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**SPIE.**

# An imaging and analysis toolset for the study of *Caenorhabditis elegans* neurodevelopment.

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## ABSTRACT

How an entire nervous system develops remains mysterious. We have developed a light-sheet microscope system to examine neurodevelopment in *C. elegans* embryos. Our system creates overlapping light sheets from two orthogonally positioned objectives, enabling imaging from the first cell division to hatching (~14 hours) with 350 nm isotropic resolution. We have also developed computer algorithms to computationally straighten nematode embryos, facilitating data comparison and combination from multiple animals. We plan to use these tools to create an atlas showing the position and morphology of all neurons in the developing embryo.

**Keywords:** *Caenorhabditis elegans*, neurodevelopment, light sheet imaging.

## 1. INTRODUCTION

A significant amount of effort has been devoted to understanding how nervous systems form, from the molecules directing the growth of individual axons and dendrites to larger-scale organizing principles. Researchers have made substantial progress in identifying molecules responsible for directing the growth and movement of individual cells and axons<sup>1-3</sup>, but the organizing principles by which entire nervous systems are assembled are less clear<sup>2,3</sup>. The ability to study neurodevelopment at a whole systems level - encompassing all cells in a nervous system - would be of great value in identifying and understanding these principles. However, due to nervous system complexity (with mammalian nervous systems containing millions or billions of cells<sup>4,5</sup>), and difficulties in efficient imaging of large tissue volumes, such a systems-level view has proved elusive. One way to get around such difficulties is to first examine systems-level neurodevelopment in much simpler model systems, such as the nematode *Caenorhabditis elegans*. The *C. elegans* embryo has 222 neurons<sup>6,7</sup>, the adult morphology and connections of which are known<sup>8</sup>. The small number of neurons, known structure of the adult nervous system, and genetic tractability of this system raise the possibility that for each neuron, the location, morphology, and potentially molecular mechanisms regulating development could be tracked, providing a systems-level view of how the embryonic nervous system is developing. However, use of the nematode embryo to study neurodevelopment has been limited because of embryonic sensitivity to photobleaching and photodamage<sup>9,10</sup>, fast embryonic movements leading to motion blur<sup>10</sup>, and a complex, twisted shape that inhibits the detailed visualization of cells within the live embryo. Using a combination of custom imaging<sup>10,11</sup> and software technologies (described herein), we have been able to obviate the difficulties in neurodevelopmental analysis caused by embryo movement and twisting, allowing individual neurodevelopmental events to be followed from the birth of a cell through hatching of the embryo. We are planning to use these technologies to follow the development of all 222 neurons in a *C. elegans* embryo, creating an “atlas” of *C. elegans* neurodevelopment. This atlas will provide, to our knowledge, the first comprehensive look at

the development of an entire nervous system, and provide a foundation for a systems-level understanding of mechanisms regulating neurodevelopment.

Creating an atlas showing the development of all cells in the embryonic *C. elegans* nervous system poses challenges, both from the inherent complexity in assembling a reference containing hundreds of neurons over hundreds of time points, and from problems in image acquisition and data analysis caused by embryo movement and twisting. At first pass, the simplest method by which to generate a neurodevelopmental atlas would be to label all cells in the embryo, and image their development *in toto* in one imaging session. However, due to technical limitations this is not currently possible (brainbow-type techniques have not been demonstrated yet in nematodes). Instead, neurons must be labeled either singly or in small groups to allow detailed examination of the development of each neuron. While individual labeling is possible with existing technology, this approach requires a method to combine data from multiple imaging sessions and to locate single neurons with respect to each other, a nontrivial task when the embryo is transparent and lacks obvious fiducial markers. Embryo movement and twisting as the embryo elongates also pose complications, as it results in a shift in the location of a labeled cell from time point to time point, making it unclear whether observed shifts in cell position reflect a cell changing location relative to the rest of the embryo, or noise from embryo twisting. Movement can also complicate morphological analysis of long structures such as neurites, as it leads to their apparent morphology changing from volume to volume. Finally, embryo movement and twisting also cause degradation in image quality due to motion blur. To deal with these issues, we have developed imaging and computational technologies that enable the labeling, visualization, and annotation of individual cells in the worm embryo, as well as permitting the combination of imaging data from multiple embryos to create a composite model of nervous system development. We describe these innovations in this paper.

