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PAPER

Why and how the nematode's early embryogenesis can be precise and robust: a mechanical perspective

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Abstract

The early embryogenesis in the nematode *Caenorhabditis elegans* is well-known for its stereotypic precision of cell arrangements and their lineage relationship. Much research has been focused on how biochemical processes achieve the highly reproducible cell lineage tree. However, the origin of the robustness in the cell arrangements is poorly understood. Here, we set out to provide a mechanistic explanation of how combining mechanical forces with the order and orientation of cell division ensures a robust arrangement of cells. We used a simplified mechanical model to simulate the arrangement of cells in the face of different disturbances. As a result, we revealed three fail-safe principles for cell self-organization in early nematode embryogenesis: ordering, simultaneity, and the division orientation of cell division events. Our work provides insight into the developmental strategy and contributes to the understanding of how robust or variable the cell arrangement can be in developing embryos.

Introduction

Caenorhabditis elegans, a widely-used model organism, is well-known for its invariant cell lineage tree [1, 2] and the conserved spatial behavior of cells such as the position of individual cells, the orientation of cell division and the cell migration trajectory in embryogenesis [3]. These features allow the use of nematode embryos as research models in which to investigate the mechanisms underlying the robustness of cell arrangement.

Embryonic development is usually studied at the biochemical level. The genetic and molecular mechanisms of cell arrangement in the early embryogenesis of *C. elegans* have been extensively studied [4–7]; for example, PAR (partitioning-related) proteins and the Wnt signaling pathway have been established as critical functional players in asymmetric division and axis formation [6, 7]. However, biochemical signaling pathways are not the sole determinants of cell arrangement. The mechanical interactions between cells and the spatial constraints of the embryo also affect cell alignment [8–10].

In the nematode embryo, the role of mechanical cues during developmental processes has been established very recently [11–13]. A mechanical model, in which cells interact via repulsive forces within the confining eggshell, has been proposed to explain the invariant cell positioning and cell migration paths [11]. Based on this model, several groups investigated the robustness and diversity of cell arrangement in the early embryogenesis of *C. elegans* [12, 13]. A limiting-component model can quantitatively capture the anti-correlation of cell volumes and cell-cycle times [12]. Combining mechanical forces with both cell volumes and cell-cycle times, the model predicts aberrant phenotypes caused by cell divisions that succeed each other too rapidly. Another model has demonstrated that the diverse patterns of four-cell arrangement are produced by the diverse shapes of eggshells [13]. Although these models represent some features of the system, they do not delve into the relationship between the mechanical

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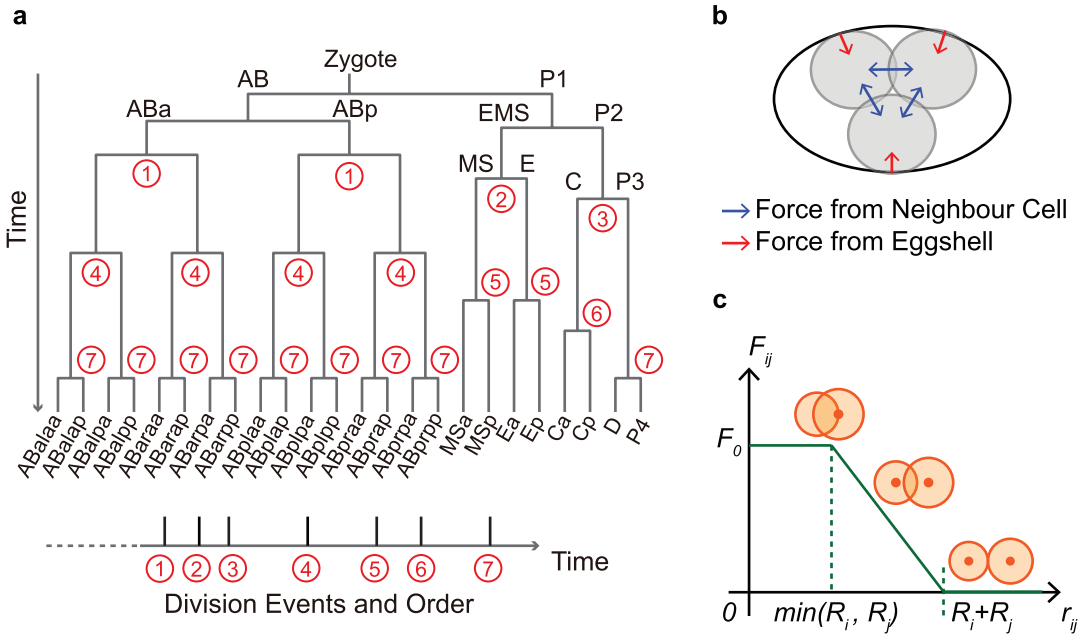

