosin-II contractility. These cells appeared as oval-shaped cells surrounded by otherwise wild-type cells, and they persisted until the end stages of closure before they were finally incorporated into a seam. To follow up on this experiment, we would be most interested in some special cases of the mosaic. By observing a single amnioserosa cell without myosin-II contractility surrounded by otherwise wild-type cells, we could isolate the viscoelastic response of a single cell in the absence of active internal forces. We could apply our viscoelastic model to this cell to investigate $|\tilde{f}(\omega)|$ as well as the frequency dependence of the response function. We predict that a cell without contractility would be relatively large and exhibit distinctive area oscillations that were the direct result of an active neighboring cell pulling on the central cell exhibiting passive elasticity. Additionally, we would expect to see the area of the cell remain essentially in the plateau region of the ingression function. A second class of mosaic experiments that would be beneficial to perform would be an otherwise wild-type cell surrounded by multiple cells lacking myosin-II contractility. If signalling of the ingression function is not affected by the compromised contractility of neighboring cells, we would expect this cell to ingress fairly rapidly as the surrounding tissue (non-contractile cells) would be more compliant to stretch. The stress that the central cell would undergo would likely be
absorbed by the neighboring cells that are acting as passive dampening cells. Additionally, if the contractile forcing mechanism is based on mechanical feedback, we would expect this cell to not exhibit oscillations.

10.2 2D Cell-Shape Changes

We currently do not explicitly account for the silent shape changes. A goal of our future research is to quantify all the forces along the periphery of a cell, i.e., $f_i(x, t)$, with subcellular resolution. A key step presented in this dissertation reduced a two-dimensional cell shape to its area, a single scalar value. The area measurement was well matched to the biophysical model, introduced in Chapter 7 and based on a spatial averaging of the forces acting on the cell’s periphery. This is a cell-centric view that does not track the silent cell-shape changes as was discussed in Chapter 4.4.1. The segmentation algorithm, however, fully characterized cell shapes as digitized cell boundaries throughout much of closure before determining its area. This research direction is based on the data set of digitized cell boundaries and extends our study to consider full two-dimensional cell-shape changes.

To extract the two-dimensional force field along cell peripheries, we need detailed knowledge of the localized stresses. Generalizing Equation (7.9) for localized forces along a cell boundary we can write:

$$b \frac{dx}{dt} = \sum_i f_i(x, t)$$

Although other constituent constructions also would be entertained. The left and right panels of Figure 10.1 illustrate the same cell at two different times. The black-boundary cell is at an earlier time than the magenta-boundary cell. This cell exhibits a translation and a complicated shape change. Although we already have the tools to determine the cell centroid, we need to extend our current method or develop new
Figure 10.1: Estimating cell-shape changes using nodes versus snake points. Left and right panels show a central cell and its neighbors at two different times. Black borders indicate the cell boundaries from an earlier time, magenta are the cell boundaries at a later time. Centroids of the black- and magenta-central cell are represented by the blue and red circles respectively. Left: node $B$ slides along snake points which exhibit no stretch. Right: edge $AB$ stretches as nodes $A$ and $B$ become $A'$ and $B'$.

methodology for extracting the explicit mapping of a point along the cell periphery from one time frame to the next.

One future approach for identifying subcellular forces is based on subcellular contrast and therefore requires additional confocal imaging (and potentially a new GFP construct). It would be ideal if we could mark the boundary of a cell with points that are fixed to the cell membrane and that were separated enough so as to be distinguishable. Promising candidates may be photoactivatable dyes or embedding beads into or between the cells. It would be great if a cell node was the result of a specific and persistent localized protein that could be labelled with GFP. This would directly enable the tracking of nodes.

Tracking nodes requires an additional numerical strategy for assessing confocal images of amnioserosa cells. There are numerical limitations in our ability to deter-
mine the velocity of a point along the periphery of a cell introduced by our contrast mechanism and our segmentation scheme. Recall we do not resolve individual DE-Cadherin proteins and instead the fluorescence we observe is the result of many molecules (Figure 2.8). Snake points are not tethered down to nodes and thus are free to rotate along the periphery of a cell from frame to frame and could not be used directly to calculate displacements along the periphery. However, additional code, guided by the initial segmentation, could be designed to track nodes in the confocal images. A careful assessment of cell-edge sliding versus stretching would then be necessary.

Two perspectives for tracking cell-shape changes are indicated in the figure. Here, decimated cartoon snake points on a common edge for the black-boundary cell are indicated. These same snake points are shown in their new locations for the magenta-boundary cell in the same color. The first perspective presented is quantification via snake points which are free to translate along the cell edge (left panel of Figure 10.1). The second perspective tracks nodes and assumes cell edges stretch (right panel of Figure 10.1). Both of these enumeration schemes, i.e., snake points versus nodes, have been chosen to illustrate a possible ambiguity introduced from using DE-Cadherin, that is the issue of sliding versus stretching boundaries.