## 2. DATA/RESULTS

Embryo movement begins around late 1.5-fold stage, and increases in magnitude and frequency as the embryo develops. From about late 1.5-fold to early two fold, the embryo undergoes infrequent twitches either in the dorsal/ventral direction or around the Z-axis (nose-to-tail axis), or in combination. These twitches are usually separated by a few minutes at first, but increase in frequency as the embryo develops. Once the late two-fold stage has been reached, motion blur becomes problematic unless imaging times are on the order of a couple seconds or less per embryo volume (approximately 50 x 50 x 50  $\mu\text{m}$ ). In addition to motion blur, excessive light exposure from conventional imaging modalities (i.e., confocal microscopy) can lead to photobleaching and photodamage, potentially causing embryo paralysis after a few hours of continuous imaging. In order to solve these issues, we have turned to light-sheet microscopy as an imaging system with the speed and gentleness required to capture continuous *C. elegans* embryonic development.

Conventional (confocal or compound) microscopes focus light onto the imaging plane in an hourglass shape, so sample regions outside of the imaging plane are exposed to excess light causing photobleaching or photodamage (Figure 1A). In contrast, light-sheet microscopes expose only the current imaging plane to a thin sheet of light (Figure 1B). This results in decreased photobleaching/photodamage<sup>12</sup>, enabling sensitive samples to be imaged for longer periods than with conventional microscopes. The parallel nature of image acquisition in light sheet microscopy also allows the acquisition of volumes more than 10x faster than with conventional microscopes<sup>11</sup>, minimizing motion blur and thus allowing clear, dual-color imaging of moving nematode embryos. Because light-sheet microscopes generate sheets coincident with the imaging plane, an orthogonally placed objective is used to capture emitted light from the sample. We have adapted this orthogonal geometry to create a dual-view system: instead of using one objective to create the light sheet and the second objective to capture emitted light, each objective can both create a light sheet and capture emitted light (Figure 1C). The views captured by each objective can then be computationally fused, creating an imaging volume with isotropic resolution. Using this system, we have achieved 330 nm isotropic resolution<sup>11</sup> while simultaneously imaging volumes in 0.5 s<sup>11</sup>, fast enough to allow imaging of twitching nematode embryos with minimal motion blur and with enough spatial resolution to capture the movement of neurons and neurites throughout all 14 hours of embryogenesis.

Even if motion blur, diffractive blur, and phototoxicity are reduced with dual-view light sheet microscopy, embryo movement and labeling limitations still impose challenges when creating a 4D neurodevelopmental atlas. It is currently infeasible to examine the development of all neurons in the embryo in a single imaging session. This limitation results in the need for a cell-specific labeling approach, in which neurons are labeled and studied individually in separate

embryos and imaging sessions, and the data derived from each embryo is combined to create a composite atlas. Such an approach creates a set of interrelated problems:

1. Even without movement or twisting, a method is needed to identify where a specific cell of interest is located with reference to the rest of the worm body. As the *C. elegans* embryo is transparent, it is not immediately obvious where a single labeled cell is located within a developing embryo.
2. Limitations in visualizing more than a small subset of neurons creates the need for a method by which data from multiple embryos can be combined. One difficulty is that embryos may differ in size and shape, so whichever system is used to combine data must be able to handle these differences.
3. The problem of locating individual cells and combining data is made more difficult by embryo movement and twisting. Embryo movement causes displacement of a cell from one volume to the next, complicating the problem of locating a cell in relation to the rest of the worm and confusing whether an observed change in position represents cell migration/developmental movement, or just an apparent positional shift due to twisting. Twisting can also affect the location of long structures like neurites, both in relation to the rest of the worm and in relation to the rest of the cell the neurite belongs to. Any difficulties in identifying cell location from volume to volume within one unique animal has corresponding effects on a researcher's ability to combine multiple datasets, all of which include movement-induced distortions.

These problems can be solved if there is some way in which to define the body of the worm - with this reference individual cells can be placed in their bodily context, and computational strategies can be used to straighten the worm, thus circumventing the problems of twisting and movement. In our case, fluorescent proteins expressed in specific cells within the worm provide the markers necessary for defining the worm body.

In general, the nematode body can be thought of as a tube containing cells (and some fluid-filled space). Conceptually, an atlas of cell positions (like our neurodevelopmental atlas) can be created just by defining an origin point with relevance to the worm body (e.g. the nose/beginning of the tube), and then calculating the position of a cell of interest in relation to the origin point. As long as the origin point stays fixed, and the dimensions of each worm are relatively constant, an aggregate cellular atlas can be assembled from multiple embryos by simply finding the position of each cell relative to the origin point, and combining the resulting positions. As described above, this concept for building an atlas is complicated by the transparency of the worm and movement of cells from time point to time point due to embryo movement and twisting. To control for these problems, we have labeled the nuclei of seam cells (which comprise 10 pairs of cells positioned along the sides of the worm body), junctions between hypodermal cells, and the worm gut/midline with green fluorescent protein (GFP). These labels, in combination, function as fiducial markers that allow us to build a lattice which defines the nose, tail, left and right sides of the worm, as well as capture bends in the worm body (Figure 2C). These fiducials can be used to generate a model of how, for each volume, the embryo bends and twists. Our implementation of this approach depends on a user manually identifying and tagging the fluorescent markers, with a computer algorithm then using the manually tagged marker points to generate a model of the worm body. Once the model has been defined, it can then be computationally straightened, and the same transformations applied to the model can be applied to the image volume, thus "untwisting" the data so that it can be analyzed in a straightened form.

