Video Article

Tracking Morphogenetic Tissue Deformations in the Early Chick Embryo

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Abstract

Embryonic epithelia undergo complex deformations (e.g. bending, twisting, folding, and stretching) to form the primitive organs of the early embryo. Tracking fiducial markers on the surfaces of these cellular sheets is a well-established method for estimating morphogenetic quantities such as growth, contraction, and shear. However, not all surface labeling techniques are readily adaptable to conventional imaging modalities and possess different advantages and limitations. Here, we describe two labeling methods and illustrate the utility of each technique. In the first method, hundreds of fluorescent labels are applied simultaneously to the embryo using magnetic iron particles. These labels are then used to quantity 2-D tissue deformations during morphogenesis. In the second method, polystyrene microspheres are used as contrast agents in non-invasive optical coherence tomography (OCT) imaging to track 3-D tissue deformations. These techniques have been successfully implemented in our lab to studythe physical mechanisms of early head fold, heart, and brain development, and should be adaptable to a wide range morphogenetic processes.

Video Link

The video component of this article can be found at http://www.jove.com/video/3129/

Protocol

1. General Experimental Preparation

1. Prepare tissue culture medium in the laminar flow hood.
    1. Dilute Dulbecco's Modified Eagle's Medium (DMEM)(1 L bottle with 4.5g/L glucose, sodium bicarbonate, and L-glutamine). Add 10 mL penicillin / streptomycin / neomycin antibiotics.
    2. Remove 100 mL of DMEM with a sterile transfer pipette and replace with 100 mL of chick serum.
    3. Aliquot the DMEM/10% chick serum/1% antibiotics into sterile 15 mL conical vials and freeze.
2. Prepare phosphate buffered saline (PBS) supplemented with calcium and magnesium. Mix 100 mL 10X PBS, 900 mL deionized water, and 1 mL of a concentrated calcium and magnesium solution (100 mg/mL $CaCl_2$•$2H_2O$, 100mg/mL $MgCl_2$•$6H_2O$).
3. Incubate eggs in a longitudinal orientation to desired stage as defined by Hamburger and Hamilton[1] (i.e. 23-25 hours for HH stage 5, or 42-48 hours for HH stages 11-12).
4. Remove embryos from the egg using the filter paper carrier method.
    1. Crack the bottom of the egg on the side of a150 mm petri dish. Pull apart the shell from the bottom and empty the contents of the egg into the dish.
    2. Remove the thicker, viscous albumen from the egg using blunt forceps.
    3. Cut circles of Whatman #2 filter paper 2-3 cm. in diameter. Using a single hole punch, punch holes in the middle of the filter paper. Place one of the filter paper rings on the yolk in a manner that keeps the embryo visible through the central hole in the ring.
    4. Cut around the outer diameter of the filter paper with microscissors. Remove the paper from the yolk with fine forceps.
    5. Gently rinse the embryo in PBS to remove adherent yolk particles. (HH5 embryos may need to soak as long as 15-20 minutes to remove residual yolk). Remove the filter paper assembly from the bath and place it ventral side up in a 35 mm culture dish. Place a second filter paper ring atop the first, sandwiching the embryo between the rings. Put a metal ring around the outside of the filter paper sandwich (≈ 1.5 - 2 cm diameter) to secure the embryo.
    6. Submerge the embryo in culture medium. Verify proper morphology and embryonic stage with a bright field microscope.
5. Use a pipette puller to prepare fine tip glass needles (1.5 mm inner diameter) (World Precision Instruments, Sarasota FL) for subsequent tissue dissections and labeling.