Figure 1. (a) Cell lineage illustrating the distinct order and simultaneity (data from Guan *et al* [3]). 1, 4, 5, and 7 indicate simultaneous multicellular division events. (b) Schematic of the mechanical model showing the repulsive forces between cells (blue arrows) and between cells and the eggshell (red arrows). (c) Relationship between spring-like repulsive force (F_{ij}) and cell–cell distance (r_{ij}) of two cells of different radius (R_i, R_j).

properties of the embryo and its intrinsic lineage, such as how mechanical interactions between cells and the coordination of conserved division events influence the final cell arrangement.

In this work, we focused on the mechanical robustness of cell arrangement against perturbation of the ordering, simultaneity, and orientation of cell divisions up to the 24-cell stage in *C. elegans*. The purposes of this study were: (1) to elucidate why cell divisions are separately grouped by ordering and simultaneity, (2) to test whether the division orientation is adjusted to a targeted direction with limited variability, and (3) to determine whether and how the cell division events account for the precision of cell arrangement.

Results

During the early embryonic development of *C. elegans*, the timing of divisions from the 4- to the 24-cell stage exhibits elaborate temporal coordination [3]. In addition, the direction of cell division is not arbitrary and is well controlled within a certain range [3]. Here, we selected 3 single-cell division events to study how division orientation influences the cell arrangement, and 4 sets of simultaneous multicellular division events and 6 pairs of events that maintained an invariant order to investigate why cell divisions are separately grouped by ordering and simultaneity (figure 1(a), supplementary tables S1 and S2 (stacks.iop.org/PhysBio/17/026001/mmedia)). Note that in this work, all experimental data and the grouping of division events were based on our previous work [3].

In order to elucidate the robustness of the cell arrangements, we used an existing mechanical model to study the roles of order and orientation of divisions in cell self-organization at the mechanical level. The model developed by Fickentscher *et al* assumes that cells in early embryos can be approximated as soft spheres and only considers repulsive forces between the cells and between the cells and the eggshell [11, 12]. Given the direction, timing, and volume ratio of cell division, this model can reproduce the cell arrangements and migration trajectories of developing embryos up to the 24-cell stage. In this report, we used the same systematic parameters as used by Fickentscher *et al* (segmentation ratio of cell volume and the force coefficient) to simulate cell arrangements (see Methods for details) [11]. Besides, cell–cell neighboring relationships were defined based on the presence of repulsive forces. To measure the differences in cell arrangement between normal and abnormal conditions, we defined accuracy as N_s/N_t (N_s: number of the contacted and uncontacted cell pairs which are the same as the normal case, N_t: total number of the possible cell pairs). It is worth noting that the model we used is not sensitive to the parameters. We have tested force parameters and found that only simulation time changes, which do not affect our final conclusion (figure S4).

Reversal of order between two division events

First, we set out to determine whether the order of cell division can be changed to achieve a specific cell arrangement. After using the three strategies of normal

