Establishment of morphological atlas of *Caenorhabditis elegans* embryo with cellular resolution using deep-learning-based 4D segmentation

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Cell lineage consists of cell division timing, cell migration and cell fate, which are highly reproducible during the development of some nematode species, including *C. elegans*. Due to the lack of high spatiotemporal resolution of imaging technique and

reliable shape-reconstruction algorithm, cell morphology have not been systematically characterized in depth over development for any metazoan. This significantly inhibits the study of space-related problems in developmental biology, including cell segregation, cell-cell contact and cell shape change over development. Here we develop an automated pipeline, CShaper, to help address these issues. By quantifying morphological parameters of densely packed cells in developing *C. elegans* embryo through segmentation of fluorescence-labelled membrane, we generate a time-lapse framework of cellular shape and migration for *C. elegans* embryos from 4- to 350-cell stage, including a full migration trajectory, morphological dynamics of 226 cells and 877 reproducible cell-cell contacts. In combination with automated cell tracing, cell-fate associated cell shape change becomes within reach. Our work provides a quantitative resource for *C. elegans* early development, which is expected to facilitate the research such as signaling transduction and cell biology of division.

1 Introduction

2 Embryogenesis in metazoans is a spatio-temporal biological process formed by a series
3 of multicellular structure evolution including proliferation and morphogenesis. As “eu-
4 tely” *C. elegans* has invariant and reproducible cell lineage consisting of division tim-
5 ing, migration trajectory and fate specification for each cell¹, it has been wildly used as

an animal model for developmental biology research². Thanks to advanced imaging equipment with single-cell resolution as well as automatic cell-tracing softwares³⁻⁵, a few researchers have made great effort to quantitatively reconstruct its developmental atlas in several dimensions of developmental properties, including cell division timing⁶, gene expression and morphogenesis^{7,8}, cell-cell contact mapping and signaling^{9,10}. Despite all this, little is known about cell morphology experimentally and systematically, due to lack of high-resolution cell membrane signaling marker and reliable imaging-based algorithm for cell segmentation, in particular for late stage which has hundreds of cells^{11,12}. Cell morphology (e.g. cell shape, cell volume, cell-cell contact) is also a set of critical developmental properties and information for metazoan embryogenesis, which is tightly correlated to cell-cycle control¹³⁻¹⁵, spindle formation¹⁶, cell-fate symmetry breaking and differentiation^{17,18}, intercellular signal transmission^{10,12,19,20}, cytom mechanics and morphogenesis²¹⁻²⁴, etc.

Recent studies also emphasized the necessity of 3-dimensional cellular segmentation aside from the nucleus^{25,26}. With large quantities of volumetric data involved in the embryogenesis of *C. elegans*, visual inspection on these images is time-consuming and error-prone. Computer-assisted analysis accommodates the difficulty in large-scale image segmentation, paving the way to efficient and accurate researches. Compared to manual annotation, automated image analysis has better objective quantification, consistency and

25 reproducibility. Confocal microscopy is popularly used in 3D imaging, which allows op-
 26 tical section in tissue or even cells at different depth. Whereas large quantities of works
 27 have been proposed to segment nuclei or cells in 2D^{27–30}, cell’s morphological features
 28 varies from those in 3D. Without information between slices, simply stacking 2D seg-
 29 mentations into 3D volume may yield misalignment in the depth direction. Some recent
 30 methods have addressed cell tracing based on nucleus information³¹, however, they can
 31 hardly characterize cell geometry information (e.g. cell volume, cell surface area, cell
 32 topology, cell-cell neighbour relationship). The performance of 3D cell segmentation suf-
 33 fers from three factors. First, unlike the nuclei, which are thick and separated ellipsoid
 34 components, cell membranes are thin planar structures, forming complicated networks
 35 by contacting with each other. Second, compared to plant tissue, highly dynamic cellular
 36 morphology in *C. elegans* limits the application of diverse techniques based on deformable
 37 model. Furthermore, as shown in Fig. S1A, laser attenuation makes the segmentation more
 38 challenging in deeper slices. Theoretically, large exposure times can improve the image
 39 quality. Poisonous laser rays, however, could harm or even kill cells in time-lapse imaging
 40 process.

41 In the last few decades, Several attempts have been made to leverage the segmenta-
 42 tion performance on microscopy. Classical techniques are based on pre-defined model and
 43 image intensity features. Among these, active contours and level sets are two of the most

44 compelling methods in segmenting microscopy. Active contour deals with segmentation
45 as a energy minimization process where the image forces pull the contour toward the ob-
46 ject boundary and internal forces resist the deformation. Different evolution equations,
47 mediating the internal and external forces, are designed to control the deformation process
48 precisely^{32–35}. To diminish the difficulty in finding desirable forces representation, level set
49 is used to embed the boundary curve as a real-valued solution of an equation, which makes
50 it straightforward to follow topology changes, such as splits and holes. By using coupling
51 constrains in level set evolution, Nath et al. proposed a computationally efficient method
52 to segment hundreds of cells simultaneously²⁷. Kiss et al. used level set to segment plant
53 tissues at multiple scales, which reduced the error at blurry surface effectively³⁶. Instead
54 of processing multiple slices in 3D simultaneously, Sharp et al. described the possibility
55 of inferring 3D shape features indirectly from 2D images in a statistical way³⁷. In practice,
56 however, the performance of level set is limited by considerable computational complexity
57 and incomplete cell boundary. Methods driven by energy functions could be problematic
58 due to the presence of artifacts and lack of strong edge information. Xing et al. provides
59 a compressive review about classical cell segmentation techniques³⁸. Data-dependent and
60 parameterized pre-processing stage is always needed in these methods, otherwise the sys-
61 tem would be exposed to under-or-over segmentation errors.