These are several examples of plausible future research areas that address questions raised in the dissertation. The segmentation of individual amnioserosa cells has in the past proved to be difficult. However, with the development of our current segmentation method we and others have the potential to explore numerous future research directions in addition to those covered here.
Conclusions

We have investigated amnioserosa cell-shape changes in quantitative detail from cellular and tissue perspectives. We developed a novel segmentation method to quantify cell shapes from confocal images collected during dorsal closure. We have described the kinematics of individual cells in terms of oscillations and ingression, the latter of which is the dominant mechanism for loss of apical area in the amnioserosa. We developed an empirical model that quantifies the kinematics of the ingression processes of a substantial fraction of the amnioserosa cells. We found that cells preferentially complete ingression along the periphery of the dorsal opening, amending the standing view. Additionally, we found an invariant property of dorsal closure in the rate at which cells complete ingression as well as an area constant of proportionality describing the geometry of the dorsal opening.

Applying signal analysis techniques, for the first time, to the oscillating amnioserosa cells we uncover elements of the forcing function and have investigated the connection between oscillations and the ingression process. We found that the ingression process dominates the loss of apical area of the amnioserosa cells, while the oscillations remain largely reversible and do not significantly drive net loss of
apical area. Using a biophysical model we estimated the “net” forces that drive area change and find the emergence of a band of oscillation frequencies extending from $\sim 2.4 - 9.0 \text{ mHz}$ and an ingression function which is dominated by frequencies less that one millihertz. We find two strong correlations between the oscillations and ingression. The first is that oscillations always precede an ingression process, and the second that the amplitude of the oscillation is proportional to the average apical area of the cell. We have accounted for these results by applying a classical biophysical model, placing constraints on candidate dynamic mechanisms.

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Note: The original Figures 1, 2, and 3 have been renamed Figures A.1, A.2, and A.3, respectively in this appendix.

**Thermal vapor bubble and pressure dynamics during infrared laser ablation of tissue**

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**Abstract:** Free-electron laser irradiation can superheat tissue water, driving thermal vapor bubbles confined by tissue matrix and leading to mechanical tissue failure (ablation). Acoustic transients propagating from an ablation cavity were recorded with a polarization quadrature, interferometric vibrometer. For 3.0 \( \mu m \) infrared irradiation, the shocklike
transients with peak pressures in the megapascal range indicate amplification due to bubble collapse. In contrast, for 6.45 µm irradiation, elastic transients with peak pressures in the 0.1 MPa range indicate tissue failure during bubble growth.

Water thermodynamics can be an essential contributor to the overall mechanisms of laser-tissue interaction\textsuperscript{1,2} For example, highly focused laser pulses in the near infrared (IR), visible, or near UV have been used to drive plasma formation and cavitation dynamics in water, both in bulk\textsuperscript{3} and as confined by biological matrix.\textsuperscript{4} In addition, mid-IR lasers have been used to superheat tissue water, leading to explosive vaporization, while also heating tissue proteins, leading to reduced collateral thermal injury.\textsuperscript{5,6} Here we report our investigation on pressure dynamics associated with tissue ablation by a tunable mid-IR free-electron laser (FEL).\textsuperscript{7} We find that the pressure transients propagating from the ablation site into the tissue exhibit a strong wavelength (λ) dependence, which we attribute to λ-dependent confinement of vapor bubbles. Our experimental method is based on polarization quadrature, interferometric vibrometer measurements of acoustic transients that have passed through millimeters of coronal rat brain following surface ablation by a FEL. A summary of the experimental methods follows; a detailed description (and a partial survey of the data) has been published previously.\textsuperscript{8} The FEL was tuned either to λ = 3.0 µm (predominantly absorbed by the OH stretch mode of water) at a spot size of ∼ 50µm or to λ = 6.45 µm (absorbed both by waters bending mode and by proteins amide II mode) at a spot size of ∼ 75 µm, and then focused to the top surface of a brain tissue slice. Previous research demonstrated the advantages of 6.45 µm irradiation relative to other mid-IR wavelengths for human brain surgery due to both the ablation rate and the minimal collateral thermal damage,\textsuperscript{5,7} and an investigation of mid-IR tissue ablation exhibited a damage pattern indicative of pressure wave propagation well
Figure A.1: Acoustic transients due to single-macropulse ablation with [(a)-(c)] 3.0 µm FEL wavelength (2 mJ, 50 µm spot size) compared with [(d)-(f)] 6.45 µm (4 mJ, 75 µm spot size)