Tagging of fiducial markers and volume straightening is implemented in the MIPAV programming environment, created by the NIH's Center for Information Technology (CIT). To untwist a worm embryo containing the markers described above, the user first loads a volume (in the form of a .tiff file.) and renders it, facilitating analysis of the dataset in 3D (Figure 2A). The user then creates a lattice, a computational construct defining the location of the nose, tail, and sides of the embryo, for a worm volume interactively in the volume renderer (Figure 2B, 2C). The lattice comprises a series of user-assigned points, with each pair of points specifying the location of the left and right sides of the embryo. The first and last pair of points in the lattice specifies the position of the embryo nose and tail, respectively. The user adds lattice points in the 3D volume by clicking with the mouse, usually starting out by positioning lattice points coincident with seam cell locations, and then adding additional lattice points if necessary during an editing step (Figure 2B). Mapping the 2D position of the mouse cursor to a 3D location in the volume is key to creating the lattice. When the user clicks on a point in the volume, a ray is generated originating from the clicked point and going through the volume. A point for the lattice is placed at the voxel of maximum intensity in the volume that intersects the ray. By manipulating the volume in the 3D renderer, the user can sequentially build a lattice, starting from the nose and moving sequentially along the left and right seam cells, from nose to tail. Once the lattice is complete (Figure 2C), the user can adjust the position of the points they placed for greater accuracy, and add additional points to capture changes in the

worm body that a lattice built from seam cells alone cannot fully capture. The program also features the ability to add annotation points, for which the program records the X, Y, and Z-coordinate in relation to a user-defined origin point (typically the nose, see also Figure 2C), and then output that location information in a spreadsheet file. The annotation points can be used to define the exact position of a cell or cellular structure in each worm volume so spatial displacement can be measured over time, and also allows a user to examine the position of a specific cell or structure of interest in multiple worm volumes, seeing how stereotyped the position of that cell is during embryo development.

Once the user builds a lattice and adds annotation points, the program uses the lattice to create a 3D model of the worm, and uses that model to untwist the worm. The first step in generating a 3D model of the worm is to create curves defining the midline and sides of the worm based on the user-created lattice points, interpolating to define the regions between the lattice points. The algorithm first calculates a centerline for the volume by identifying the midpoint between left and right lattice points. Once the centerline has been calculated, natural splines are used to generate curves corresponding to the left side, right side, and midline of the worm. Once the midline, left, and right sides of the worm volume have been determined, the algorithm uniformly samples the volume along the midline, with one-voxel spacing. Corresponding positions on the left and right curves are determined, and this combination of midline, left, and right points are used to determine a 2D sampling plane for each position along the midline. Once the 2D sampling planes have been determined, each sampling plane is constrained by an elliptical model of the worm cross-section with limits defined by the left- and right-side curves. These constraints are used to prevent overlap in areas where the worm contacts itself or folds back on itself. For regions of the worm where the ellipses overlap, the voxels corresponding to the region of the overlap are removed and the ellipses adjusted to prevent overlap. The new 2D contours are then expanded outward from the ellipse, until they either contact an expanding contour line from another region of the worm or reach a preset distance. This process defines the 2D sampling planes used to create a straightened volume. Once the sampling planes have been defined, the straightened volumes are generated by intersecting each sampling plane with the imaging volume, and exporting the voxels within the sampling plane into a corresponding output slice. Voxels present in the twisted-up volume that do not intersect with a sampling plane do not appear in the final, untwisted volume. By concatenating the voxel data from the 2D sampling planes, a final 3D volume is created corresponding to the straightened worm (Figure 2D).