62 Recently, deep learning based methods provide a promising tool for recognition

tasks, such as denoising^{39–42}, tumor segmentation^{43,44} and image synthesis^{45–48}. Compared to classical methods, convolutional neural network (CNN) shows remarkable improvement on biological image analysis by mining subtle texture and shape changes. Since U-net was proposed by Ronneberger et al.⁴⁹, this kind of encoder-and-decoder structure has extensively promoted learning-based segmentations on medical images⁵⁰. For fluorescent images, the ability of deep learning in discriminating and filtering useful information is further verified^{48,51}. To mitigate the complexity in cellular networks, the segmentation is usually decomposed into multiple intermediate tasks, such as nucleus detection and membrane segmentation^{2,52,53}. 3D convolution attracts increasing attention because of its advantage in combining multi-directional information. However, 3D deep network relies heavily on computation resource and training data. Some works are proposed to alleviate the computational complexity in 3D^{54–57}.

In this work, we propose a complete pipeline CShaper for analyzing cellular shapes in *C. elegans*. Deep learning is utilized to accommodate the complications associated in volumetric *C. elegans* embryo. First, instead of segmenting cells as a binary classification task directly, CShaper generates the discrete distance map from the membrane stack image with a distance regularized neural network DMapNet. Second, Delaunay triangulation is employed to construct a weighted graph based on the binary segmentation extracted from the DMapNet. Local minima are clustered as different seeds for watershed segmentations.

82 Last, nucleus images are used to filter out hollow regions among cells. Automatic seeding
83 procedure precludes substantial computations involved in most current works due to the
84 over-segmentation problem. After adjusting position variations in wide-type embryos, we
85 establish a spatio-temporal morphogenesis reference for *C. elegans* embryogenesis during
86 4- to 350-cell stage. Both individual evaluation and experimental verification on previous
87 conclusions demonstrate the unprecedented performance of CShaper.

88 **Results**

89 By measuring the consistency between segmentations of prevalent methods and manual
90 annotations, CShaper outperforms regarding both accuracy and robustness. Besides, based
91 on the segmentations, cell volume and cell surface area, which usually get involved in cell-
92 cycle control and cell-fate specification^{13–18}, were firstly investigated and found to have
93 high reproducibility among individuals.

94 **Dataset** In *C. elegans* embryo, nucleus and membrane were stained in vivo with mCherry
95 marker on nucleus and GFP marker on cell membrane, respectively. 4D (space + time)
96 nucleus and membrane stacks from two channels were collected by Leica SP8 confocal
97 microscopy at 1.5-min interval. All images with a resolution $512 \times 712 \times 70$ (voxel size
98 $0.09 \times 0.09 \times 0.43\mu\text{m}$) were resized into isotropic volume images with a resolution $205 \times$

99 285×134 (voxel size $0.22 \times 0.22 \times 0.22 \mu\text{m}$) before analysis. Different wide-type embryos
100 are used in different stages as listed in **Supplementary Table. S1**.

101 Manual annotations are needed to train the DMapNet and compare different meth-
102 ods. In plant tissue, cells are encased within cell walls that physically adhere to their
103 neighbors, yielding better image quality and uniform size. In the animal embryo, howev-
104 er, irregular cells are separated by thin membrane, making it much challenging to segment
105 each cell accurately. Besides, since only 2D slice can be shown on computer screen, full
106 annotation of volumetric data is very tedious. Therefore, the gold standard is produced
107 by semi-automatic software with the results revised by experts. Membrane stacks are
108 pre-segmented by 3DMMS⁵⁸ first, and then checked by two experts with ITK-SNAP⁵⁹
109 slice-by-slice. Nucleus image is also imposed to prevent invalid gaps between cells. Please
110 note that annotations are composed by cell-wise regions, which can be transformed into
111 membrane mask with morphological operations. Most annotations have less than 100 cells
112 to prevent annotation quality deteriorating with the image quality and segmentation error
113 of 3DMMS. Although DMapNet is trained on frames within 150-cell stage, experiments
114 show that it is able to process the embryo in 350-cell stage with high quality owing to the
115 multi-scale input features. All datasets companied with annotations or segmentations will
116 be publicly available.

117 **Comparison with other method** Here a through comparison among CShaper and other
 118 state-of-the-art methods is discussed. To be a fair comparison, watershed algorithm is
 119 also used as a postprocessing procedure in 3D U-net and FusionNet where only binary
 120 membrane segmentation is available. However, different from CShaper, the seeds are
 121 collected from the nucleus locations generated by AceTree, which theoretically produces
 122 more accurate estimation on real nucleus images. In order to compare the CShaper without
 123 distance learning strategy, the CShaper was changed to a naive binary segmentation by
 124 replacing the last layer of DMapNet (see Methods) with two channel filters, while other
 125 parameters kept the same as that in CShaper.

