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Whole-organ cell shape analysis reveals the developmental basis of ascidian notochord taper

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ABSTRACT

Here we use *in toto* imaging together with computational segmentation and analysis methods to quantify the shape of every cell at multiple stages in the development of a simple organ: the notochord of the ascidian *Ciona savignyi*. We find that cell shape in the intercalated notochord depends strongly on anterior–posterior (AP) position, with cells in the middle of the notochord consistently wider than cells at the anterior or posterior. This morphological feature of having a tapered notochord is present in many chordates. We find that ascidian notochord taper involves three main mechanisms: Planar Cell Polarity (PCP) pathway-independent sibling cell volume asymmetries that precede notochord cell intercalation; the developmental timing of intercalation, which proceeds from the anterior and posterior towards the middle; and the differential rates of notochord cell narrowing after intercalation. A quantitative model shows how the morphology of an entire developing organ can be controlled by this small set of cellular mechanisms.

Introduction

Embryonic morphogenesis involves the fine spatial and temporal control of many cell parameters, including numerous aspects of cell shape and motility. The ascidian *Ciona* has a stereotyped chordate body plan with a notochord and hollow dorsal neural tube in the context of an embryo small enough to be imaged *in toto* in a single field of view at high resolution. This has led to the emergence of *Ciona* and related ascidian species as model systems for image-based, quantitative studies of chordate morphogenesis *in toto* (Munro and Odell, 2002; Sherrard et al., 2011; Tassy et al., 2006).

Manual image segmentation in 3D involves the laborious tracing of cell outlines in all of the planes of an image stack, and can rapidly become prohibitively time-consuming for more than a modest number of cells. Automated 2D segmentation methods have recently become powerful tools for high throughput image-based screening of cultured cells (e.g. Carpenter et al., 2006; Thomas, 2009), but fully automated 3D segmentation tools are still being developed e.g. Dufour et al. (2005) and Zanella et al. (2009). Here we take a middle path, using an interactive, semi-automated method to segment more than 2000 ascidian notochord cells in 3D.

The ascidian notochord consists of exactly 40 cells that intercalate to form a single-file column that acts as a stiffening element in the center of the tail (Munro et al., 2006). The notochord is one of the defining features of the chordate body plan and, although transient in many species, is typically the first organ to develop (Stemple, 2005). The cell lineages for the ascidian notochord are well established, though there is known to be a transition from completely stereotyped to partially stochastic cell behaviors during notochord cell intercalation (Nishida, 1987). After intercalation is complete, the notochord cells change from being shaped like thin, flat disks to become longer in the anterior to posterior dimension and narrower in the mediolateral dimension (Miyamoto and Crowther, 1985). This process is poorly understood, but is known to involve actomyosin contractility (Dong et al., 2009, 2011). The notochord cells subsequently undergo complex rearrangements that result in them forming an inflated hollow tube running the length of the tail (Dong et al., 2009, 2011).

Our initial goal was to quantify the 3D shape of every notochord cell from the end of intercalation until the onset of tubulogenesis, so as to determine how cell shape varies both spatially within the embryo and temporally from stage to stage. Upon identifying an extremely consistent taper in the intercalated notochord from a wide middle towards narrower tips, we then switched from discovery-driven to hypothesis-driven experiments to determine the cellular mechanisms underlying this phenomenon.

Materials and methods

Imaging

Ciona savignyi eggs were fertilized and dechorionated by standard methods (Veeman et al. 2011). Embryos were fixed in

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2% EM grade paraformaldehyde in seawater, stained with Bodipy-FL phallacidin (Molecular Probes), cleared through an isopropanol series and mounted in Murray Clear (1:2 benzyl alcohol and benzyl benzoate). They were imaged on an Olympus Fluoview 1000 laser scanning confocal using a 40×1.3 na objective. Images were collected with a voxel size of 155 nm in *X* and *Y*, and 300 nm in *Z*.

Staging

The timepoints examined were more closely spaced than the stages of the standard *Ciona* staging series of Hotta (Hotta et al., 2007), so we have presented timepoints as actual minutes of development using the first timepoint in each dataset as $t=0$. Approximate Hotta stages are also given for comparison between datasets. Each of our three main time series datasets (post-intercalation, during intercalation and wt versus *aim*) was generated from a single fertilization with a narrow 10 min fertilization window, giving rise to extremely synchronized embryos. All embryos were grown at approximately 18 °C.

Marker generation

Initial testing showed that marker-assisted watershed produced a good segmentation of notochord cell boundaries. Two types of markers were used: a short line inside each notochord cell and a rough shell around the outside of the notochord. The outer shell did not need to be particularly close to the notochord, but it needed to intersect all of the notochord's neighboring cells without intersecting the notochord itself.