In order to facilitate the combination of cell positional information, our algorithm also allows a user to define annotation points (corresponding to a specific voxel in the 3D volume), which can act to label cells or cell components (like axons) of interest. After untwisting, the 3D position of each annotation point relative to a user-defined reference point are calculate, allowing the specific X, Y, and Z - coordinates of each point to be calculated. This information is written to a spreadsheet, with each individual point assigned a row and the X, Y, and Z - coordinates displayed in columns (Figure 3A). For cases in which a specific cell/annotation point is tracked over multiple volumes, we have developed a custom MATLAB script which searches through the individual spreadsheet files for each volume, identifies the annotation points by name, and rewrites the data to a new file organized by the name of each annotation point and time. Once organized in this manner, a user can examine how the position of a given cell or cellular component changes as the worm is developing (Figure 3A), and in cases where a structure is annotated in multiple embryos, can compare the position of that structure across the different volumes (Figure 3B, 3C). We are currently working on developing an integrated rendering capability which will graphically display the position of the annotation points over time, allowing a user to directly visualize how the various annotation points relate to each other in the developing embryo (Figure 4). While under development, this capability should be functioning shortly and should allow a user to identify the position of a neuron or cell of interest throughout development, and display how that position relates to the rest of the worm and changes over time.

### 3. FUTURE DIRECTIONS

Future plans include examining stereotypy of cell positions during embryo development - assembly of a neurodevelopmental atlas will be significantly easier if we find that cells occupy the same relative positions in multiple embryos, and more difficult if cell position proves to be variable when looked at from embryo to embryo. Currently we believe that cell position (at least for seam cells) is fairly stereotyped, but this needs to be confirmed. Once we understand how similar cell position is across embryos, we can begin assembling the neurodevelopmental atlas by identifying the 3D position of each neuron in the embryo, as well as any neurites that extend from those cells. By averaging positional information for the same cell across multiple embryos, we should be able to get an idea of how the “average” cell

develops, and by combining this positional information for multiple cells and rendering that positional information with our rendering software, we should be able to create a computational atlas showing the development of the entire *C. elegans* nervous system.

### FIGURES

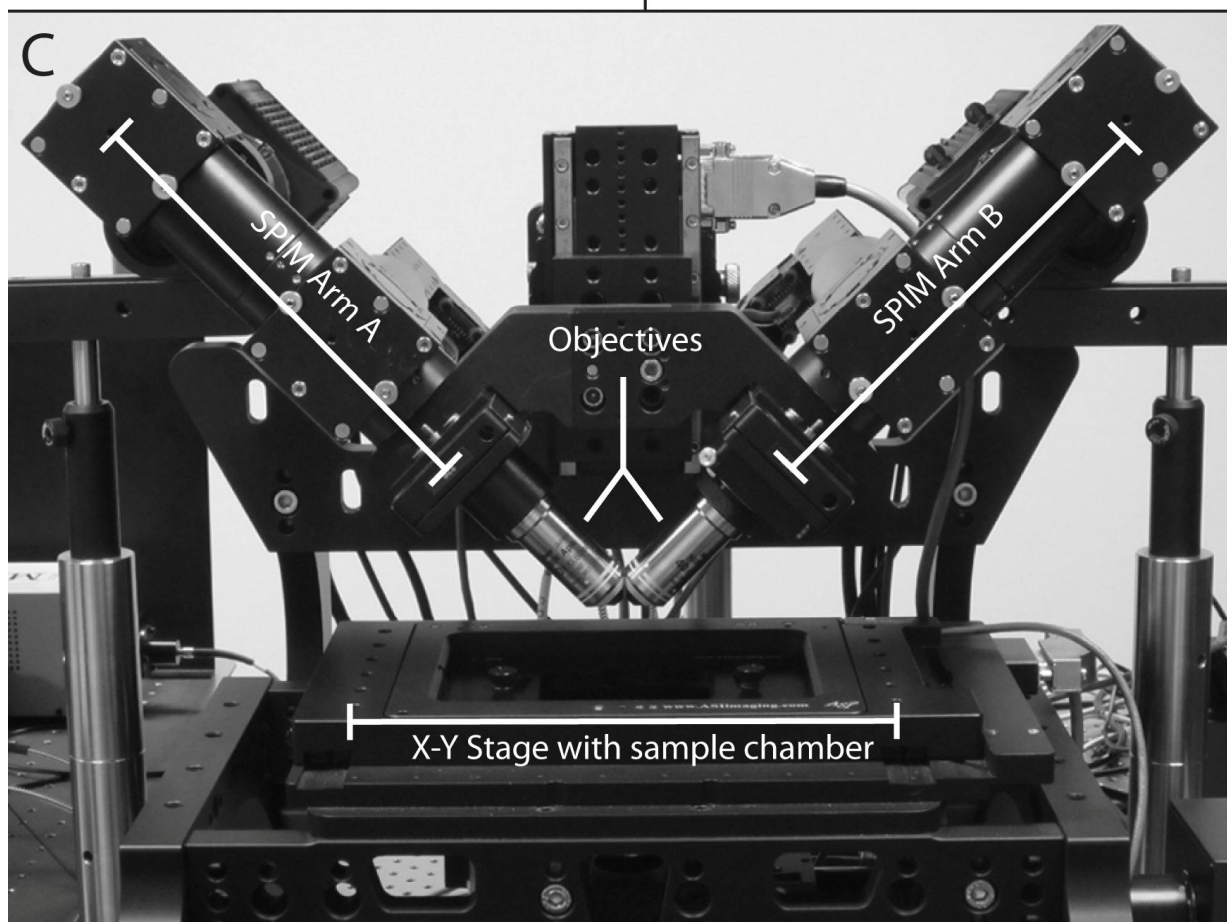
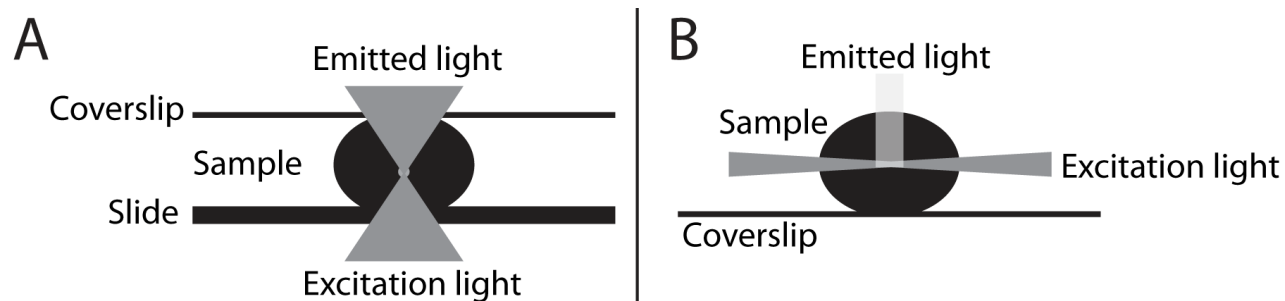