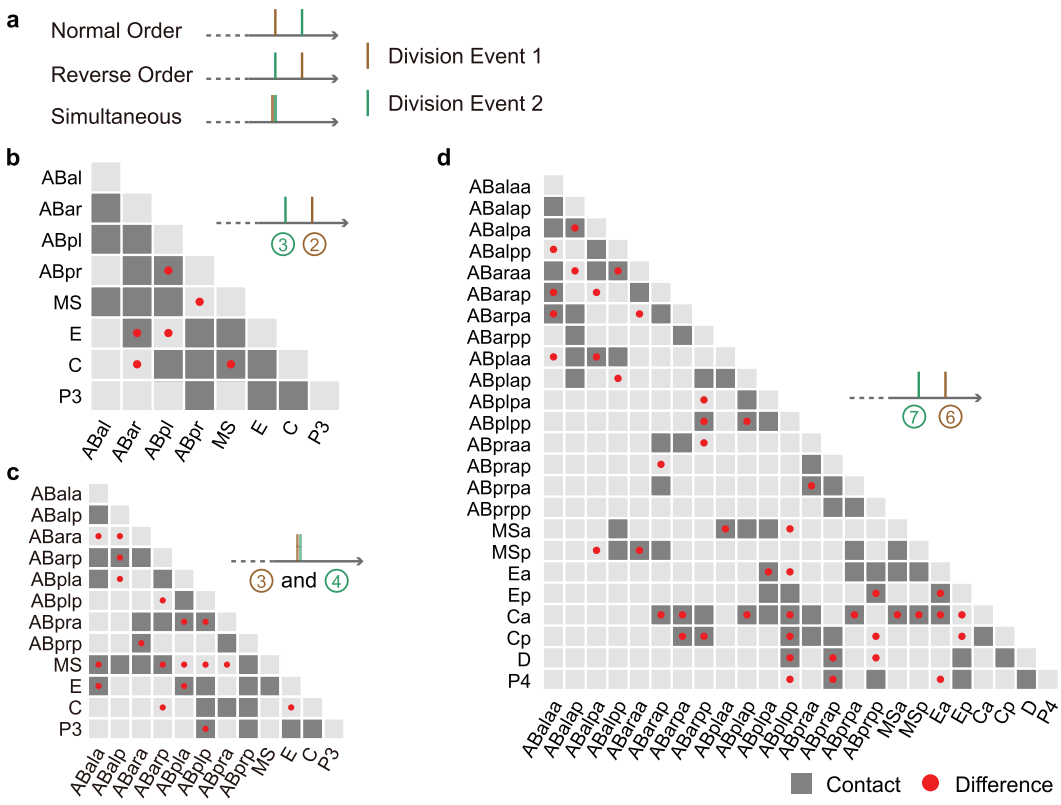

Figure 2. Simulation of perturbation of division ordering. (a) Three permutations for two division events. (b) Cell–cell contact map of 8-cell stages after changing the sequence of events 2 and 3. Gray squares, contact between cells; light gray, no contact; red dots, difference from normal order. (c) Cell–cell contact map of 12 cell stages after synchronizing events 3 and 4. (d) Cell–cell contact map of 24 cell stages after reversing the sequence of events 6 and 7.

Table 1. Changes of division sequences.

Division events[a]	Check stage	Accuracy of model prediction[b]	
		Reverse order (%)	Simultaneity (%)
1,2	7 cells	80.95	100
2,3	8 cells	78.57	100
3,4	12 cells	84.85	72.73
4,5	14 cells	93.41	95.60
5,6	15 cells	90.48	90.48
6,7	24 cells	83.70	84.78

[a] Sequence number of the division event as in figure 1(a).
[b] Accuracy is defined as N_s/N_t).

Table 2. Synchronous division events.

	Division event	Ratio of dividing cells[a]	Tolerance time (s)[b]
1	ABx (1)	2/4	>1080
2	ABxx (4)	4/8	45
3	MS&E (5)	2/12	309
4	ABxxx&P3 (7)	9/15	16

[a] Ratio of dividing cells: (Number of cells that are about to divide) / (Number of total cells in the embryo).
[b] Tolerance time is the maximum interval between the first and last dividing cell that can achieve the same cell pattern as the synchronous division case in all possible division sequences.

order, reverse order, and simultaneity (figure 2(a)) to simulate six pairs of division events, we found that only two pairs produced the same structure as the normal order in the case of simultaneous division, while all others led to changes in cell positioning and the relationship of cell–cell contact (table 1). Note that although we did not make continuous changes in time in this part of the simulation, which we have done in the next section, we can expect to see a sudden change in accuracy. The reason for this is that, for a cell, there may be a relatively sensitive location on its expected motion trajectory, so that the interference caused by the division of another cell will be amplified around at this particular time.

Although *C. elegans* develops in a lineage-based manner, i.e. the cell-fate decisions are made upon cell division as the lineage tree expands, cell–cell contact is another essential mechanism involved in fate decision in early development. Abnormal contacts would influence the fate of the cells, and form a chain-reaction of errors. Some important embryonic induction events, such as the primary induction of left-right asymmetry at the 12-cell stage, need the robust and continuous contact of specific cells, e.g. the MS cell uses Notch signals to instruct the fate of the ABalp but not the ABala cell [14, 15]. A change of the order of division between

