Mapping the Development of the Zebrafish Embryo

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Morphogenesis, the process by which an organism develops into shape, is a phenomenon whose genetic influences are fairly well understood, yet the mechanics and dynamics behind this physical process are still unknown. We observed embryos of zebrafish (*Danio rerio*), during the developmental process of gastrulation using *in toto* multiview light sheet microscopy to generate movies of its development. From this data, we analyzed embryonic flow fields, investigated similarities and differences between the embryonic layers, and aimed to correlate these physical observations with the expression of different genes and proteins. We demonstrated that zebrafish tissues appear to follow three flow fields during development, and these flow fields are consistent throughout the different tissue layers. This research increases our knowledge of zebrafish embryo flow patterns and the transitions between distinct developmental stages. More broadly, this work improves our understanding of macroscopic flow behavior during morphogenesis and furthers our goal of constructing a quantitative framework of development.

I. INTRODUCTION

Living matter changes autonomously over time. During morphogenesis, cells rearrange themselves and form different structures. This process has been the subject of much study; however, the mechanics and dynamics behind it are still largely unknown. We are investigating the early morphogenetic event of gastrulation [1]. By studying this, we aim to improve our understanding of the mechanics responsible for dynamic cellular motion and to develop a sense of the general process behind morphogenesis.

A. Morphogenesis

Morphogenesis is the developmental process by which cells differentiate and form tissues, organs, and organ systems. This process begins shortly after fertilization with cleavage of the embryo. Specifically, the embryo undergoes rapid cellular divisions that split up the cytoplasm. Following this, the embryo begins gastrulation. In this phase, the cells begin to travel around the embryo and the three germ layers are formed: the ectoderm, mesoderm, and endoderm [2]. The construction of these layers is important because a cell's position within these layers determines its ultimate fate. The cells located in the ectoderm will eventually become the skin and the nervous system; those in the mesoderm will become bones, muscles, and tissues; and those in the endoderm will line the digestive and respiratory systems and help form several organs [3]. After the emergence of these layers, there is differentiation across the embryo and the formation of the different body axes. This is followed by the development of specific organs and organ systems [2].

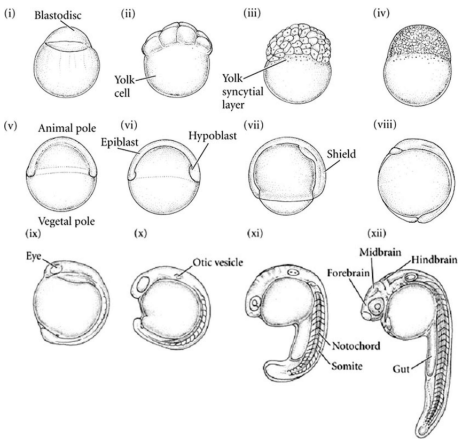

FIG. 1. Diagram depicting early development in the zebrafish embryo. Image i) shows the embryo shortly after fertilization and images ii)-iv) depict cleavage. Gastrulation begins in image vi) [4].

B. Zebrafish as a Model Organism

We are investigating morphogenesis by observing and analyzing gastrulation in zebrafish (*Danio rerio*). We are using this species as a model organism for the fol-

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lowing reasons. First, zebrafish embryos are transparent. This allows for better imaging and analysis of internal cellular motion and tissue development. These embryos are also externally fertilized which allows us to observe the entire developmental process. They are easy to genetically manipulate so we can look at different strains. They even develop rapidly: gastrulation occurs only six hours after fertilization, and the embryos hatch after only three days [5]. This developmental time span is ideal as it allows enough time to image the different morphogenetic processes within a reasonable time frame. Lastly, zebrafish embryos are approximately 700 microns in diameter. This allows us to image the entire embryo and analyze it as a self-contained system.