Figure 1: Implementation of light-sheet imaging in the diSPIM system

A. A diagram showing sample light exposure in conventional imaging modalities (compound or confocal microscopes). Notice how large areas of the sample not directly in focus are exposed to either excitation or emitted light (grey cones). This exposure to out-of-focus light can cause sample photobleaching and photo damage, leading to reductions in sample viability and available imaging time.

B. A diagram showing sample light exposure in a light sheet imaging setup. A thin light sheet (dark grey) is created optically and scanned through the sample a plane at a time. When compared to conventional imaging modalities (panel A), the amount of sample exposed to light is significantly smaller, leading to less bleaching and damage and longer available imaging times.

C. A photograph of the diSPIM dual light-sheet microscope, with major components labeled. Each SPIM arm (A and B) houses optics for generating a light sheet, as well as a camera for capturing images. The objectives are oriented orthogonally (90 degrees); a light sheet is generated in one arm and the objective on the other arm captures emitted light from the sample. As the objectives and optics are the same for each arm, light sheets can be generated sequentially and provide two orthogonal views of the sample. The sample imaging chamber is mounted in an x-y motorized stage, allowing selection of individual embryos for imaging. For additional information on this design, please see<sup>11,13</sup>.

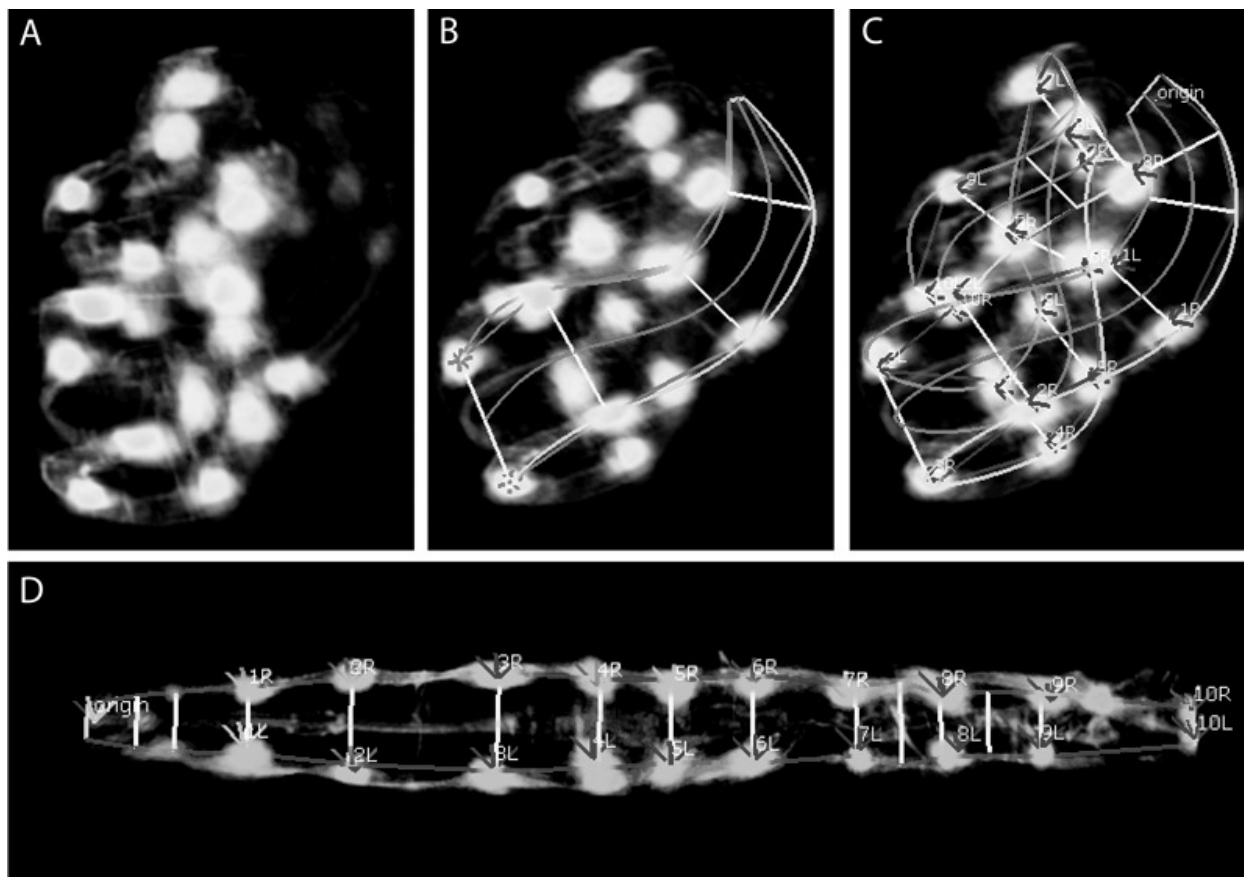


Figure 2: Untwisting an embryo volume in MIPAV

A: A volume corresponding to a twisted-up nematode embryo, displayed in the MIPAV volume renderer. Seam cell nuclei, junctions between hypodermal cells, and the worm midline are labeled, but due to twisting the shape of the embryo is not immediately clear.

B. Starting with the nose, a user has begun adding lattice points at places where seam cells are located. Using the positions of the lattice points, the algorithm develops a model of the worm body (defining left and right sides of the worm, as well as modeled dorsal/ventral extent), which tracks the growing lattice.

C. A completed version of the lattice, extending from the nose of the worm to the tail, with a lattice point placed at each seam cell and additional lattice points added when necessary to capture curves in the embryo. In addition, annotation points have been placed on each seam cell (and the origin point placed at the nose) to allow the position of these cells in the embryo to be identified. Seam cells are labeled using an XL / XR scheme, where X is a number denoting the pair the seam cell belongs to (measured relative to the nose) and L/R denotes which side of the body the seam cell lies on.

D. The final untwisted, annotated volumes. The nose is to the left, and is localized by the annotation point labeled “origin.” The untwisted volume extends from the nose to the last pair of seam cells (10R/10L); labeling beyond the last pair of seam cells appears to be of variable visibility from time point to time point, so the exact tip of the tail cannot be regularly identified.