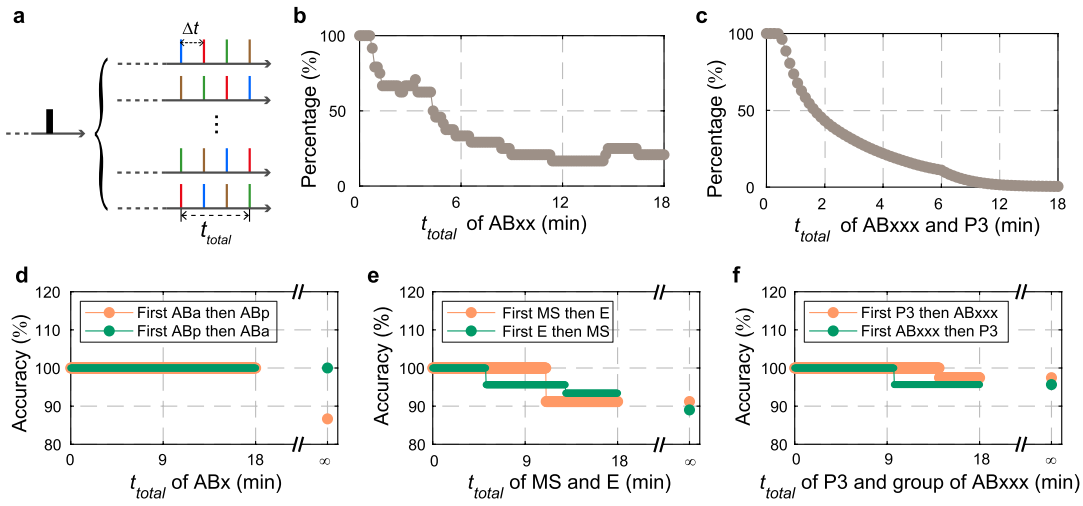

Figure 3. Simulations of perturbation of division simultaneity. (a) Schematic of the perturbation of synchronous divisions. All possible permutations of dividing cells were considered. In each sequence, any two consecutive division events were equally spaced (Δt is equal). The t_{total} is defined as the total time interval between first and last dividing cell. (b) and (c) Percentages of scrambled division sequences, that led to the same final cell arrangement as in the synchronous division case, among all possible permutations with respect to the total time interval (t_{total}). Division event 4 (ABxx) in b; division event 7 (ABxxx and P3) in c. (d) Division event 1 (ABx). In this case, order 2 (first ABp and then ABa) was more robust than order 1 (first ABa and then ABp). ∞, time required for the system to reach a steady state. At this point, no changes in cell position are possible. (e) Division event 5 (MS and E). In this case, order 1 (first MS and then E) was more robust than order 2 (first E and then MS). (f) The division simultaneity of the P3 cell with the ABxxx cells is more relaxed compared with that of ABxxx cells themselves.

P2 and ABxx caused both the ABalp and ABala cells to contact the MS (figure 2(c)), which may lead to an ABalp-fate not only in ABalp itself but also in the ABala cell (figure 2(c)). What is more, the well-known signaling from C to ABar by the Wnt/β-catenin pathway, which functions in spindle formation [7, 17], was interrupted when the division ordering between EMS and P2 was reversed (figure 2(b)). Our simulation results provide theoretical evidence for the importance of an invariant order of division.

Simultaneity-breaking in division events with more than one cell

In the wild-type, each of the founder cells (AB, MS, E, C, D, and P4) establishes a lineage with a distinct cell-cycle period. Most of the descendants of any one founder cell subsequently undergo roughly equal and synchronous divisions, especially in the AB lineage (table S2). Nonetheless, there are cases in which the descendants of different founder cells enter mitosis nearly synchronously, although they have very different cell cycle lengths. For example, the P lineage expresses longer cell-cycle periods than the AB lineage, while P3 and ABxxx cells divide at almost same time [3] (figure 1(a)).

To determine whether the simultaneity can be broken without affecting the final cell arrangement, we made the synchronous divisions asynchronous. Specifically, we increased the time interval of the synchronous division group so that the divisions in the group take place asynchronously within a time window. We performed simulations on four groups of synchronous division events and found that the cell arrangement did not change in a limited time window (table 2, figure 3).

We exhausted all the possible sequences of division for each set of multi-cellular division events. As the number of dividing cells increased, the maximum time interval that still resulted in a normal cell pattern became smaller. Besides, the final structure was sensitive to the order and the time interval of cell division. For example, the sequence MS and then E division events had a larger maximum time interval to achieve the normal cell pattern than the sequence E and then MS. Interestingly, in agreement with this model prediction, the experimental data have shown that although MS and E divide at almost the same time, 70.7% of wild-type samples showed that MS cells divide ahead of E and none the other way around [3] (figure 3(e)).