C. Genetics, Mechanics, and Dynamics

The study of morphogenesis can be approached from three different directions. The first of these is to study genetics. Genes provide the embryo with developmental instructions and direct the motion of the cells. The next approach is to investigate mechanics. In particular, to investigate proteins and the other molecules that are responsible for moving the cells and tissues. The last approach is to look at dynamics, by analyzing the motions of cells and tissues that occur as a result of these genetics and mechanics.

In this research we investigated dynamics and genetics. From the dynamic standpoint we analyzed the flow fields that emerge during gastrulation, and from the genetic standpoint we looked for the presence of morphogen gradients. A morphogen is a specific protein that is secreted from a group of cells and forms a gradient across the target tissue. Different genes are expressed in the presence of different concentrations of a morphogen, and such a gradient would allow for spatial patterning of gene expression [1]. This could play a role in coordinating development across the embryo.

II. METHODS

In order to analyze zebrafish development we imaged live zebrafish embryos using *in toto* light sheet microscopy, and imaged fixed zebrafish embryos using confocal microscopy.

A. Imaging Embryonic Development: Dynamics

Prior to this summer, only one zebrafish embryo had been imaged and analyzed to visualize tissue flow fields. In order to see if these flow fields were consistent across the species we used an *in toto* light sheet microscope to image two additional zebrafish embryos during gastrulation. The general procedure for imaging a zebrafish embryo is as follows:

1. In toto Light Sheet Microscopy: Preparation

One day prior to imaging, we place a male and a female zebrafish in a tank where they are separated by a divider. The next morning, we pull the divider and let the fish mate. After 10-15 minutes the fish are returned to their respective tanks, and the tank water is poured through a mesh sieve to separate out the embryos. These embryos are then placed in a petri dish with water and incubated for approximately 6 hours.

During development, the zebrafish embryo is surrounded by the chorion, also known as the egg envelope. This envelope protects the embryo from nearby mechanical disturbances, from changes in the water, and from dehydration [6]. This envelope can be taken off without disrupting the embryo's development and we remove it before imaging because the chorion would add an additional scattering layer in the laser's optical path. The embryos were dechorionated using one of two techniques. The first is to treat the embryo with pronase. This chemical will break down the chorion very quickly; but, unfortunately, will also disintegrate the embryo if left for too long. We can also dechorionate the embryo mechanically using a pair of tweezers to carefully tear a hole in the chorion and then pull it off the embryo. Both techniques require care and attention and the one we use depends on the number of embryos we have and their relative ages.

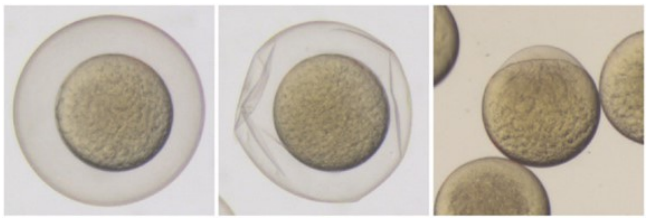

FIG. 2. Images of embryo before, during, and after dechorionation with pronase [7].

Once the embryo has been dechorionated, a small cone is made out of a transparent agarose gel containing multispectral beads. This cone will serve as the holder for the embryo. Once the embryo is carefully placed inside the cone we are ready to image with the *in toto* light sheet microscope.

2. In toto Light Sheet Microscopy: Imaging

An *in toto* light sheet microscope aims a Gaussian beam at a pair of galvanometric mirrors that rotate and create a digital sheet. This sheet scans the embryo layer by layer —producing a image stack of the embryo along a single axis. Upon completion of these scans, the sample is rotated and this process is repeated. We scan the embryo from a total of eight directions in order to achieve isotropic illumination and create a clear view of the entire surface tissue. When imaging a live sample, we repeat

this multiview data acquisition process every five minutes during the imaging window (typically several hours long).

It is noteworthy to mention that this type of imaging is beneficial because there is a large source to noise ratio, and the laser only illuminates the portion of the sample in the focal plane. This reduces bleaching and the overall damage to the embryo. It is especially beneficial for imaging live organisms because it reduces the likelihood of accidentally killing the organism or changing its morphology with extensive light exposure.