126 Experimental results are reported in Fig. 1. It shows that CShaper achieves much
 127 higher accuracy on three different wide types at various time points (Fig. 1A), which
 128 validates the accuracy and generality of our approach. Such improvement benefits from:
 129 1). CShaper allows segmentation on weak boundaries because of the distance constrained
 130 learning strategy (**Supplementary Fig. S1**); 2). In RACE and FusionNet, segmentation-
 131 s are implemented slice-by-slice. Although slices are combined into stack statistically
 132 during the post-processing stage, few inter-slice information, if any, in raw images are uti-
 133 lized to establish a promising result; 3). In contrast to naive binary segmentation methods,
 134 DMapNet reaches a compromise at the membrane boundary by constructing a relatively s-
 135 mooth transition between the membrane and the background. This encourages the network

136 to learn more morphological features around the membrane.

Figure 1: Analysis on the segmentation results. **(A)** Comparison on the Dice ratio of the segmentations from 3D U-Net, FusionNet, RACE, CShaper and naive binary. All values are calculated based on seven manual annotations of three wide types, respectively. **(C)** The distribution of cell volume consistency ρ_c with respect to time t_c in four embryos. **(D)** The distribution of lost nucleus with respect to time in four embryos. **(D)** Comparison of the segmentations from RACE, 3DUnet, FusionNet and CShaper.

As Fig. 1D shows, RACE and FusionNet suffer severe leakage at the bottom layers of the embryo, where the membrane signal is too weak to be discriminated because of laser attenuation. 3DUnet provides better feature extraction by applying convolution between slices. However, partial membrane, especially at the boundary of the embryo, is still too weak to be recognized, which deteriorates the lost cells in the periphery. Under the distance map constraint, CShaper learns to depict weak or lost membrane as annotated in the training data.

Robustness on extensive datasets To measure the performance of CShaper on more datasets, whose annotations are not available, we quantitatively measured the volume consistency, as well as the lost nucleus, of serial segmentations. CShaper segments each embryo independently, frame by frame, without capturing typical temporal patterns across time. Successive imaged cells, theoretically, should have temporally consistent volume, or with limited variance when considering biological dynamics. Therefore, we can examine the performance of CShaper on extensive time-lapse stacks by analyzing series information. Besides the membrane images, their corresponding nucleus stacks were processed by AceTree, which can be used to identify cell's name and the lifespan of each nucleus. We defined volume consistency and ratio of lost nuclei to estimate the error of segmentations in four wide-type embryos with 200 frames in each, which are used for following spatio-temporal reference reconstruction. A index matrix $V_{tc|t \in [1,2,3,\dots,200], c \in C}$ was constructed

such that “0” represents the existence of cell c at time t , otherwise the element is kept as NaN (invalid value), where C is the collection of cell names. We assembled cell’s volume of all segmentations of one wide-type embryo into the matrix $\mathbf{V}$. Then for each cell c , the volume consistency ρ_c is defined as the ratio of the standard deviation and mean applied to volume series $\mathbf{V}_{t,c|t=1,2,\dots,200}$. Note that all invalid values NaN were not taken into consideration. The start time point of cell c is also labelled as t_c in order to discriminate the error at different developmental stages. Larger ρ_c means the segmentation of cell c has better temporal consistency, yielding higher performance. The distribution of consistency coefficients (t_c, ρ_c) of the four wide-type embryos is plotted in Fig. 1B. In these segmentations, most cells have relative small volume variation through the development, although temporal information are not applied to CShaper in the segmentation procedures intentionally. With the number of cells increasing over time, the cell volume becomes smaller and the signal-to-noise ratio decreases dramatically, which makes it more challenging to be segmented precisely. Simultaneously, because nuclei are not involved in the segmentation stage, nucleus information processed by AceTree can be used to justify the accuracy. In Fig. 1C, we show the ratio of lost nucleus (see **Supplementary note 1**) at different time points. Few cells before 200-cells stage are lost in the four embryos. With the number of cells increasing and density of fluorescence shrinking, the number of cells that are lost becomes larger when entering the eighth round of cell divisions. However the lost cells

175 in the entire embryos only occupy a small proportion (around 11.9% at 350-cells). There-
176 fore, we can safely conclude that CShaper has superior ability on segmenting extensive
177 time-lapse embryo images.

178 **Establishment of spatio-temporal morphogenesis reference** Using experimental meth-
179 ods and quality-control standards described before^{6,60}, 4 wild-type embryos within 350-
180 cell stage were used to construct the morphogenesis reference of early *C. elegans* embryo.
181 All the 17 embryos (4 embryos with both nucleus and membrane markers and the other 13
182 embryos with only nucleus marker) were first linearly normalized to minimize their posi-
183 tional variation according to a proposed computational pipeline⁶⁰; secondly, translation in
184 yz plane and rotation around *x* axis were successively performed on the 4 embryos with
185 membrane marker, to ensure the compressed contact faces were parallel to *xz* plane; third-
186 ly, translation in *xz* plane and rotation around *y* axis were successively performed on the
187 4 embryos, so that their projection to *xz* plane could be embedded by a centralized ellipse
188 with minimum area; after that, the 4 embryos were rescaled to the same length in *x*, *y*, *z*
189 directions; at last, the other 13 embryos were normalized to the nucleus-position averages
190 of the 4 embryos using methods proposed previously⁶⁰ (**Supplementary Fig. S2**).

191 **Morphological developmental properties at single-cell level** Using the cell-segmentation
192 algorithm designed above, a total of 226 cells ($\approx 70\%$) among AB4-128, MS1-MS16,

193 E1-E8, C1-C8, D1-D4, P3 and P4 were recorded with complete lifespans and segmented
194 without any error in all the 4 wild-type embryos, generating 4-dimensional morphological
195 dynamic information at single-cell level with high confidence, such as cell shape (e.g. cell
196 volume, cell surface area, topology) and cell-cell contact (e.g. contact duration, contact
197 area, neighbour relationship) (Fig. 2, **Supplementary Fig. S3**)(Table. S2).