The occurrence of an abnormal cell arrangement is due to changes in the force fields. On the one hand, in the case of synchronous division events, daughter cells are situated in somewhat unstable, stressed positions immediately after the division. Increasing the time interval between two consecutive divisions provides more time for daughter cells to relax. On the other hand, when the division interval increases, the position of the next dividing cell will also change accordingly. This change will have a different effect depending on the division sequences. For example, in the case of division event 1, the sequence ABp and then ABa had no effect on the final cell arrangement, while the sequence ABa and then ABp did have an effect (figure S1). Briefly, the cells were exposed to different force fields and this eventually led to an incorrect cell arrangement. Thus, embryogenic failures can emerge via a slightly altered

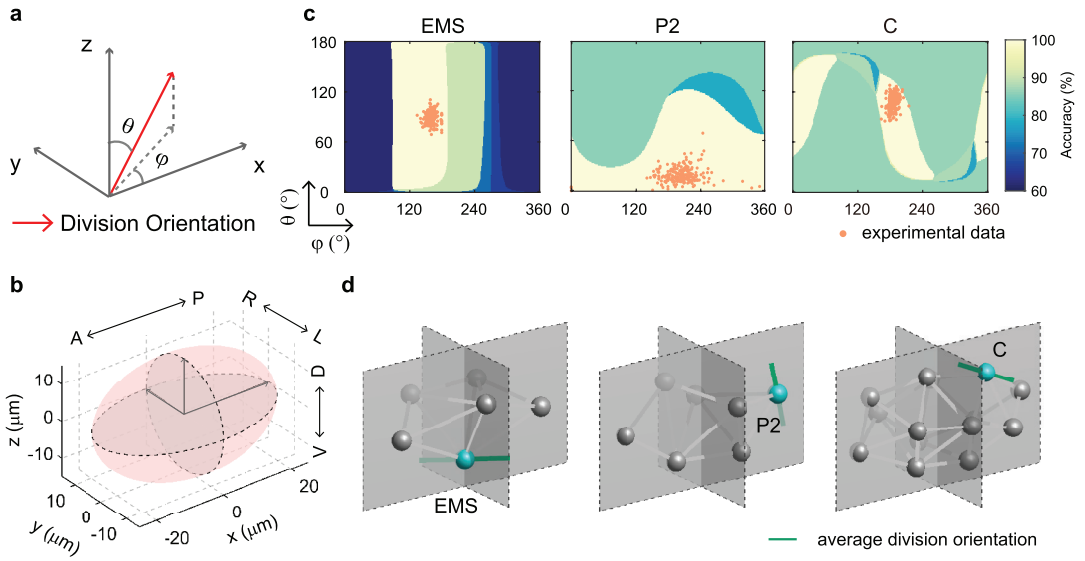

Figure 4. Simulation of perturbation of division orientation. (a) Schematic representation of the division orientation. φ, counterclockwise angle in the *x*-*y* plane measured in degrees from the positive *x*-axis; θ, angle in degrees between the positive z-axis and division orientation. (b) Schematic representation of the eggshell and three body axes. A–P, anterior–posterior axis; D–V, dorsal–ventral axis; L–R, left–right axis. (c) Accuracy map for perturbation of division orientation of the single-cell division events (EMS, P2, and C). Also plotted are the experimental data of 222 wild type embryos from [3] (orange dots). (d) Cell pattern at the time of the division event of the green cell. Green axis indicates the average division orientation of 222 embryos.

timing of cell division. Our simulation results suggest that nematodes have evolved a strategy to establish a specific reproducible cell pattern based on force fields.

The tolerance time window for the division event 7 (ABxxx and P3) is very small, a mere 16 s (table 2 and figure 3(c)). Note that the P3 cell is not neighboring most of the ABxxx cells. We thus investigated whether this narrow window of tolerance is due to the requirement of simultaneity within the ABxxx division events or between ABxxx and P3. We divided these nine cells into two groups by lineage, one group for the eight ABxxx cells and the other group for the P3 cell. Cells were assumed to divide simultaneously within each group. We observed that the tolerance time window between the two groups' division events was much longer (figure 3(f)), implying that the division simultaneity within the ABxxx group (which are also closely spaced spatially with each other) is a much stricter requirement.

Orientation change of cell division axis

The orientation of division is one of the important features in morphogenesis. Previous studies have shown that the variability of orientation in the early embryo development of *C. elegans* is small. However, the relationship between the direction of cell division and cell arrangement has not been fully elucidated. We therefore tested whether the division orientation is adjusted to a targeted direction with limited variability to robustly achieve a specific cell arrangement. In order to exclude the effects of other division events, we studied perturbation of the division axes of single-cell division events (EMS, P2, and C). In all the cases, we calculated the accuracy of cell arrangement for each division vector (φ, θ). The simulations showed a limited number of possible and stable terminal states of cell arrangement (figure 4).