Once imaging has concluded, the data is organized according to the time of acquisition, the imaging channel, and the laser angle. These images are then run through a program to detect and register the position of the multispectral beads contained within the gel sample holder. These beads have a constant position in relation to the embryo, so we can calculate the transformation needed to map one set of beads onto another and apply this transformation to the entire image. This allows us to fuse all the different views together, creating a multiview image. These images are then strung together to make time lapse videos of embryonic development.

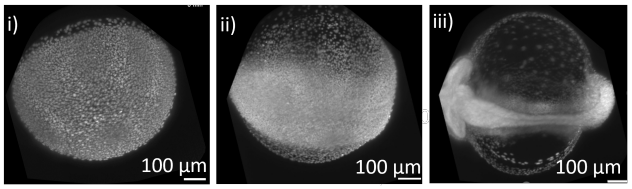

FIG. 3. Images of a zebrafish embryo at different times in development taken using *in toto* multiview light sheet microscopy. Image i) shows the embryo at the beginning of the shield stage, and images ii) and iii) respectively show the embryo 300 and 615 minutes later.

B. Imaging Embryonic Development: Genetics and Mechanics

In order to look at the genetics and mechanics behind morphogenesis, we looked for morphogen gradients using both an *in toto* light sheet microscope and a confocal microscope (see next section). We specifically looked at the expression of E-cadherin and phosphorylated myosin. E-cadherin is a protein primarily located in the tight junctions of the epithelium and assists in cell-cell adhesion. As a result, its presence is associated with increased tissue stiffness and reduced cellular motion [8]. Phosphorylated myosin is a protein that is used as a nuclear expression reporter for bone morphogenetic protein —BMP for short. Studies have shown that BMP gradients play a role in the formation of the germ layer and the different body axes [9].

1. *Confocal Laser Scanning Microscopy: Preparation*

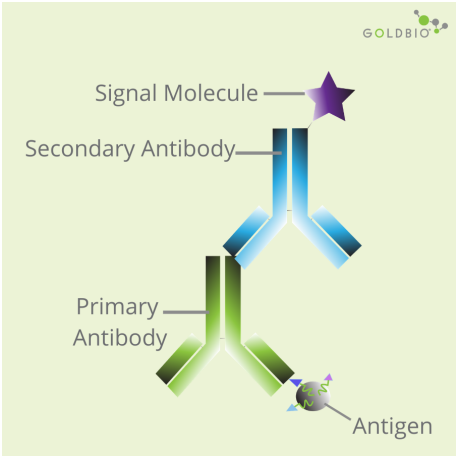

FIG. 4. Diagram showing the interaction between a target antigen and the antibodies applied in order to image it. The target antigen is not visible on the microscope, so a primary antibody is applied. This antibody bonds with the antigen, but is also not visible. A secondary antibody that contains a signal molecule —and is therefore visible under the microscope —is then applied. This bonds with the primary antibody and therefore shows the antigen's location when imaged [10].

We can use the confocal microscope to image fixed samples. In order to prepare these samples, we follow the same mating and incubating process as above. It is relevant to note that we split the embryos into different groups, and depending on what we want to study, will sometimes apply a gene inhibitor or a specific stain to some of these groups. These different groups will be fixed at different times so we can see how the embryo changes during development.

After fixation, the embryos undergo antibody staining. During this process, two different antibodies are applied to the sample which will render the expression and position of the desired protein and/or cell feature visible under the microscope. From start to finish, this process takes four days. The embryo can then be mounted by placing it in agarose gel on the coverslip of a petri dish. For our phosphorylated myosin and E-cadherin images we positioned the embryo so that the animal cap was pointed down on the coverslip of a petri dish.

