Supplementary online material

Materials and Methods

Fluorescent labeling of cells within the segmental plate

Fertile White Leghorn chick eggs were acquired from a local supplier (Lakeview Farms) and incubated at 38^{o}C until approximately the 8-12 somite stage (ss) of development (29) . To label cells within the paraxial mesoderm, eggs were injected with a solution of Bodipy Ceramide (D-3521; Molecular Probes) made by dissolving the fluorescent dye in DMSO to a stock solution concentration of 4 mM, then diluted 1:200 in warmed (38^{o}C) Howard Ringer's solution. Bodipy-ceramide is a vital dye which labels cell membranes allowing the outline of cellular boundaries to be visualized with confocal microscopy. After windowing the egg shell, a hole was cut in the vitelline membrane, using a fine tungsten needle, and dye solution was injected into the segmental plate using a picospritzer with a quartz micropipet (Sutter Instrument Co.) positioned posterior to the latest forming somite and just lateral to the segmental plate. To label small subpopulations of cells within the segmental plate, DiI (Molecular Probes; D-282) was made up in 0.5% stock solution in 100% ethanol, and focally iontophoretically injected in ovo into the segmental plate. Injected eggs were then resealed with adhesive tape and incubated at 38^{o}C prior to time-lapse imaging.

Time-lapse confocal microscopy

All embryos were visualized using an inverted confocal microscope (either a Biorad MRC-600 or a Zeiss 410). The microscope (Zeiss) was surrounded by cardboard (4 mm thick) covered with thermal insulation (Reflectix Co.; 8 mm thick) which enclosed a warming heater (Lyon Electric Co., Inc. #115-20) that maintained the environment at 38^{o}C for the duration of time-lapse filming, with only mild temperature fluctuations. The fluorescent dye, Bodipy-ceramide, was excited with the 488 nm laser line using the BHS filter set intended for fluorescein. DiI was excited with the 560 nm laser line using the YHS filter set intended for rhodamine. For Bodipy-ceramide labeled embryos, the aperture of the confocal microscope was mostly opened such that with a 40X objective (Zeiss), numerical aperture (NA) 0.75, each optical section represented about 10-15 um in z-height. Although this large aperture sacrificed some resolution, it allowed for a maximum optical section thickness so that we could capture a majority of cell movements over time. During a typical time-lapse imaging session (n = 52), we took 3-dimensional (x,y,t) data sets by collecting and recording an image every 2 minutes onto 1 GB Jaz disks (Iomega Corp. #10410) using the COMOS software package. Images were analyzed and played back as a SMovie after conversion (using macros from H. Karten; University of California, San Diego) using the image processing and analysis program, NIH Image 1.62.

Culture chamber preparation for time-lapse imaging

To visualize local cell movements in time during somite segmentation, a special culture chamber was designed such that individual labeled cells could be distinguished in high-

magnification (40X-63X) while maintaining the living embryo. A petri dish was prepared with a small hole cut in the bottom and a glass coverslip was attached with silicon grease (Dow Corning #79810-99) underneath the dish to cover the hole. Whole embryo explants were then pipetted and spread out on the surface of the glass. Embryos were oriented with the ventral surface in contact with the glass, leaving the dorsal surface exposed to the atmosphere. In order to maintain a humid environment around the embryo and allow for oxygen transfer, a teflon membrane cover was constructed and placed over the embryo and sealed to the petri dish with silicon grease. The height of the teflon membrane cover (0.5 cm) allowed for space between the dorsal surface of the embryo and the teflon membrane. The teflon membrane was assembled on a cylindrical acrylic ring (2.2 cm inner diameter by 2.6 cm outer diameter by 0.5 cm in height) and is made of a high-sensitivity, oxygen permeable membrane (Fisher #13-298-83; 3.8 cm by 7.5 cm by 15 um). To attach the acrylic ring to the teflon membrane, the acrylic ring was dipped into warmed white beeswax (Eastman Kodak Co. #1126762).

Comparing cell positions and gene expression in the same animal

In order to compare the positions of labeled cells and gene expression patterns during somite shaping in the same animal, embryos from post time-lapse imaging sessions (n=24) were fixed at specific time points within the 90-minute somite shaping window. Bodipy-ceramide labeled embryos were time-lapse imaged then fixed on the microscope stage by adding 4% paraformaldehyde into the culture chamber. Just prior to fixation, z-sections were taken through the segmental plate in the vicinity of the last formed somite, starting from the ventral side of each embryo. Embryos were then in-situ hybridized with

EphA4 and *cMeso-1* probes (kind gifts from Dr. David Wilkinson, and Dr. A. Buchberger, respectively) and the gene expression patterns were imaged with 2.5X-40X magnification using an inverted microscope (Zeiss-Axioplan) outfitted with a high-resolution camera (Roche). Images of the in-situ embryos were taken at the notochord and neural tube levels and then matched with z-sections of the Bodipy-ceramide labeled embryos taken at the last time point before fixation. Images of the bodipy-ceramide labeled cells at the last point in the time-lapse prior to fixation were then overlaid with the images of gene expression for direct comparison of gene expression patterns and cell positions in the same animal.

To compare the spatial position of the 2 genes in the same animal, we split embryos (n=24) along the anteroposterior midline of the neural tube using a glass needle, prior to fixation. Each side was then fixed and an in-situ hybridization performed with one of the 2 gene probes.

Serial sectioning by surface imaging microscopy

Chick embryos (n=8) were processed for surface imaging microscopy at Resolution Sciences Corporation (Corte Madera, California, USA: http://www.resolve3d.com) by standard methods. The embryos were stained for 6 hours in Resolution Standard Stain, then rinsed through 3 changes of PBS, and dehydrated through a series of either methanol or ethanol (25%, 50%, 75%, 100%). Following dehydration, embryonic tissues were equlibrated with a mixture of Resolution Standard Embedding Polymer and an opacifier for 6 hours to allow for cellular infiltration. The embedding polymer was cured for 8 hours at 70°. The cured block was loaded into a surface imaging microscope, sections

were removed by a diamond knife, and images were collected of the blockface that remained. These images were then reconstructed into a digital volume and analyzed in ResView 3.0.