We found that the variable range of cell division axis depended on the cell's location in the embryo. For example, the EMS cell divided at 6-cell stage, and it was located at the ventral side (figure 4(d)). So the division orientation was not sensitive in the θ direction. The φ direction, which determines the positions of the daughter cells along anterior-posterior and left-right axes, was more important. In the case of P2 division, it happened at 7-cell stage, and P2 was located at the posterior end. As long as the daughter cell C was located more dorsal than the daughter cell P3, which is determined by θ direction, the final cell pattern was guaranteed. As for the C cell, it was located in the upper middle part of the embryo surrounded by more cells. Its range of division orientation was more confined. As a test of our model, we plotted the experimentally observed division orientation for 222 embryos [3] in figure 3(d). They all fell into the allowed tolerance ranges of the model.

The accuracy map of C cell showed perfect symmetry while EMS and P2 did not. The main reason was the different volume of daughter cells and the way we named them. In the case of the C cell, the daughter cells had the same cell volume, and Ca cell was always more anterior, so the vector (φ, θ) had same accuracy as vector $(180° + \varphi, 180° - \theta)$. However, in the case of P2 and EMS, their daughter cells had different volumes due to the asymmetric division, so even though we named the daughter cells based on their relative positions, not on the division vector (oriented from P3 to C or E to MS), there was still some asymmetry (figure S2).

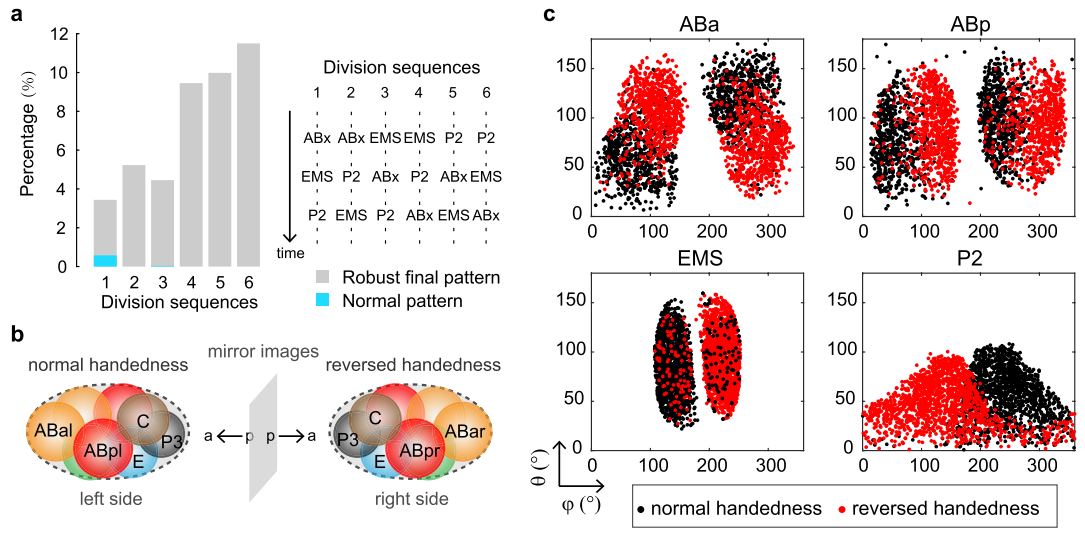

Figure 5. (a) Quantification of reproducible final 8-cell pattern in six division sequences. Left, percentages of the parameter sets that produced a robust cell pattern among 500 000 sets of parameters for each division sequences. Gray, all possible robust cell patterns; blue, the normal cell pattern. Right, six division sequences. (b) Schematic representation of the cell arrangement at the 8-cell stage with different handedness. (c) Distribution of division vectors that produce a normal cell arrangement (black dots) or its mirror image (red dots), for the division sequence ABx, EMS, and then P2.

This asymmetry disappeared only if the daughter cell volumes were also set to be equal.

What is more, to quantify the division-angle variation, we defined the largest cone region surrounding the division vectors that produced the normal cell arrangement. The half angle of the cone region was 50° in EMS, 69° in P2, and 29° in C. Interestingly, we found that their experimental values were 26.10°, 36.73°, and 28.32°, respectively [3]. These experimental values were smaller than the simulation predications, suggesting the existence of a biological mechanism controlling the orientation of division. In fact, a number of different biological mechanisms controlling the direction of division have been reported [4–7, 16–18]: maternal effect mutations in five par genes result in errors of spindle position and orientation [4–7]; contact-dependent mitotic spindle orientation appears to work by establishing a site of the type described by Hyman and White in the cortex of the responding cell [16–18]. These works have studied the mechanism of cell division direction and explored the importance of the correct division direction from the perspective of cell fate differentiation. Our numerical simulations give some insight from a mechanical perspective to help us understand the importance of cell division for proper cell alignment.