beyond the ablation cavity. Here the macropulse energies ranged from 1.0 to 5.0 mJ in 0.5 mJ increments, where a single macropulse (∼ 5 µs burst of picosecond pulses at 2.85 GHz repetition rate) ablated tissue to a depth of tens of microns or less, and measurements were made on 1.0, 1.5, 2.0, 2.5, 3.0, or 4.0 mm slices of tissue at room temperature. Tissue ablation was confirmed by visual inspection of the resulting cavity and by collection of the ejected tissue. A continuous wave HeNe probe beam was focused by a 10× objective (through ultrasound gel) onto a square millimeter of 0.5 mil aluminum foil, coated with retroreflective paint, which adhered to the bottom tissue surface. The displacement of the bottom tissue surface was measured
with a time-resolved quadrature laser interferometer, where the quadrature signals were recorded by two channels of a 150 MHz bandwidth digital oscilloscope. The research was performed at the Duke University FEL Laboratory and the W.M. Keck FEL Center at Vanderbilt University.

Figure A.1 compares the acoustic transients due to ablation by a single macropulse at either $\lambda$. Figures A.1a, A.1d present displacements $z_\lambda$, with five sequential measurements at the same site on the tissue surface. The pressure transient impinging on the bottom surface (tissue thickness $r$) is

$$P_{\text{acoustic},\lambda}(r, t) = \rho_o c \dot{z}_\lambda \left[ 1 + \frac{\dot{V}}{r \ddot{V}} \right]^{-1}$$  \hfill (A.1)

where $\rho_o \ (1039 \text{ kg/m}^3)$ is the density and $c \ (1526 \text{ m/s})$ is the speed of sound for brain tissue. The second term in the square brackets is the near-field correction ($\sim 10^{-3}$ for the 3.0 $\mu m$ results reported below), where $V$ is the volume of the vapor bubble. Figures A.1b, A.1e present $P_{\text{acoustic},\lambda}(r, t) = \rho_o c \dot{z}_\lambda$, where the arrival of the spherical acoustic wave at the foil introduces an insignificant 2080 ns ambiguity in resolving the pressure. Figures A.1c, A.1f present the Fourier transforms of $P_{\text{acoustic},\lambda}(r, t)$, where the insets demonstrate the asymptotic power law dependencies. The line shapes of the pressure transients [Fig. A.1b] and the high-frequency asymptotic power law ($f^n; n = 1.1 \pm 0.2$) evident in the inset of Fig. A.1c are the signatures for vapor bubble collapse. Figures A.2, A.3 investigate further the radiated acoustic pressure due to ablation by a single macropulse with $\lambda = 3.0 \mu m$. Figure A.2 addresses the attenuation of $P_{\text{acoustic},3.0}(r, t)$ for increasing tissue thickness (2.0 mJ macropulse energy). Figure A.3 addresses the changes in $P_{\text{acoustic},3.0}$ in a 3.0 mm tissue slice due to a systematic increase in the macropulse energy, where Fig. A.2 indicated a substantial attenuation of $P_{\text{acoustic},3.0} \ (3.0 \text{ mm}, t)$.

A model for thermal bubble dynamics is summarized in the inset of Fig. A.1e.
The dotted line represents 3.0 µm tissue ablation corresponding to the conditions in Figs. A.1a, A.1b, A.1c. FEL irradiation superheats the tissue water via the OH stretch mode on the nanosecond timescale. The open circle indicates the superheated tissue water (theoretically $T_o = 302$ °C, $P_o = 9.2$ MPa). At this initial condition, the metastable volume $V_{3.0,o} \approx \pi r_{3.0}^2 z_{3.0}$ depends on $r_{3.0} = 25\mu m$ (half the FEL spot size) and $z_{3.0} = 4.3\mu m$ (at the conclusion of a FEL macropulse, the depth at which the tissue water has been heated to $T_o$ as determined by Beers law and thermal diffusion$^6$).

Following irradiation, the metastable $V_{3.0}(t)$ includes water undergoing spontaneous spinodal decomposition to the vapor phase, nonaqueous cell components, and ex-
tracellular matrix. As it expands, \( P_{\text{bubble},3.0}(R) = P_o(3V_{3.0,0}/4\pi R^3)\gamma \), and the vapor is constrained by the tissue matrix \( P_{\text{atm}} + 2S/R \) (solid line).\(^{12}\) \( \gamma = 1.3 \) and \( P_{\text{atm}} \) is the atmospheric pressure. \( S \) is the sum of the surface tension attributable to any liquid water at the bubble surface \( (S_{\text{water}}) \) and the surface tension due to the confining tissue \( (S_{\text{matrix}}) \). Since \( S \) is an interfacial elastic constant, and thus essentially a function of density and the speed of sound, we use a value for \( S \) (0.05 N/m) that is typical for a vapor-condensed phase interface.\(^{11,12}\) We view the expanding \( V_{3.0}(t) \) as fledgling vapor bubbles that coalesce during the phase transition to form a vapor bubble with nonaqueous impurities constrained both by the surrounding tissue matrix and by a cap of tissue matrix at the air-tissue interface. The superheated \( V_{3.0,0} \) expands along the dotted line from the initial effective radius (open circle), where the stretching of the confining tissue matrix is characterized by the solid line. Mechanical equilibrium is reached at the intersection of these two lines \( (R_{E,3.0} = 24.8 \mu m) \).