2. DiI Labeling of HH Stage 5 Embryos using Magnetic Iron Particles

1. Add a few drops of saturated DiI in alcohol to a small quantity of iron powder (iron reduced, Mallinckrodt Baker, Inc., Phillipsburg, NJ) at room temperature and let dry. (This usually causes the particles to cake together once the alcohol has evaporated.) Use the tip of a pulled micropipette to break up the clumped iron particles, transfer them to a microcentrifuge tube, and rinse them several times and cover with deionized water.
2. To label ectodermal cells in a HH stage 5 embryo, place the harvested embryo (i.e., the entire filter paper assembly) dorsal side up in a 35 mm culture dish covered with a generous layer of culture media and put the dish under a dissecting microscope. Use a glass needle to remove the vitelline membrane and expose the ectodermal cells. (Take care to not pierce embryo.)
3. Draw a column of labeled iron particles (in deionized water) into a Pasteur pipette. Using the dissecting microscope, submerge the pipette tip beneath the fluid surface and position just above the embryo. Rolling the pipette slowly back and forth between the fingertips, jostle the particles out of the pipiette and sprinkle them across the embryo.
4. Once the embryo is covered with particles, carefully place it in a 37°C incubator for approximately 10 minutes. Take embryo out of the incubator and use a strong magnet to remove the particles.
5. Use a fluorescent microscope with attached video camera and appropriate filter settings to acquire both bright field and fluorescent images of the embryo, as shown in Figure 2A,B.
6. Note the label density. If a denser distribution is desired, use additional particles (as described above) to label the embryo again and extend the incubation time accordingly. If the labels are too dense, add a small quantity of unlabeled iron particles to the microcentrifuge tube filled with already labeled particles and mix the tube contents. Repeat the labeling process with a new embryo and again note label density.

3. Polystyrene Microsphere Labeling for Optical Coherence Tomography (OCT) Imaging of HH11-12 embryos

1. Prepare a solution of black 10 µm diameter microspheres (Polysciences Inc., Warrington, PA) in PBS in a 35 mm petri dish. (Beads should be large enough to generate contrast for OCT imaging, but small enough such that bead size is on the order of individual cells. Specific values will vary depending on the resolution of your OCT system and the tissues being studied). One drop of the stock solution (2.6% solids-latex) per mL of PBS is generally sufficient. Pull this solution into a 10cc syringe using a 22 gauge needle. Vortex the solution for 30 seconds to ensure a uniform bead distribution.
2. Bevel the tips of your pulled glass needles using a computer hard drive[2]. Tip diameter should be large enough that beads can fit through the opening, but at the same time, as small as possible to minimize tissue wounding during injection.
3. Fill a beveled glass needle with the bead solution using the syringe. Lightly flick the micropipette to ensure that the solution has reached the tip.
4. Using a micromanipulator, place the glass needle in the same field of view as the embryo. Beads should be flowing out of the pipette tip.
    1. If no beads exit the micropipette, there may be a clog. In this case, repeat steps 3.2-3.4 with a new glass needle.
    2. If too many beads cloud your field of view, your pipette tip may be too wide. Repeat steps 3.2-3.4 with a new pipette and a new embryo.
5. Insert the pipette tip into the lumen of the brain tube. Be sure not to pierce the ventral floor of the tissue. You will see a transient swelling of the tissue as beads and fluid enter the lumen. This indicates a successful intubation; now, quickly remove the glass needle.
6. Verify a sufficient bead density in the inner lumen of the brain tube using a bright field microscope. Let the beads settle for 20 - 30 minutes before proceeding to Sect. 4.

4. Data Acquisition

* The following methods are well suited for the fluorescence labeling technique described above. However, with the microsphere labeling technique, beads can dislodge when transferring samples between incubators and imaging systems. In this case it is best to proceed to step 4.2 (which avoids sample movement/agitation altogether). In both methods, embryos are cultured while submerged in liquid culture medium. If the embryo is not completely submerged (as is the case with some conventional tissue culture techniques), tissue geometry is greatly altered by abnormally high surface tension loads and strain distributions will fail to accurately capture normal 3-D morphogenesis[3, 4]. Moreover, submerging the tissue prevents the bright contrast observed at the liquid-gas interface during OCT imaging from obscuring embryonic morphology and marker coordinates.