Although a significant improvement over manually tracing the outline of every notochord cell, hand drawing the outer shell remained laborious as it required a rough outline to be drawn in hundreds of *Z* slices. To address this problem, we took advantage of the essentially curvilinear sausage-like shape of the notochord, which can be approximated by fitting a spline to its midline together with a value for the radius at each point on the spline. This can be thought of as the Minkowski sum of the midline spline dilated by a sphere of varying radius. As the notochord is locally quite smooth, only a small number of control points are required to achieve a good approximation by cubic spline interpolation. We implemented a Matlab GUI that allows the user to make a small number of line selections in a 3D slice browser and then builds the Minkowski sum accordingly. New control points can be added interactively to refine the mask as needed, with a typical image requiring one at each end of the notochord and 3–4 in the middle.

The GUI also allows the user to generate the inner markers by making a line selection in the center of each cell. A line across the nucleus was found to give a more consistent segmentation than a point, as it spanned the perinuclear actin blobs that otherwise occasionally perturbed the segmentation.

Cell segmentation

Image volumes were resized by cubic spline interpolation to give isotropic 0.3 μm voxel sizes, and then smoothed by coherence enhancing diffusion (CED) (Weickert, 1999) using the implementation by Kroon and Slump (2009) modified to enhance planar rather than curvilinear features. Notochord cells were segmented by the seeded watershed transform (Meyer and Beucher, 1990; Vincent and Soille, 1991), using markers interactively generated as discussed above. The seeded watershed algorithm then propagates away from those markers to identify the notochord cell boundaries. When necessary, the markers were manually refined until an acceptable segmentation was achieved. The algorithm returns a label matrix *L* in which the voxels belonging to each of *J* watershed domains are labeled $1\ldots J$, and the watershed surfaces between domains are labeled 0. For convenience, we reorder the label matrix so that the index $j=1$ for the anteriormost cell through $j=40$ for the posteriormost cell, with the "not-notochord" segment labeled $j=41$.

Cylindrical model fitting

For post-intercalation stages, we fitted a cylindrical model to each cell to robustly measure height and diameter in three dimensions. Principal component analysis (PCA) was used to identify the cylinder axis, now denoted $\hat{\mathbf{e}}_{\mathrm{cyl}}$, of each cell as the eigenvector best aligned with the vectors between that cell's centroid $\mathbf{c}$ and its anterior and posterior neighbors. For each cell j we used binary morphology to subsegment it into its anterior and posterior surfaces (the tops and bottoms of the cylinder, which contact other notochord cells) and its lateral surfaces (the sides of the cylinder, which contact the flanking tissues).

$$L_j^{\mathrm{lat}} = (L_j \oplus B) \cap (L_{41} \oplus B)$$

$$L_j^{\mathrm{ap}} = (L_j \oplus B) \cap (L_{j+1} \oplus B) \cup (L_j \oplus B) \cap (L_{j-1} \oplus B)$$

where B is a $3\times3\times3$ structuring element and $\oplus$ is binary dilation.

Mean height was measured by calculating twice the mean of the closest distance from each point in the anterior and posterior surfaces to a plane through the cell centroid orthogonal to the cylinder axis.

$$\overline{h} = \sum_{m=1}^{M} \frac{2\left|\hat{\mathbf{e}}_{\mathrm{cyl}}\cdot(\mathbf{c}-\mathbf{q}_m)\right|}{M} \text{ for } \mathbf{q}_m = \begin{bmatrix} x_m \\ y_m \\ z_m \end{bmatrix} \in L_j^{\mathrm{ap}}$$

Mean radius was measured by calculating the mean of the closest distance from each point in the lateral surfaces to a line defined by the cell centroid and the cylinder axis.

$$\overline{r} = \sum_{k=1}^{K} \frac{\left\|\hat{\mathbf{e}}_{\mathrm{cyl}}\times(\mathbf{c}-\mathbf{p}_k)\right\|}{K} \text{ for } \mathbf{p}_k = \begin{bmatrix} x_k \\ y_k \\ z_k \end{bmatrix} \in L_j^{\mathrm{lat}}$$

Scripts for these analyses were all implemented in Matlab. As the first and last notochord cells are shaped more like bullets than cylinders, they were excluded.

Microsurgery

For tail cut experiments the tail was cut in half at its approximate midpoint using a fine glass needle. The resulting fragments were gently moved apart so they did not reattach and then cultured for two hours before being fixed, stained and imaged.

Intercalation timing

Each cell was manually scored as being intercalated if its contacts with non-notochord cells formed a closed ring, and not intercalated if these contacts formed only a segment of arc. For this series of images, we measured notochord diameter manually in ImageJ by fitting circular regions of interest (ROIs) to orthogonal notochord cross-sections resliced at a given cell position.

Notochord taper in other chordates

Notochord taper was examined in lamprey and amphioxus larvae using commercial prepared specimens, and in zebrafish larvae kindly provided by Jungho Kim and Michael Liebling.