A consideration based on the Rayleigh-Plesset equation indicates \( R_{E,3.0} \) is uniformly less than \( R_{\text{critical}} \) (condition for explosive cavitation) for all values of \( V_{3.0,0} \) and that the bubble then collapses “violently.”\(^{11,12}\) As the bubble radius \( R \) decreases during collapse, \( P_{\text{bubble},3.0}(R) \) increases dramatically, generating cavitation noise during the final stages of collapse, i.e., radiated acoustic pressure. The experimental line shapes [Fig. A.1b] and the asymptotic power law [Fig. A.1c] compare quite favorably with previous measurements of cavitation noise in pure water (see Figs. 3.18 and 3.20 in Ref. 12). Bubble collapse also is consistent with the experimental dependence of \( P_{\text{acoustic},3.0} \) on macropulse energy (Fig. A.3), where \( z_{3.0}, V_{3.0,0}, \) and \( R_{E,3.0} \) all scale with macropulse energy and the radiated acoustic pressure is a function of \( R_{E,3.0} \).\(^{11,12}\) Based on these observations, we attribute the 3.0 \( \mu m \) pressure dynamics to thermal vapor bubble collapse.

The dashed line in the inset in Fig. A.1e represents 6.45 \( \mu m \) tissue ablation corresponding to the conditions in Figs. A.1d,A.1e,A.1f, where the initial condition
Figure A.3: (a) $P_{\text{acoustic},3.0}$ and (b) its Fourier transform due to 3.0 $\mu m$ FEL ablation (single macropulse) in 3mm thick tissue for increasing macropulse energy [(indicated in (a)].

now is indicated by the solid circle. For this case, $r_{6.45} = 38 \mu m$ and $z_{6.45} = 42.0 \mu m$ and, consequently, $V_{6.45,o}$ (solid circle, dashed line) is larger than $V_{3.0,o}$ (open circle, dotted line), displacing the state equation. While $R_{E,6.45}(70.4 \mu m)$ also is uniformly less than $R_{\text{critical}}$, in Figs. A.1e,A.1f the line shapes for $P_{\text{acoustic},6.45}(r,t)$ are characteristics of an elastic pressure transient with a clear absence of any signature for bubble collapse. The experimental values for $P_{\text{acoustic},6.45}(r,t)$ are in the 100 kPa range, suggesting that bubble failure occurs for values of R closer to $R_{E,6.45}$ than that associated with the escalating pressures of bubble collapse. Furthermore, the characteristic frequencies evident in the pressure transients [Fig. A.1f] are orders
of magnitude below the resonant frequencies associated with bubble oscillations at about $R_{E,6.45}$,\textsuperscript{11} which indicates bubble/cap failure per se is not involved and tissue cap failure alone may be responsible for the radiated acoustic pressure. Based on these observations, we attribute the 6.45 $\mu m$ pressure dynamics to mechanical tissue failure during the growth phase of the thermal vapor bubble to $R_{E,6.45}$, where previously it was proposed that 6.45 $\mu m$ FEL irradiation drives protein chemical kinetics that promote brittle failure of tissue matrix.\textsuperscript{6,8} During bubble growth, the expanding tissue cap, and especially its periphery, would be subject to relatively large stresses, therefore promoting brittle fracture, releasing the bubble vapor to the air, and compromising the bubble altogether as ablation proceeds.

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Biography

Adam Sokolow was born on September 9th, 1982 in Rochester, New York. He received two Bachelor of Science degrees in 2005 from the State University of New York at Buffalo. The first was in Applied Mathematics and the second in Computational Physics. Following his Bachelors degree, he was accepted to the Physics Department at Duke University where in 2008 he received his Master of Arts in Physics. He is expected to receive a Doctor of Philosophy degree in Physics from Duke University under the supervision of Glenn Edwards in May, 2011. During his enrollment at Duke University, he received a fellowship from the University’s own Center for Theoretical and Mathematical Sciences, which supported him for one calendar year.

During his science career he has co-authored a number of research papers that resulted in publication. These include: Visco Jr et al. [2004], Sokolow et al. [2005], Job et al. [2005], Sen et al. [2005, 2007], Sokolow and Sen [2007], Job et al. [2007], Sokolow et al. [2007], Simion et al. [2009], Wagner et al. [2009] and Sokolow et al. [to be submitted]