2. *Confocal Laser Scanning Microscopy: Imaging*

A confocal laser scanning microscope images a stationary specimen by scanning an illumination beam across the sample [11]. The wavelength of this beam is chosen so that it will illuminate the desired fluorescent markers. The beam is focused on a specific z-plane, and typically multiple planes along the z-axis are imaged to encom-

pass the entire sample. The resulting image stack can be collated to create a single view image of the sample.

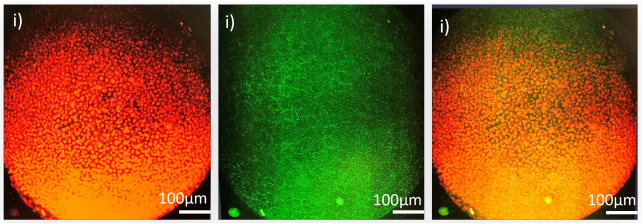

FIG. 5. Maximum Intensity Projection images of a zebrafish embryo taken using a confocal microscope. Image i) shows the distribution of phosphorylated myosin. Image ii) shows the distribution of E-cadherin. Image iii) shows the distribution of both proteins overlaid together in the same image.

III. RESULTS

The following analysis sections look at data that was collected using the *in toto* light sheet microscope.

A. Analysis of Embryo as a Whole

As previously stated, the multiview images of the zebrafish embryo were complied into a movie to show tissue movement and embryonic development over time. In order to facilitate surface tissue analysis, we performed tissue cartography on this data. Specifically, we used an Image Surface Analysis Environment, that was developed in Matlab, to unwrap the 3D embryo and create a 2D surface [12]. The concept is akin to producing a 2D map of Earth in order to see all the continents. At the end of this process, a series of images are produced using different cylindrical projections which provide a standardized way to view the embryo. Different images allow us to study different portions of the embryo with minimal distortions. For the following data analysis we used images produced using the cylindrical projection.

It is important to note that in this analysis we are looking at the maximum intensity projection of the data. What this means is that the images being examined are composed of pixels that do not correspond with any particular layer within the embryo. Instead each pixel is the maximum intensity value at that particular x and y position, from all the different layers in the z-stack. Studying the data in this format allows us to visualize the overall tissue motion.

After the 2D surface has been constructed, the tissue flow can be studied using particle image velocimetry. The program that performs this takes the video of the 2D surface and identifies the local displacement of pixels between different time points. It then constructs the tissue's changing instantaneous velocity field, allowing for visualization of the tissue's evolving flow.

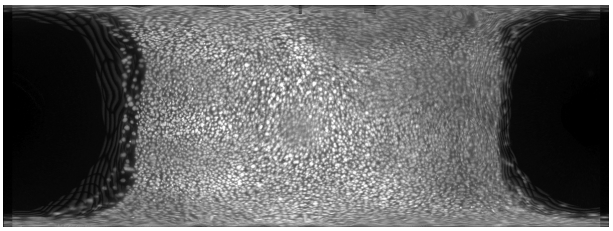

FIG. 6. Maximum Intensity Projection of a zebrafish embryo in the shield stage, as a 2D surface produced by the Image Surface Analysis Environment. There is some size and angle distortions along the edges of this image.

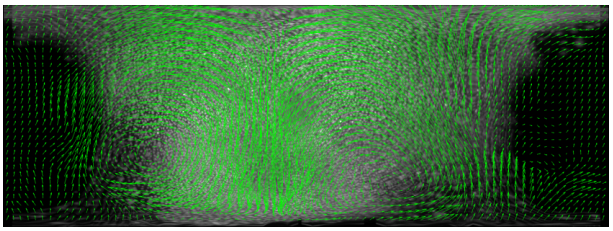

FIG. 7. Instantaneous velocity field for the zebrafish embryo in the previous figure, constructed using particle image velocimetry.

For the one embryo that has been completely analyzed, it appears that there are intervals within the imaging period in which the velocity fields become consistent. Specifically, there were three intervals lasting between 1.5 and 2 hours in which fixed points emerged in the velocity field and the tissues repetitively followed a particular trajectory around them.