1. Embryo Culture (general)
    1. After acquiring your first images (bright field, fluorescence, or OCT), put up to seven 35 mm culture dishes (with embryos) in a 150 mm Petri dish and place the entire assembly in a small plastic bag. Add a couple of drops of deionized water to the bag for humidification and fill the bag with a 95% O_2 / 5% CO_2 mixture. Place the embryos into the incubator at 37°C (Queue Stabiletherm, Asheville, NC) for the desired time until the next imaging timepoint. Using this method, experiments can be performed on multiple embryos in the same day.
2. Time-lapse culture (automated)
    1. Place your labeled embryos into a Delta T Dish (35 mm outer diameter, 23 mm central aperture with tapered side wall, 0.17mm thick glass; Bioptechs, Butler, PA) containing 1.2 mL of culture medium.
    2. Cover the dish with a glass lid kept at 37°C using a Bioptechs Delta T4 Culture Dish Controller (to prevent condensation). Superfuse the embryo with a 95% O_2 / 5% CO_2 gas mixture supplied from a Mini-Pump Variable Flow device (Fisher Scientific). Prior to reaching the embryo, heat and humidify the gas by bubbling it through deionized water on a warm (<100°C) hot plate. A schematic of this experimental set-up is shown in Figure 1.
    3. Set up the software to automatically acquire images at user-specified time intervals. For our fluorescence microscope, we use Openlab software (PerkinElmer, Waltham, MA), and for our OCT system, we use swept source Thorlabs software (Newton, NJ).Because this

method is currently only capable of handling one time-lapse experiment at a time, it is often best to allow these types of cultures to run overnight. One could circumvent this limitation by installing an automated translatable stage compatible with both the hardware and software of the imaging system[5].

5. Representative Results:

Labels are tracked automatically (using Volocity, PerkinElmer) or manually (using the Manual Tracking plugin in ImageJ, NIH) in each experiment. In the fluorescent labeling technique, we use the Matlab routine *gridfit* to fit 2D surfaces through the marker coordinates, which enables morphogenetic surface strains to be calculated[6, 7]. Standard equations are used to transform these values into a relevant embryonic coordinate system. Alternatively, in the OCT technique, surfaces are generated from segmented image volumes (obtained via standard software such as Matlab or Caret[8]) and strains can be calculated in the direction of maximum or minimum curvature of the sample[9].

We used our iron particle technique to label and track the motion of ectodermal cells during head fold formation in the early chick embryo. As depicted in Figure 2A, fluorescently labeled cells were distributed across the entire embryo. Bright field and fluorescent images of the embryo were captured at different intervals during *ex ovo* culture. The motions of the tracked labels (Fig. 2B,C) were then used to calculate the evolving morphogenetic strain distributions during head fold formation (Fig. 2D-F).

Similarly, our polystyrene microsphere technique was used to track tissue movements at the mid-hindbrain boundary of the early chick brain. As shown in Figure 3 B-D, bead motions were tracked for 6 hours and strains characterizing tissue deformation in the longitudinal and circumferential directions were calculated from marker coordinates. Note that this method is capable of handling distinctly 3-D deformations as beads tend to stick to all sides of the inner lumen of the brain (Fig. 3A).

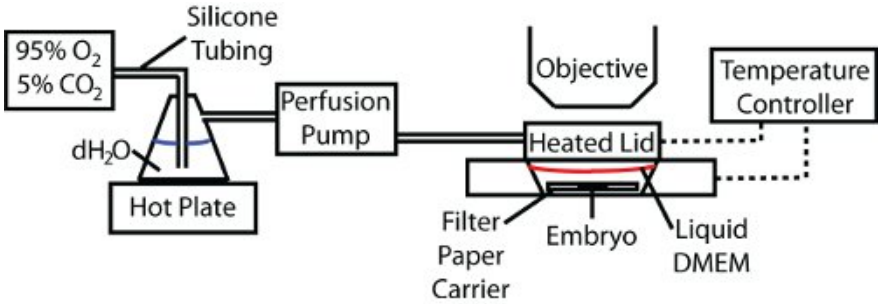

Figure 1. Schematic of set-up for time-lapse tissue culture.