198 To test the data quality and further refine the information useful to biological study,
199 here we focus on three low-dimensional but valuable developmental properties : cell vol-
200 ume, cell surface area and cell-cell contact. Cell volume and cell surface area, which
201 usually get involved in cell-cycle control and cell-fate specification^{13–18}, were firstly inves-
202 tigated and found to have high reproducibility among individuals (Fig. 2 **A,B**). Cell-fate
203 induction (e.g. Wnt signaling^{19,20} and Notch signaling^{10,61}) during embryo development
204 intimately depends on continuous, sufficient and direct physical contact between specif-
205 ic cells for interaction between receptors and ligands and consequent effective signaling
206 transmission. Based on the contact relationship acquired from raw experimental images
207 and automatic segmentation, we filtered the most reliable and valid contact between two
208 specific cells by adding three empirical criteria¹⁰, **1**) with contact area larger than 1/36 of at
209 least one cell's surface area (sufficiency, $S_{\text{contact}}/S_{\text{surface}} > 1/36$); **2**) with contact duration
210 no shorter than 3 minutes, i.e. consecutive two time points (continuity, $T_{\text{contact}} \geq 3 \text{ min}$)
211 ; **3**) be reproducible in all the 4 wild-type embryos (reproducibility, $N_{\text{replicate}} = N_{\text{embryo}}$).

At last, totally 877 contact pairs of two specific cells are identified (Fig. 2C). Cells with missing information are listed in **Supplementary Table. S3**. Please note that the criteria are set arbitrarily and can be readjusted for different research purposes.

Figure 2: Reproducibility, precision, validity of cell volume, cell surface area, and cell-cell contact. All the cells involved are completely recorded and segmented without any error during their lifespans (Table 1). (A) Highly reproducible volume of cells in the 4 wild-type embryos. Inset, variation coefficients of cell volume. (B) Highly reproducible surface area of cells in the 4 wild-type embryos. Inset, variation coefficients of cell surface area. (C) Selection filter of sufficient, continuous and reproducible cell-cell contact pairs according to a set of arbitrary criteria.

Discussion

Cell morphology is critical and useful developmental property involved with different biological process. In this paper, a complete pipeline CShaper for analyzing spatio-temporal morphological features of *C. elegans* embryo at single-cell level is proposed. The CShaper benefits from the well-defined distance learning task. By learning to capture multiple

discrete distances, DMapNet extracts the membrane mask by considering shape information, instead of just intensity features. Remarkable performance is examined by both intrinsic geometric constraints and previous notable discoveries. Based on these accurate segmentations, we merged the embryos and quantitatively generated a spatio-temporal morphogenesis reference for 4- to 350-cell stage of *C. elegans* embryogenesis, including single-cell properties such as cell shape (e.g. cell volume, cell surface area, topology), cell-cell contact (e.g. contact duration, contact area, neighbour relationship), cell positional variability, etc. In all, 226 cells with complete lifespans and dynamic morphology trajectory were produced. Furthermore, a total of 877 contact pairs between two specific cells were identified with high reproducibility, continuous contact duration and sufficient contact area, which could be a solid foundation for searching potential signaling pathways between cells. Our work provides a quantitative and statistical framework for *C. elegans* morphogenesis, which is a powerful resource to push forward multiple biological research fields like signaling transmission, fate specification and asymmetric segregation. Next, we discuss the coincidence of CShaper with three well-known experimental discoveries and inspect its valuable applications on different datasets.

Verifications on previous studies To further validate the *C. elegans* morphogenesis reference with single-cell developmental properties (e.g. cell volume, cell position, cell-cell contact), here we use our new quantitative data to repeat and verify three separate sets of

239 experimental conclusions about *C. elegans* embryonic development^{10, 13, 61–63}.

240 Firstly, Arata et al. found the power law relationship between cell cycle duration and
241 cell volume in the early *C. elegans* development, that is, AB and MS cells adopt the same
242 power exponent (≈ -0.27) while C and P cells adopt another different power exponent ($\approx -$
243 0.41)¹³. Under log-log scale coordinates system, we also performed linear fitting between
244 cell cycle duration and cell volume and found that the two exponents obtained (AB and
245 MS, -0.2990 ; C and P, -0.4244) are very close to the values proposed before (Fig. 3A, B).

246 Secondly, Li et al. uncovered the “low-high-low” dynamic pattern of cell positional
247 variability during 4- to 350-cell stage of *C. elegans* embryogenesis⁶². Using the same eval-
248 uation method on positional variability (RMSD) proposed before, we used our normalized
249 cell positions (nucleus positions) to calculate their spatial variation and reconstructed a
250 similar curve with a turning point occurring when cell number reaches around ninety (Fig.
251 3C).