4-cell stage to 8-cell stage

Above, we simulated cell positioning with different perturbations of the order and simultaneity of divisions and division orientation separately. We found that the order and orientation of division were carefully designed to prevent errors. In the next step, we considered these factors together to determine whether this well-designed developmental process is only one solution among many for an animal to achieve a specific pattern robustly and reproducibly.

We simulated cell positioning from the 4-cell stage to the 8-cell stage, during which there are three division events, ABx (in which there two simultaneous divisions), EMS, and P2. For simplicity and due to computational limitations, we only examined the influence of division sequences and division orientation. Once we had determined the division sequences, the division orientation of each cell in each simulation was randomly selected (except division order and division axis, all other parameters, such as cell size partitioning remain the same). After each division, all cells were allowed to migrate to their final stable positions before the next division event. And we tested the robustness of the cell arrangement in the selected cell division orientation with 20° variations. We defined a good solution (a set of parameters including division order and a conical region with half-angle of 20° for each cell division orientation) as one that robustly achieved a final 8-cell stage pattern.

For each division sequence, we simulated 500 000 sets of parameters uniformly sampling the division orientations and found that no more than 12% produced a robust final pattern (figure 5(a)). For example, for the division sequence ABx, EMS, and then P2, only 3.44% of the parameters produced a robust cell pattern. That means to achieve a reproducible cell arrangement requires tight control over the direction of cell division.

We then compared the results with the normal 8-cell pattern computed with the experimental parameters and found that only two division sequences produced this target pattern or its mirror image. One was the same as the normal sequence (ABx, EMS, and

then P2); the other was the sequence EMS, ABx, and then P2. Among all the parameters that produces the target pattern or its mirror image, 94.55% belonged to the normal sequence. This result suggested that, in addition to tight control over the direction of division, proper division order is equally important for achieving a specific cell arrangement. Interestingly, the most robust cell pattern (in the sense that it can be produced with the largest sets of parameters) is not the normal pattern of *C. elegans*. In total, we found there were 77 different stable cell patterns, among which there were 27 pairs of mirror image. The normal pattern was the 12th most robust pattern for all cell division orders, but the 1st for the division order #1 (the normal order).

In *C. elegans*, bilateral asymmetry first arises during the 4-cell to 8-cell stage. Previous studies have shown that the handedness of the embryo is determined by the direction of the ABa/ABp spindle skew [19, 20], and the chiral morphogenesis is timed by the cleavage furrow of EMS [20]. In line with these experimental results, our simulations showed that the handedness was affected by the division orientation of ABa/ABp (figures 5(b) and (c)). Also, the correlation between the handedness of the embryo and the division orientation of EMS/P2 suggests that the completion of chiral morphogenesis requires the proper division orientation of EMS/P2. Previous study showed that the actomyosin cortex generates active chiral torques to facilitate chiral skew event at the 4-cell stage [21]. Thus, whether the EMS/P2 division events might also be driven by the active torque generation in the actomyosin layer remains to be confirmed.

Discussion

In summary, we simulated the cell arrangements with perturbations of the order and simultaneity of divisions and division orientation. Our findings revealed that temporal and mechanical heterogeneity lead to spatial heterogeneity. To achieve a specific pattern robustly and reproducibly, cell division must be regulated and coordinated by three simple fail-safe principles, as follows: (1) cell division events must be divided into groups in a distinct order and with enough time between them to ensure that the system can stabilize; (2) cells in the same group must be coordinated to divide within a short time-window to avoid any cell occupying the expected location of another in advance; and (3) division orientation must be adjusted to a targeted direction with limited variability, to avoid the possibility of incorrect positioning and cell–cell relationships.

C. elegans has evolved mechanisms for selecting a stable path to a specific cell-positioning state, which satisfies the requirements for cell positioning and specific cell–cell contact. To balance the trade-off of proliferation rate, cell-positioning robustness and differentiation, the combination of simultaneous divisions and ordered divisions evolved and was maintained in the early embryogenesis of nematodes. In short, controlling the ordering and simultaneity of division and division orientation ensures the cell-positioning organization in *C. elegans*, both rapidly and precisely. This can be regarded as a set of simple and valuable engineering strategies to reproducibly form a multi-cellular pattern under specific spatial restraints.