B. Analysis of Individual Embryonic Layers

We are imaging zebrafish embryos during the shield stage. At this point in development there are three different layers within the embryo: from outside to inside they comprise the enveloping layer, the interbulk layer, and the yolk syncytial layer. These layers all have different densities and differently sized nuclei; therefore, it is possible that the flow patterns aren't consistent across these layers.

The images collected using the *in toto* light sheet microscope can be rearranged to look at the embryo layer by layer. When this was done it was found that the differences between the nuclear densities and nuclear sizes in each layer were clearly visible. This meant that the surfaces for each layer could be distinctly separated from each other. After separation, the maximum intensity projection was created for each layer. It is relevant to note that although it was possible to identify and separate each layer, the resulting image for the yolk syncytial layer was not complete. In this image, the layer appears to have a black hole in the middle which is an artifact of imaging. This location is too far inside the embryo for a

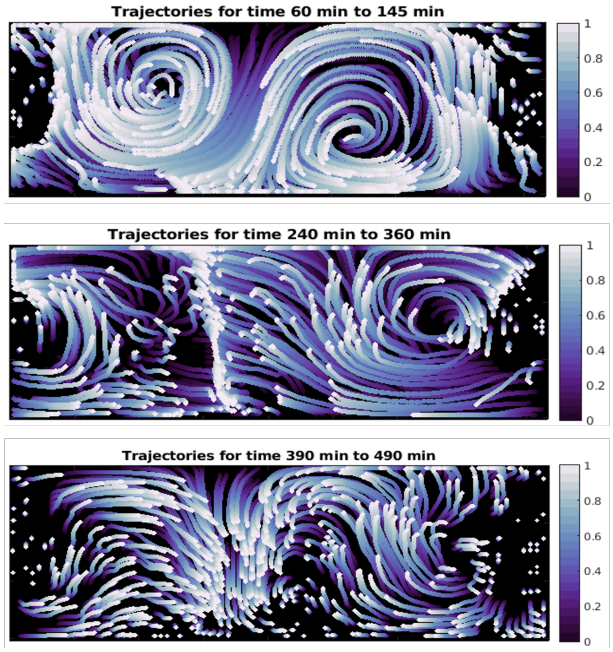

FIG. 8. Above are integrated flow trajectories, represented in pullback space, during the three windows of time where the flow was stationary. The color bar represents time: 0 is the beginning of the trajectory and 1 is the end.

robust signal to be collected due to light diffraction and reflection.

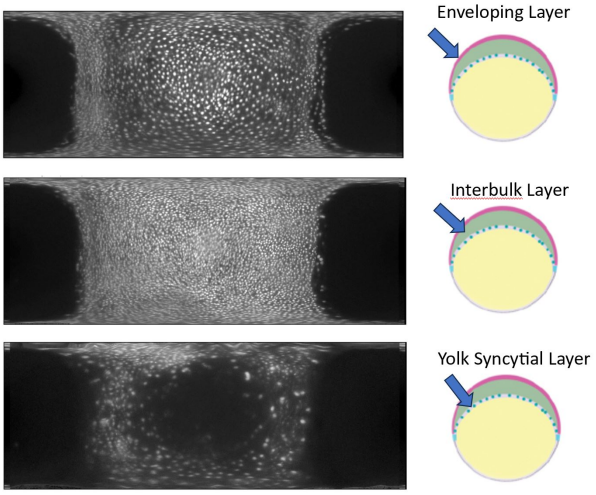

FIG. 9. Images of each embryonic layer during the first recorded time, at the beginning of the shield stage. Each layer can be clearly distinguished [13].

Particle image velocimetry was performed on each set of layers. This was successful for the enveloping layer and the interbulk layer. It was not successful for the yolk syncytial layer for two reasons. First, the images for this layer were incomplete so there were areas where the nuclei can't be traced. Second, this layer was significantly less dense then the other two layers so there are simply less pixels to trace.