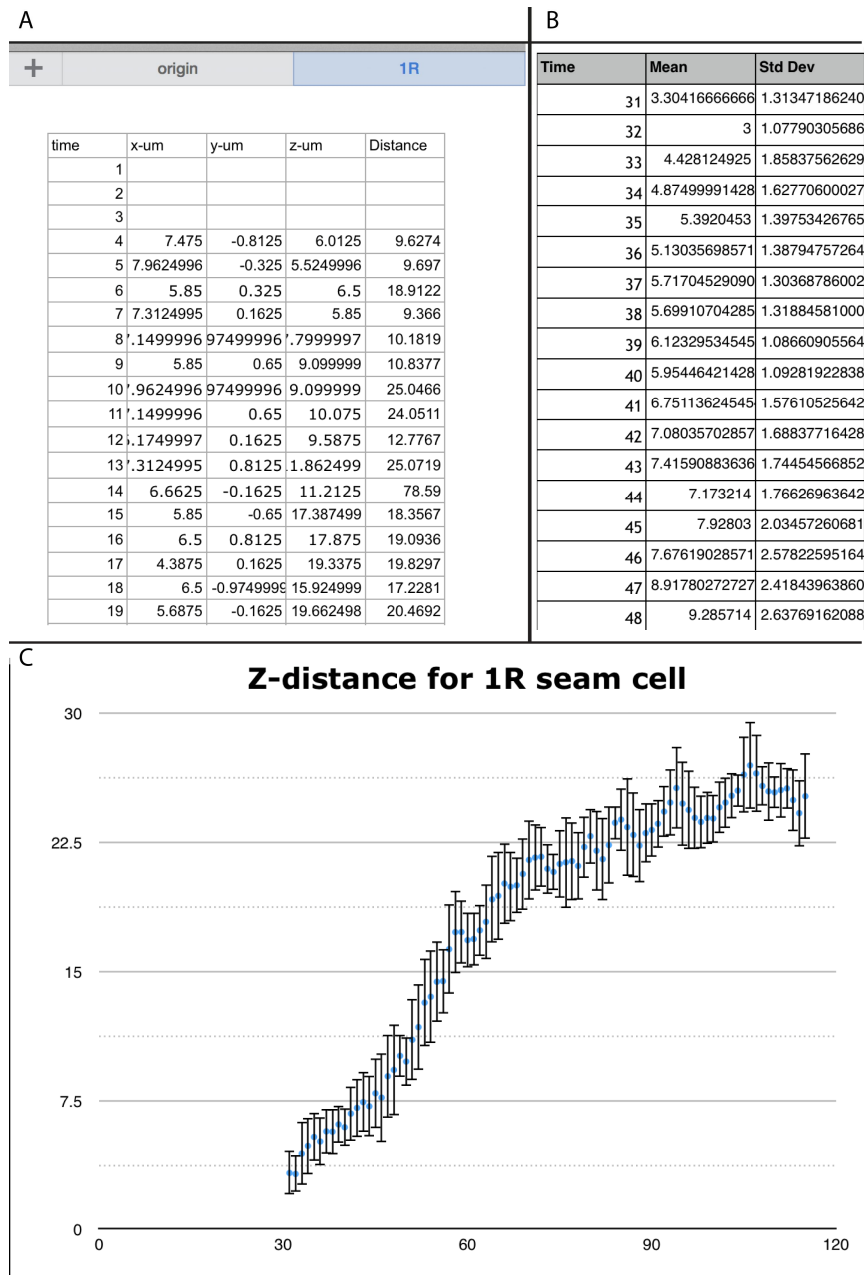


Figure 3: Annotation points allow positional (x, y, z) data to be determined for cells of interest in the embryo.

A: A spreadsheet showing the X, Y, and Z-positions (in microns) relative to the nose for the first seam cell on the right side of the animal. The data has been combined using a customized MATLAB script from individual spreadsheet files. Time represents the number of the volume analyzed in an image sequence. X-um, y-um, and z-um represent the distance (in microns), along each of these axes compared to an origin point placed at the nose. Distance refers to a 3D distance calculation for the seam cell compared to the origin point.

B: The positional data for specific cells can be combined and averaged to generate a “mean” position for any given cell in embryos of the same age. After shifting the data to align embryos by age, positions (in this case the z-distance for the first seam cell on the right of the animal) are averaged, generating a mean and standard deviation. This represents numerically how this z-position changes in an



“average” embryo. This data is derived from five different embryos, but in principle this can be scaled up to look at as many different datasets as are required.

C: A graphical representation of distance from the nose (Z-distance) vs. time for the first seam cell on the right side of the animal, graphed from the data in B. The X-axis measures time (units indicate which numbered volume in the image sequence a time point belongs to), while the Y-axis measures the distance of the seam cell from the nose in Z, with microns as the unit of measurement. As the embryo develops distance rapidly increases, before leveling off once the embryo has finished elongating.