252 Thirdly, several intercellular signaling based on accurate cell-cell contact have been
253 identified to play important roles in asymmetric segregation, spindle formation and cell-
254 fate induction^{10, 19, 20, 61, 63}. Here, we compared the known cell-cell signaling pairs with our
255 cell-cell contact dataset (877 pairs in total). The majority of known contact pairs past

256 through our filter criteria with continuous contact duration as well as sufficient contact
 257 area, except $C \rightarrow ABar$ and $MS \rightarrow ABalp$ (Table. 1). The contact between C and
 258 ABar can be found in all the 4 wild-type embryos with at least two consecutive time point
 259 (≈ 3 minutes), however, the relative contact area is smaller than the arbitrary filter criterion
 260 ($S_{\text{contact}}/S_{\text{surface}} > 1/36$) in one of the embryos (Fig. 2C), revealing that the threshold
 261 for identifying valid cell-cell contact should be reestimated and readjusted on the basis of
 262 the actual biological scenes (e.g. surface density of ligands or receptors), nevertheless,
 263 the original contact information from image segmentation is objective and can be used for
 264 different research purposes. For the other contact pair $MS \rightarrow ABalp$, one of the cells
 265 ABalp are missing (i.e. fail to be segmented) due to its location near the top of embryo
 266 and consequent dim fluorescent signal.

Figure 3: Verification of our newly proposed morphogenesis reference using experimental results from References^{13, 62}. (A) Power law relationship between cell cycle duration and cell volume in AB and MS cells, with power exponent ≈ -0.2990 . Inset, illustration in log-log scale coordinates system. (B) Power law relationship between cell cycle duration and cell volume in C and P cells, with power exponent ≈ -0.4244 . Inset, illustration in log-log scale coordinates system. (C) “Low-high-low” dynamic pattern of cell positional variability during 4- to 350-cell stage.

Table 1: Verification of our newly proposed morphogenesis reference using experimental results from references.

Cell-Cell Contact Pair	Validity & Reproducibility	Contact Duration (min)	Relative Contact Area Surface (%)	Relative Contact Area Surface (%)	Remark	Ref.
P2 → EMS	4/4	7.04	9.61±2.30	12.31±2.15	Wnt Signaling	Thorpe et al ¹⁹
MS → ABal	4/4	7.04	15.70±2.60	15.03±2.66	Latrophilin Signaling	Langenhan et al ⁶³
C → ABar	3/4#	7.04	2.37±2.57	2.60±2.79	Wnt/ β -catenin Signaling	Walston et al ²⁰
P2 → ABp	4/4	5.63	13.38±1.99	20.47±2.01	Notch Signaling (1 st)	Priess et al ⁶¹
MS → ABalp	/*	/	/	/	Notch Signaling (2 nd)	Chen et al ¹⁰ , Priess et al ⁶
MS → ABara	4/4	5.63	9.28±1.19	12.98±2.14	Notch Signaling (2 nd)	Chen et al ¹⁰ , Priess et al ⁶
ABalapa → ABplaaa	4/4	5.63	14.22±3.86	11.13±3.26	Notch Signaling (3 rd)	Chen et al ¹⁰ , Priess et al ⁶
ABalapp → ABplaaa	4/4	5.63	13.06±3.91	10.66±2.65	Notch Signaling (3 rd)	Chen et al ¹⁰ , Priess et al ⁶
MSapp → ABplpapp	4/4	5.63	12.58±3.69	13.92±3.86	Notch Signaling (4 th)	Chen et al ¹⁰ , Priess et al ⁶
MSapppp → ABplpppp	4/4	5.63	7.58±0.21	9.19±2.61	Notch Signaling (5 th)	Chen et al ¹⁰ , Priess et al ⁶¹

Without complete reproducibility in the 4 wild-type embryos under designed filter criteria.

* Including missing cell (ABalp).

Application on different datasets To evaluate the performance of CShaper on different kinds of images, the plant tissue images used by Fernandez et al⁶⁴ were segmented with our method. In the work, *Arabidopsis thaliana* stem cells were processed with MARS⁶⁵, which provides reasonable discrimination on inner parts of the tissue by fusing multi-angle acquisitions. Different from MARS, CShaper processes the stem in a more challenging way, segmenting single-direction membrane stack without the fusion stage. Because of the large shape difference between animal and plant cells, we retrained DMapNet with two segmentation results from the MARS. Negative segmentations were filtered when its volume deviates the average level too much. The comparison result is listed in **Supplementary Fig. S4**. Both MARS and CShaper show promising segmentations of cells at shallow layers. However, CShaper shows stronger results on inner parts with extremely low intensity. Although DMapNet was trained with defective reference as shown in **Supplementary Fig. S4B, C and D**, CShaper escaped these irregular errors during the inference stage.

Constrains of CShaper CShaper provides new insights into the study of *C. elegans* embryogenesis at single-cell level, both in spatial and temporal aspects. First, to promote the accuracy of automatic *C. elegans* embryo segmentation, especially at later developmental stage, some constrains of CShaper need to be emphasized here. First, as the cell shape changes with time continuously, the temporal features between consecutive frames are

supposed to elevate the segmentation performance. LSTM, originally designed for natural language process (NLP), is an obvious candidate to capture temporal features across time²⁶. However, CShaper doesn't adopt LSTM-based model, such as convLSTM⁶⁶, because of the considerable computational resources involved in 3D convolution. We also find that the segmentation errors of CShaper concentrate at the top of the embryo, where the membrane signal intensity decreases critically because of the laser attenuation. In the framework of CShaper, potential strategies could be used to normalize the quality of top half embryo based on the bottom one. For example, Generative Adversarial Networks (GAN) can be employed to transform low-quality images into the target with higher resolution^{40,45}.

Methods

CShaper consists of three phases. The first step is to extract the membrane mask by deep learning based DMapNet. After that, Delaunay triangulation is used to cluster local minimum for the followed watershed segmentation. The negative segmentations and potential gaps among cells are filtered with the nucleus channel. The final cell shape lineage is constructed based on the series information provided by the nucleus lineage. The framework of CShaper is shown in Fig. 4.