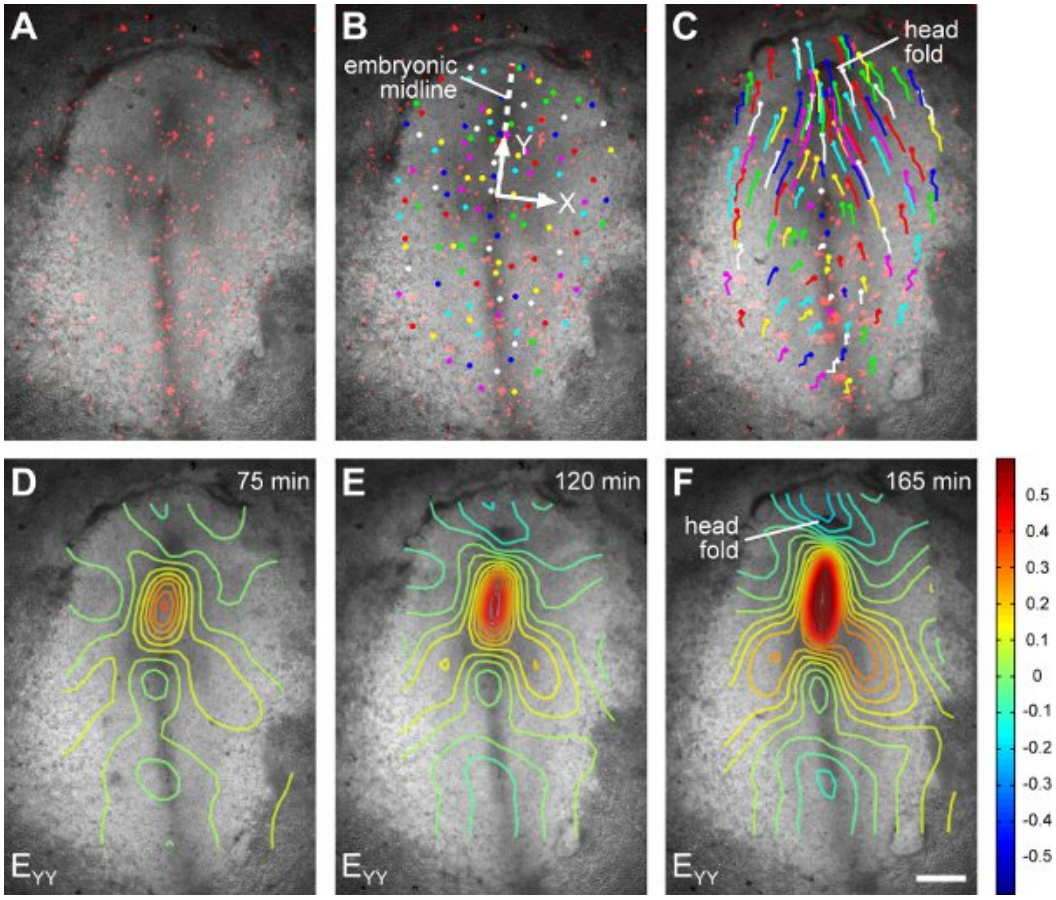

Figure 2. Quantifying 2-D tissue deformations in the early chick embryo using tracked fluorescent labels. (A) Merged bright field/fluorescent image of HH stage 5 embryo after removal of iron particles. DiI-labeled ectodermal cells (red) are distributed across the entire embryo. (B,C) The motion of labeled cells was tracked using ImageJ. Label displacements were calculated in embryo coordinates (X,Y). Note that A and B are the same image. (D-F) Contour plots of evolving longitudinal strain distributions during head fold formation. Longitudinal Lagrangian strains were calculated relative to HH stage 5 (i.e., A and B) after (D) 75 min, (E) 120 min, and (F) 165 min of incubation. These strains characterize the length changes of line elements originally oriented along the Y-axis (in the stage 5 embryo). Further details on using tracked label coordinates to calculate morphogenetic strains can be found in Filas *et al.* (2007)[6] and Varner *et al.* (2010)[7]. Note that C and F are the same image. Scale bar = 500 μm.

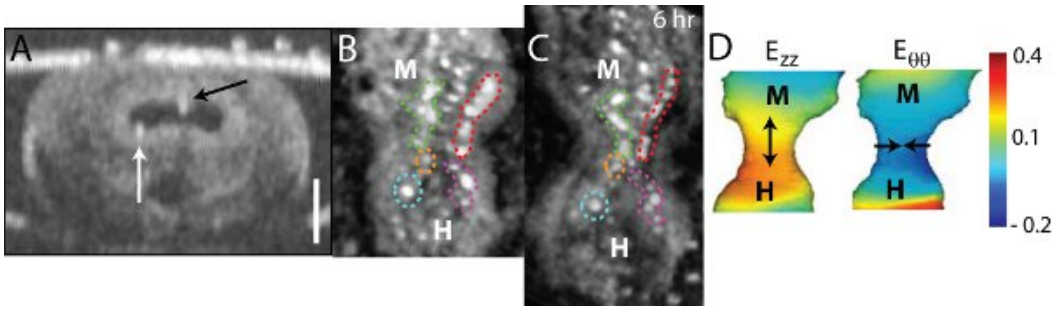

Figure 3. Quantifying 3-D tissue deformations in the early chick brain. (A) Polystyrene microspheres that adhere to the dorsal (black arrow) and ventral (white arrow) sides of the brain tube are easily discernible from surrounding tissues in transverse cross section.Ventral views of OCT reconstructions show microsphere locations near the mid-hindbrain boundary at (B) HH12 and after (C) 6 hr of incubation (M: midbrain, H: hindbrain). Several groups of beads are outlined to highlight the overall deformation of the tissue. (D) Longitudinal (E_{zz}) and circumferential ($E_{\theta\theta}$) Lagrangian strains were calculated from these deformations. Positive