In both the enveloping and interbulk layers we saw patterns emerge in the velocity fields that were similar to those seen for the entire embryo. We specifically noted this correlation during the time intervals for the first two flow fields. The third flow field was not analyzed because the layers could no longer be accurately separated. In fact, even during the second flow field, the spacing between the embryonic layers had reduced and the different layers had slightly overlapped. This occurred because at this stage, the tissue begins to be drawn together to form the spinal cord.

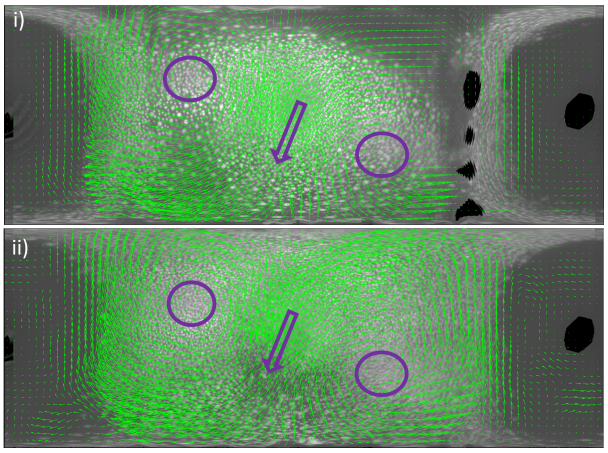

FIG. 10. Velocity field 100 minutes into imaging. Image i) is the enveloping layer and Image ii) is the interbulk layer. The purple circles show the location of fixed points in the flow field, and the purple arrow shows the relative tissue flow in a particular location. The black spots visible in these pullbacks correspond to regions that are cut off because the sample exceeds the imaging window.

C. Analysis of E-Cadherin and Phosphorylated Myosin - Stained Embryo

The embryo that was stained for E-cadherin and phosphorylated myosin expression was fixed and imaged on the light sheet microscope. Once again, tissue cartography was performed to unwrap the 3D embryo and create a 2D surface. Both a cylindrical and an anterior equidistant projection were used to create images for this embryo. The image obtained from the anterior equidistant projection can be found in Appendix A. In the image constructed from the cylindrical projection it is evident that E-cadherin and phosphorylated myosin form distinct opposing gradients across the animal cap.

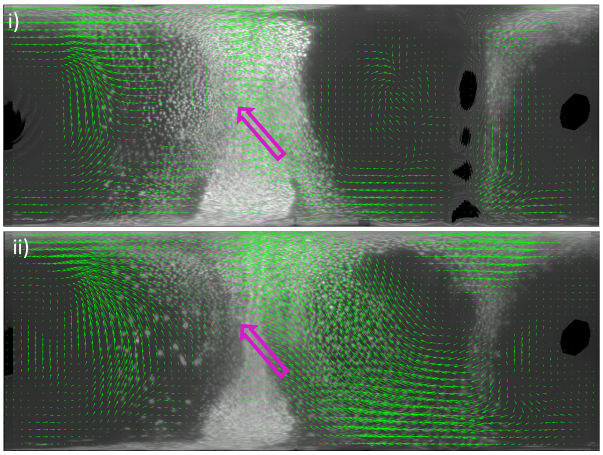

FIG. 11. Velocity field 300 minutes into imaging. Image i) is the enveloping layer and Image ii) is the interbulk layer. The pink arrow shows relative tissue flow in a particular location. The black spots visible in these pullbacks correspond to regions that are cut off because the sample exceeds the imaging window.