Finally, it is worth noting that our conclusions, which are based on numerical simulation, are consistent with previous experimental work based on mutants or using laser irradiation technology [22–24]. Those works suggested that the normal sequence of cell divisions is required for proper cellular positioning. However, those earlier mutant analyses and micromanipulation experiments were focused on embryo development after the 12-cell stage. Timing errors (only slower division rate, no change of division order haven been reported so far) before 12-cell stage would usually lead to an embryo arrest. An elaborate comparison between our simulation results and the perturbation experiments of high precision would be an interesting future direction.

Materials and methods

Construction of the model and computer simulations

The simulations of cell arrangement within the confined space of the eggshell were constructed by modifying a model developed by Fickentscher and colleagues [11]. The simulations were programmed in MatLab and the source code is available upon request.

The model considered all cells to be soft spherical balls with radius R_i ($i = 1, 2, \ldots, N$; with N denoting the total number of cells). And the center of mass was represented by position $\boldsymbol{r}_i$. The eggshell was considered to be an ellipsoid with axes $\ell_x = 25\,\mu$m, $\ell_y = \ell_z = 15\,\mu$m. The center of the eggshell was the origin, and the long axis coordinates were on the x-axis.

The model assumed that the cells moved in a viscous environment and there were only two types of repulsive force: those between neighboring cells and those from the eggshell. The cell position was calculated by a discretized overdamped Langevin equation (integration time step $\Delta t = 1$ s):

$$\boldsymbol{r}_i(t+\Delta t) = \boldsymbol{r}_i(t) + \left(\frac{\Delta t}{\gamma}\right)\left(\boldsymbol{K}_i + \sum_{j(j\neq i)}(\boldsymbol{F}_{ij})\right).$$

Here, we ignored the cell motion noise, because it interfered with our analysis of the simulation on the synchronous division and division orientation.

The cells experience a repulsive force $\boldsymbol{K}_i$ when touching the eggshell:

$$\boldsymbol{K}_i = K_0(1 - a/R_i)\boldsymbol{e}_n.$$

Here, $\boldsymbol{e}_n$ denotes a unit vector perpendicular to the eggshell, oriented into the cells, and a is the minimum

distance between cell i and the eggshell. For $a > R_i$, $\boldsymbol{K_i} = \mathbf{0}$.

The forces between any two cells i and j were defined as:

$$\boldsymbol{F}_{ij} = F_0 \boldsymbol{e}_{ij} \begin{cases} 1, r_{ij} \leqslant min\left(R_i, R_j\right) \\ \frac{R_i + R_j - r_{ij}}{max(R_i, R_j)}, min\left(R_i, R_j\right) \leqslant r_{ij} \leqslant R_i + R_j \\ 0, otherwise. \end{cases}.$$

Here, r_{ij} is the distance between their centers, $\boldsymbol{e}_{ij}$ is the unit vector pointing from cell i to cell j. We used same parameters as Fickentscher [11] ($K_0/\gamma = 0.2\ \mu$m/s and $F_0/\gamma = 0.1\ \mu$m/s).

Cell divisions and cell volumes

The division axes of all cells used in simulations were based on the average experimental data from our previous work [3]. The radii of daughter cells were chosen to be identical for symmetric divisions, while for asymmetric divisions, the volume ratio was set at 2:3 [11]. Throughout a simulation, the total volume of all cells was fixed to the egg volume.

All simulations were started at the 4-cell stage, and they were positioned at a diamond-type arrangement [4, 13]. The division times of the cells involved in the simulations were set to the averages from the previous experimental data [3]. However, it was impossible to determine the true division time after switching the order, so we assumed that before and after these division events occurred, the system stabilized in a steady state.

Classification of the cell arrangement patterns

The patterns of cell arrangement were classified based on cell–cell neighboring relationships. The cells were considered to be in contact only when there were repulsive forces between them. To measure the cell arrangement differences between normal and abnormal conditions, we defined accuracy as N_s/N_t, where N_s is the number of contacted and uncontacted cell pairs which are the same as the normal case, and N_t is the total number of possible cell pairs. For example, if there are n cells in the eggshell and the pattern is the same as the normal case, the N_t will be equal to C_n^2 and N_s will be equal to N_t, so the accuracy of the normal pattern will be 100%.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

CT and BT conceived and designed the projects. BT performed model simulations. BT and GG analyzed data. BT and CT wrote the manuscript; LT co-supervised the projects.

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