longitudinal and negative circumferential strains at the mid-hindbrain boundary reflect an axial lengthening and circumferential shortening of this region during culture. Further details on calculating morphogenetic strains for complex surfaces during morphogenesis can be found in Filas *et al.* (2008)[9]. Scale bar = 200 μm.

Discussion

Two tissue labeling techniques are presented for the *ex ovo* culture of early chick embryos. The first uses fluorescent lipophilic dyes delivered via magnetic iron particles to simultaneously label hundreds of cells. However, this method is currently not compatible with optical coherence tomography, as fluorescent dyes generally show little contrast from surrounding tissues using OCT[10]. Hence, we show an alternative technique using polystyrene microspheres to label tissues for time-lapse OCT analysis. This technique yields 3-D datasets but care must be taken not to dislodge the beads from the tissue. Using either one of these methods in the appropriate experimental setting should provide reliable tissue labeling and *ex ovo* development in early embryos. Both methods use readily available, relatively low-cost materials (e.g., iron powder, polystyrene microspheres) and enable tissue deformation to be quantified during morphogenesis. The tissue markers in both cases can be easily and reproducibly applied to embryonic tissues and do not require specialized equipment, making these experiments accessible to newcomers in the field. Visualizing and analyzing the resulting data (e.g., by computing morphogenetic strain maps) should help illuminate the mechanics of morphogenesis in your model system[6, 7, 9, 11, 12].

Disclosures

No conflicts of interest declared.

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