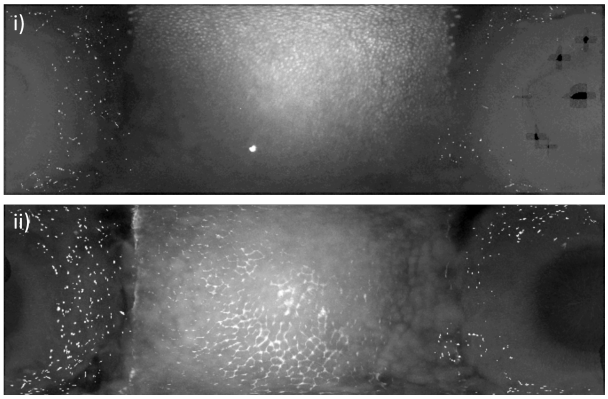

FIG. 12. Pullbacks for the stained embryo. Image i) shows phosphorylated myosin expression and Image ii) shows E-cadherin expression. The bright spots on the edges of both these images are fluorescent beads from the surrounding agarose gel (Note: the bright spot in front of the embryo in image i) another one of these beads)

D. Analysis of Individual Embryonic layers of E-Cadherin and Phosphorylated Myosin - Stained Embryo

The E-cadherin and phosphorylated myosin data can be rearranged using the same technique as above to view the embryonic layers individually. When this was done, it was evident that E-cadherin is largely restricted to the epithelium layer, while phosphorylated myosin extends further into the embryo. It's relevant to note that the gradient formed by phosphorylated myosin is visible in each layer.

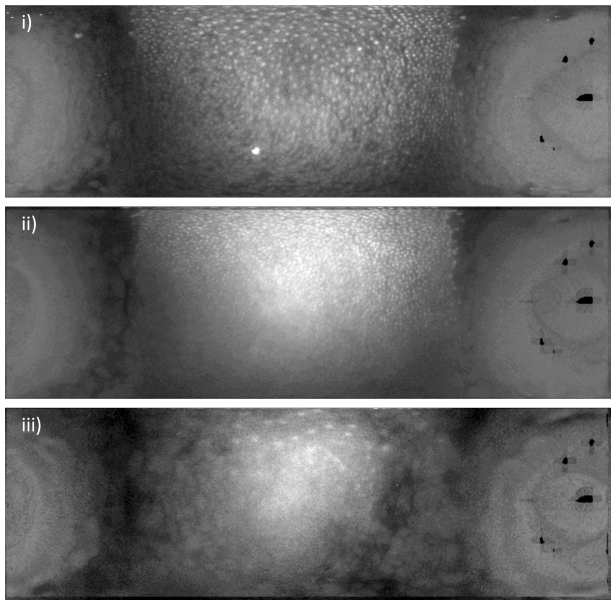

FIG. 13. Pullbacks for the different layers for the phosphorylated myosin embryo. Image i) is the pullback for the enveloping layer, Image ii) is the pullback for the interbulk layer, and Image iii) is the pullback for the yolk syncytial layer.

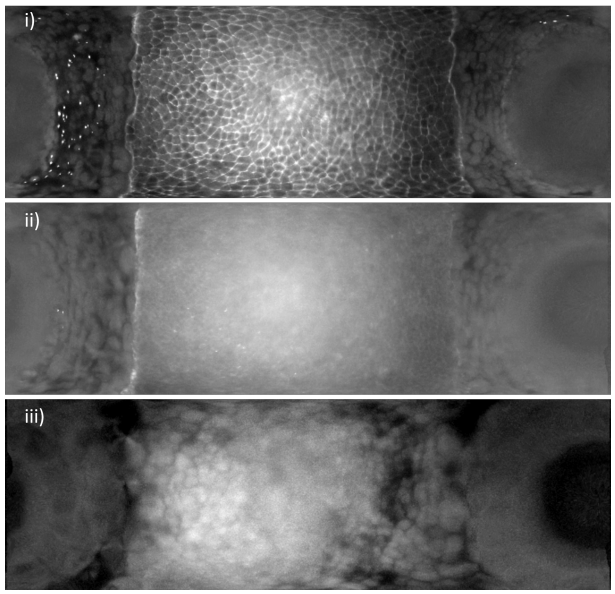

FIG. 14. Pullbacks for the different layers for the E-cadherin embryo (same embryo as FIG. 13). Image i) is the pullback for the enveloping layer, Image ii) is the pullback for the interbulk layer, and Image iii) is the pullback for the yolk syncytial layer.