Figure 4: The framework of CShaper. Serial nucleus and membrane stacks are imaged simultaneously. For membrane image, CShaper is applied to segment the embryo at single-cell level automatically. Nucleus stack is processed by AceTree, which tracks nucleus through the entire development process. Finally, CShaper embedded cellular shape into the nucleus lineage.

303 **Distance Constrained Learning** The noise and physical imaging limitation degrade the
 304 quality of automatic segmentations. This problem prevails in embryo segmentation since
 305 the membrane enclosing a cell can be hardly imaged completely. DMapNet adopts a
 306 distance constrained learning to address the problem in segmenting noisy embryo images.
 307 By implicitly learning the distance map, the DMapNet is able to discriminate weak or even
 308 lost membrane signal.

309 With the membrane labelled as front label 1, the binary membrane annotation M^B
 310 was prepared as discrete distance map M^D with

$$M^D(x, y, z) = \begin{cases} \min_{M^B(i,j,k)=1} ((x-i)^2 + (y-j)^2 + (z-k)^2) & \text{if } M^B(x, y, z) = 0 \\ 0 & \text{if } M^B(x, y, z) = 1 \end{cases} \quad (1)$$

$$M^D = \max(M^D) - M^D \quad (2)$$

312 In Eq. (2), we reversed the distance map to keep it monotonically decreasing from
 313 the membrane to the background. To relieve the burden on learning the distance where
 314 pixel was too away from the membrane to have recognizable signal, M^D was clipped to
 315 prevent long-range spatial dependencies. Then M^D was further discretized nonlinearly
 316 into M^{dmap} , the learning target, with smaller intervals around the membrane mask. The
 317 cross-entropy loss was used to evaluate the learning progress. However, different from
 318 traditional multi-classification, DMapNet should ideally provide a class distribution such

that the predicted class closer to the real class has higher probability than that is further away. For example, the penalty of predicting $l = 1$ as $l = 2$ should be smaller than that of the prediction $l = 15$. Therefore, the cross-entropy loss is weighted by the class distance as

$$l = \sum_{i=1}^N \sum_{k=1}^K \xi_k w_{i,k} (t_{i,k} \log p_{i,k} + (1 - t_{i,k}) \log(1 - p_{i,k})) \quad (3)$$

where ξ_k is the weight for each interval, $w_{i,k}$ is the class distance weight for each pixel i , $t_{i,k}$ is the k -th element of the one-hot target vector at pixel i , and $p_{i,k}$ is the k -th channel of the network output at pixel i . N and K are the number of pixels and distance intervals, respectively. The K -th interval denotes the mask at the center of the membrane, while 0-th class represents the background far away from the membrane. Larger weight w_k on classes closer to the membrane helps the network put more attention on cell boundary. The class distance weight $w_{i,k}$ is calculated as

$$w_{i,k} = \exp\left(\frac{|k - D_i^{dmap}|}{K}\right) \quad (4)$$

where D_i^{dmap} is the real class at pixel i .

Network structure The structure of DMapNet is shown in **Supplementary Fig. S5**. Taking the fully convolutional network as the backbone, DMapNet is constructed by considering some specific problems in fluorescent images. In order to reduce 3D computational complexity, DMapNet utilizes inter-slice information by using $3 \times 3 \times 1$ and $1 \times 1 \times 3$ con-

335 volution successively. The dilation convolution, from the output of inner-slice convolution
 336 to its input, is added to enlarge the receptive field. The residual block is composed of two
 337 convolutional layers⁶⁷. Parametric Rectified Linear Units (PReLU)⁶⁸ are used as the acti-
 338 vation layer. To help the higher layers retain the information from pixel, the input is scaled
 339 down and concatenated with the feature map, which also helps to train the network on cells
 340 in different sizes⁵¹. This is essential for segmenting cells when annotations corresponding
 341 to late development stage are not reliable. Feature maps at lower resolution are upsampled
 342 and concatenated together to generate the probability map. The membrane image volume
 343 is split into multiple overlapped slice series $I_{D \times W \times H}$, which are processed by DMapNet
 344 individually. The final prediction of whole volume $M_{K \times (D-8) \times W \times H}^{prop}$ is achieved by pack-
 345 ing these predictions sequentially as discussed in Distance Constrained Learning. Then the
 346 discrete distance map can be derived by $M^{pred}(z, x, y) = \max_{c=[1, \dots, K]} M^{prob}(c, z, x, y)$.

347 **Automatic seeds clustering based on Delaunay triangulation** Till now, we have just
 348 obtained the discrete distance map M^{pred} from the DMapNet, as shown in Fig. 4. Wa-
 349 tershed segmentation is well suited for separating individual cells from the map, where
 350 black cell interiors are surrounded by bright boundaries. The learned map M^{pred} , includ-
 351 ing multiple discrete distance intervals, approximates the distance transformation on the
 352 latent binary membrane mask. With the holistic information embedded in the ordered
 353 intervals, cell boundaries can be extracted. However, watershed algorithm cannot be ap-

plied on M^{pred} directly because of redundant local minima and low distance resolution. In this part, Delaunay triangulation is employed to detect seeds for watershed segmentation automatically.

First, by selecting the K -th interval as the foreground mask M_K^{pred} , a reversed EDT was applied to M_K^{pred} , yielding I^{edt} . All local H-minima in I^{edt} are noted as $S = \{s_i\}_{i=1,\dots,S}$, where S is number of local minima. In order to filter s_i s that belong to the same cell or background, a weighted graph G is constructed. Edges $E = \{E_1, E_2\}$ in G come from two sources: one is the Delaunay triangulation on S , noted as E_1 ; another one is the edges E_2 among all local minima locating on the boundary of the volume. Weight of the edge e_{ij} is defined as

$$W(e_{ij}) = \begin{cases} \sum_{(x,y,z) \in e_{ij}} M_K^{pred}(x, y, z) & e_{ij} \in E_1 \\ 0 & e_{ij} \in E_2 \end{cases} \quad (5)$$

where $(x, y, z) \in e_{ij}$ represents all points on the edge e_{ij} . The edge is moved from E if its weight is greater than the OTSU⁶⁹ threshold on W . Finally, vertexes M were clustered based on their connectivity. All s_i s in one cluster are regarded as one seed in the watershed segmentation stage. In the seeding procedure, a seed could possibly locate in a hollow gap between cells, giving rise to fake cell regions. Thus, nucleus stack was used to modify these errors. Owing to the impressive performance of DMapNet and automatic seeding, only intensity normalization was needed for nucleus image. Regions were set as

371 background when the cumulated intensity in the region is smaller than a threshold.

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Author Contributions H.Y., Z.Z., C.T. conceived and coordinated the study. J.C. designed the cell-segmentation algorithm ; G.G. analysed the segmentation results. M.W., L.C. performed imaging and embryo curation. Z.Z. provided reagents and experimental methods. J.C., G.G. wrote the manuscript ; H.Y., Z.Z., C.T. revised the manuscript. All the authors reviewed the results and approved the final version of the manuscript.

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Supplementary

Figure S1: Comparison of CShaper and naive binary segmentations. (A) Raw image. (B) and (C) are segmentations of naive binary and CShaper, respectively.

Figure S2: Spatio-temporal reference of wild-type *C. elegans* embryonic morphogenesis. Take the 8-cell stage for example and illustration. x , y , z axes represent anterior-posterior (A-P), left-right (L-R), dorsal-ventral (D-V) axes respectively. Each color represents one specific cell identity, noted in legend. (A) 3D structure, y projection and x projection of cell morphology in 4 wild-type embryo samples. (B) Distribution of nucleus position in 17 wild-type embryo samples. Each point represents a cell's nucleus position in one embryo sample. (C) Spatial deviation and cell-cell contact mapping. Radius of sphere represents spatial deviation Δr_{STD} defined by $\Delta r_{\text{STD}} = [(\sum_{i=1}^N |\mathbf{r}_i - \bar{\mathbf{r}}|^2) / N]^{1/2}$; gray lines represent reproducible and effective contact between cells, under arbitrary filter criteria ($S_{\text{contact}} / S_{\text{surface}} > 1/36$; $T_{\text{contact}} \geq 3\text{min}$; $N_{\text{replicate}} = N_{\text{embryo}}$).

Figure S3: 4-dimensional morphological evolution during *C. elegans* embryogenesis. Take ABp and its daughters ABpl, ABpr as example and illustration. x, y, z axes represent anterior-posterior (A-P), left-right (L-R), dorsal-ventral (D-V) axes respectively. Green, ABa ; red, ABpl ; blue, ABpr. Evolution dynamics is shown in rows which represent different developmental timing noted on left (Tabel. S2). The first column is nucleus-position distribution from 17 wild-type embryo samples. The second to fifth columns are reconstructed cell morphology from the 4 wild-type embryo samples.

Figure S4: The application of CShaper on plant tissue. (A, H) are segmentations of MARS and CShaper shown in 3D. (B-D, (E-G) and (I-K) are three orthogonal sections of MARS's segmentation, raw image and CShaper's segmentation, respectively.

Figure S5: Network structure of DMapNet. Multiple neighboring slices are processed by three residual blocks at three different levels consecutively. Feature maps at high levels are resized into the same size as the input image with linear interpolation. In order to remedy the lost information, raw images are downsampled and concatenated into the feature maps. The complete distance map of one volume is achieved by combining multiple predictions.

Table S1: Datasets (with membrane marker) description

Dataset	Name of Wild-Type Embryo	With Annotation	Usage
Training	170704plc1p2{27}, 181215plc1p1{27}	Yes	Training DMapNet
Testing	170704plc1p2 {7}, 181210plc1p3{7}, 190314plc1p3 {7}	Yes	Comparing methods
Reference construction	170704plc1p2 {200}, 181210plc1p1 {200}, 181210plc1p2 {200}, 181210plc1p3 {200}	No	Extensive evaluation and spatio-temporal reconstruction

Note: Number in the bracket {*} represents the frames in the wide type.

Table S2: Time segmentation on developmental process from 4- to 350-cell stage.