IV. DISCUSSION

We saw that during development, zebrafish tissues appear to move according to three different flow patterns. Currently, only one zebrafish embryo has been analyzed for the presence of these flow patterns. In order to make

these results more robust data analysis needs to be performed on at least two additional data sets.

We also demonstrated that it is possible to identify and separate the different embryonic layers based on the density and size of the embryo's nuclei, and that in these early stages the flow fields across these layers appear to have similar tissue movements. In the future it would be beneficial to look for a way to quantify the similarities between these different layers. This would allow for a more precise understanding of the similarities and differences between these flows.

Finally, we were able to visualize the location of both phosphorylated myosin and E-cadherin in the embryo. This showed that these two proteins form opposing gradients. We hope in the future to look further into the locations of these gradients and into the general genetics and mechanics that cause these tissue flow patterns.

This research increases our knowledge of zebrafish embryo flow patterns and therefore helps improve our understanding of macroscopic flow behavior during morphogenesis. This is beneficial as it will assist in the overall goal of constructing a quantitative framework for development.

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Appendix A: Additional Images

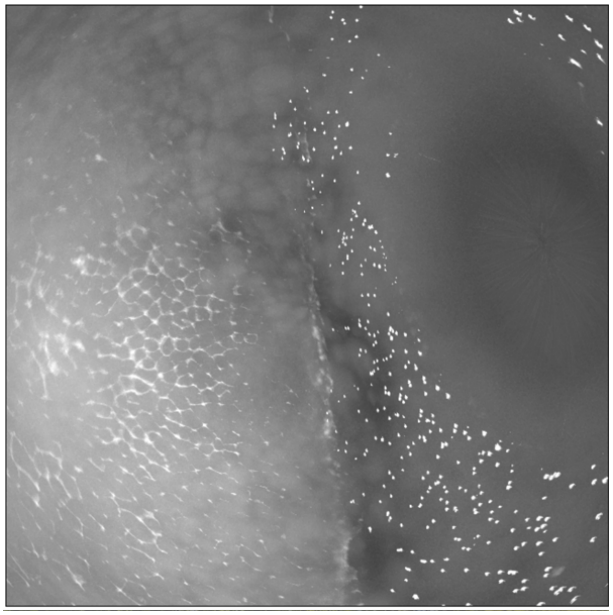

FIG. 15. Image of surface E-cadherin expression obtained from the anterior equidistant projection using tissue cartography. This embryo was stained for both E-cadherin and phosphorylated myosin.

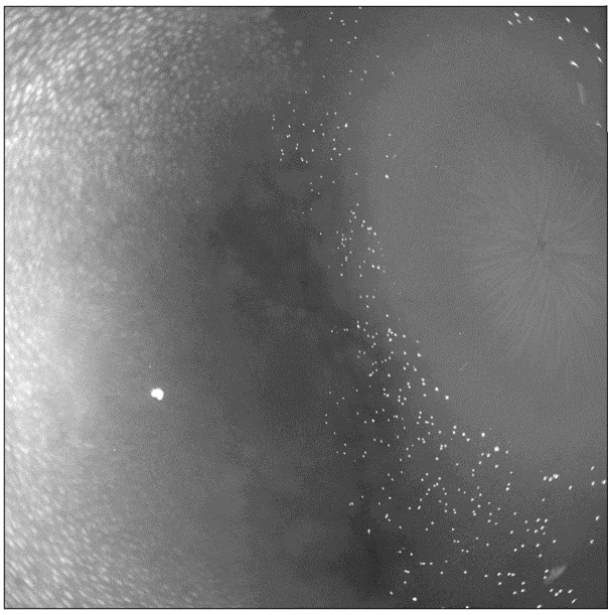

FIG. 16. Image of surface phosphorylated myosin expression obtained from an anterior equidistant projection using tissue cartography. This embryo was stained for both E-cadherin and phosphorylated myosin.

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