Division Event	First Moment (min)	Last Moment (min)	Division Event	First Moment (min)	Last Moment (min)
AB2	/	0.00±0.00 {01}	E1	4.88±0.86 {04}	22.29±1.82 {10}
AB4	1.53±0.26 {02}	15.45±1.49 {07}	E2	23.74±1.87 {12}	62.21±4.53 {25}
AB8	17.67±1.30 {08}	32.25±2.83 {15}	E4	65.69±5.01 {26}	106.92±5.34 {39}
AB16	35.21±3.22 {18}	56.47±3.80 {23}	E8	113.14±5.66 {41}	175.11±6.86 {52}
AB32	60.92±4.11 {24}	84.84±5.15 {32}	E16	191.30±6.79 {54}	/
AB64	92.25±5.15 {34}	118.51±5.60 {44}	C1	8.85±1.21 {06}	25.92±2.60 {13}
AB128	134.41±6.13 {46}	159.97±4.64 {50}	C2	27.37±2.61 {14}	49.85±3.47 {21}
AB256	190.98±4.70 {53}	/	C4	51.91±3.65 {22}	81.15±4.85 {31}
EMS	/	3.43±0.82 {03}	C8	88.20±5.55 {33}	115.85±6.12 {43}
P2	/	7.40±1.22 {05}	C16	143.16±7.27 {47}	/
MS1	4.88±0.86 {04}	21.18±1.71 {09}	D1	34.38±2.90 {16}	68.21±5.97 {28}
MS2	22.63±1.76 {11}	42.00±3.12 {19}	D2	69.66±5.98 {29}	112.53±6.83 {40}
MS4	43.96±3.43 {20}	67.75±4.55 {76}	D4	115.77±7.34 {42}	155.45±7.15 {49}
MS8	72.20±4.91 {30}	97.58±5.76 {35}	D8	163.81±7.13 {51}	/
MS16	104.74±6.14 {38}	130.54±6.05 {45}	P3	8.85±1.21 {06}	32.93±2.88 {16}
MS32	153.27±6.10 {48}	/	P4	34.38±2.90 {17}	99.90±6.89 {36}
			Z2/Z3	101.35±6.90 {37}	/

Note: Number in the bracket{*} represents the order of the 54 division events (frames).

Table S3: Cells with missing information during 4- to 350-cell stage in the 4 embryos expressing membrane marker (30.7%).

AB Lineage (81 / 254, 31.9 %)	'ABalp'	'ABalpp'	'ABaraa'	'ABplap'	'ABplpp'	'ABalpap'	'ABpraaa'
	'ABpraap'	'ABprpap'	'ABalapap'	'ABaraaaa'	'ABarappa'	'ABarpaaa'	'ABarpaap'
	'ABarppaa'	'ABarpppa'	'ABplapaa'	'ABprapaa'	'ABprapp'	'ABalaaaal'	'ABalaaaar'
	'ABalaaapp'	'ABalaapaa'	'ABalaapap'	'ABalaappa'	'ABalapaaa'	'ABalapaap'	'ABalapapa'
	'ABalappaa'	'ABalappap'	'ABalapppa'	'ABalpaaaa'	'ABalppaap'	'ABarapaaa'	'ABarapapa'
	'ABarappaa'	'ABarappap'	'ABarpaaaa'	'ABarpaaap'	'ABarpaapa'	'ABarpaapp'	'ABarpapaa'
	'ABarpapap'	'ABarpappa'	'ABarppaaa'	'ABarppaap'	'ABarppapa'	'ABarppapp'	'ABarpppaa'
	'ABarppppa'	'ABarppppp'	'ABplaaaa'	'ABplaaaap'	'ABplaaaapa'	'ABplaaapaa'	'ABplaaapap'
	'ABplapaaa'	'ABplapaap'	'ABplapapa'	'ABplapppa'	'ABplapppp'	'ABplpaaaap'	'ABplpapppp'
	'ABplppapp'	'ABplpppaa'	'ABplpppap'	'ABplppppp'	'ABpraaaaa'	'ABpraaaap'	'ABpraapaa'
	'ABpraapap'	'ABprapaaa'	'ABprapaap'	'ABprapapa'	'ABprapapp'	'ABprapppa'	'ABprapppp'
	'ABprpaaap'	'ABprppppp'	'ABprppaaa'	'ABprppppp'	/	/	/
4 MS Lineage (6 / 31, 19.4 %)	'MSap'	'MSaap'	'MSaaap'	'MSaapp'	'MSappa'	'MSppap'	/
E Lineage (5 / 15, 33.3 %)	'Ealp'	'Eara'	'Earp'	'Epla'	'Epra'	/	/
C Lineage (6 / 15, 40 %)	'Ca'	'Cp'	'Cap'	'Caap'	'Capa'	'Cpap'	/
D Lineage (2 / 7, 28.6 %)	'D'	'Dap'	/	/	/	/	/
Others (('EMS', 'P2', 'P3', 'P4')) (0 / 4, 0 %)	/	/	/	/	/	/	/

Note: For AB2, EMS, P2 with incomplete lifespan recorded, only duration between 4-cell stage and 8-cell stage was considered ; for AB4-AB128, MS1-MS16, E1-E8, C1-C8, D1-D4, P3, P4 which have complete cell cycle in all the 17 wild-type embryos, all the time points in their lives were taken into account ; for each cell, if the membrane signal is too dim at any time point and cause failure in precise cell segmentation, the cell would be regarded invalid with missing information.

Note 1: In CShaper, there are three discriminative situations where nucleus derived from AceTree cannot be found:

1. The boundary between two cells (not sisters) is too weak to be extracted by DMapNet, therefore, two cells are segmented as one cell during the watershed transformation;
2. Membrane signal is lost at boundary of the embryo, which leads to the leakage of the background into the embryo;
3. In CShaper, we determine the accomplishment of division stage by checking the signal intensity on the line between sister cells' nuclei. However, when the intensity drops at the middle of lifespan, the sister cells may be combined as their mother cell, leading to missing cells.

We exclude these mistakes by combining the nucleus lineage from AceTree and segmentations from CShaper.