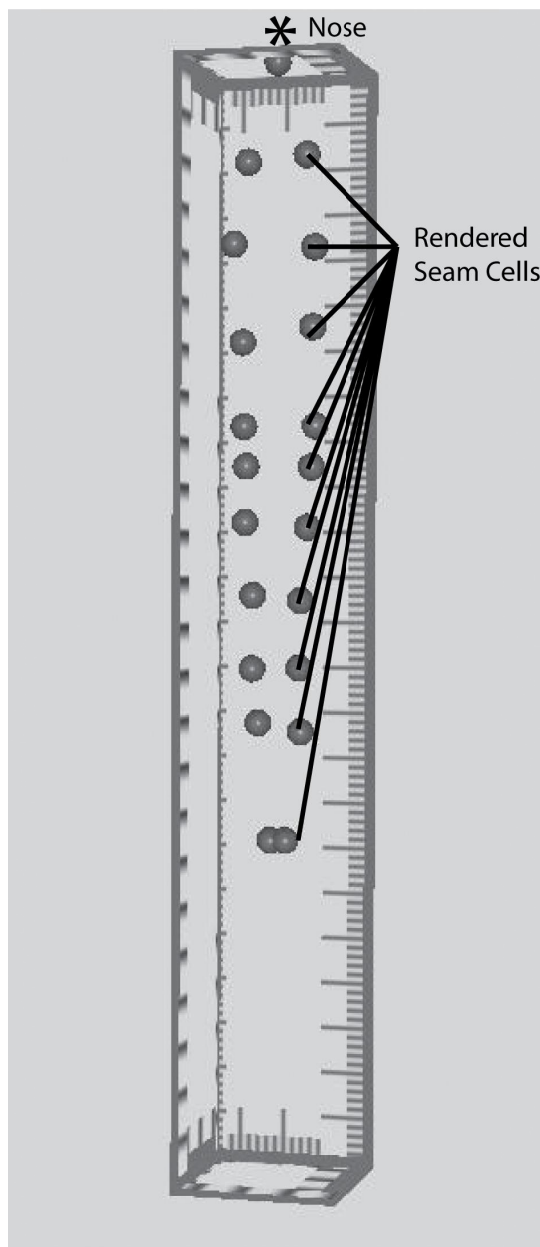


Figure 4: MIPAV allows rendering of positional data to graphically display worm development. MIPAV can take numerical positional information (as displayed in Figure 3A), and display that information in a graphical format. In this case, the positions of seam cells in a developing embryo are being displayed. An asterisk marks the nose of the animal, and the 3D positions of the seam cells on

the left and right sides of the animal are denoted by rendered spheres. Currently the rendered spheres are the only way of displaying points, but we are planning to add additional display options in the future.

## FOOTNOTES

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## REFERENCES

- [1] O'Donnell, M., Chance, R.K., Bashaw, G.J., "Axon growth and guidance: receptor regulation and signal transduction," *Annu. Rev. Neurosci.* 32, 383-412 (2009).
- [2] Tessier-Lavigne, M., and Goodman, C.S., "The molecular biology of axon guidance," *Science* 274(5290), 1123-1133 (1996).
- [3] Chilton, J.K. "Molecular mechanisms of axon guidance," *Dev. Biol.* 292(1), 13-24 (2006).
- [4] Herculano-Houzel, S., Mota, B., Lent, R., "Cellular scaling rules for rodent brains," *Proc. Natl. Acad. Sci. U.S.A.* 103(32), 12138-12143 (2006)
- [5] Azevedo, F.A.C., Carvalho, L.R.B., Grinberg, L.T., Farfel, J.M., Ferretti, R.E.L., Leite, R.E.P., Jacob Filho, W., Lent, R., Herculano-Houzel, S., "Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain," *J. Comp. Neurol.* 513, 532-541 (2009)
- [6] White, J.G., Southgate, E., Thomson, J.N., Brenner, S., "The structure of the nervous system of the nematode *Caenorhabditis elegans*," *Phil. Trans. R. Soc. Lond. B.* 314, 1-340 (1986)
- [7] Sulston, J.E., "Neuronal cell lineages in the nematode *Caenorhabditis elegans*," *Cold Spring Harb. Symp. Quant. Biol.* 48(2), 443-452 (1983)
- [8] Sulston, J.E., Schierenberg, E., White, J.G., Thomson, J.N., "The embryonic cell lineage of the nematode *Caenorhabditis elegans*," *Dev. Biol.* 100, 64-119 (1983)
- [9] Bao, Z., and Murray, J.I., "Mounting of *Caenorhabditis elegans* embryos for live imaging of embryogenesis," *Cold Spring Harb. Protoc.* 2011(9) (2011)
- [10] Wu, Y., Ghitani, A., Christensen, R., Santella, A., Du, Z., Rondeau, G., Bao, Z., Colón-Ramos, D.A., Shroff, H., "Inverted selective plane illumination microscopy (*i*SPIM) enables coupled cell identity lineaging and neurodevelopmental imaging in *Caenorhabditis elegans*," *Proc. Natl. Acad. Sci. U.S.A.* 108(43), 17708 - 17713 (2011)
- [11] Wu, Y., Wawrzusin, P., Senseney, J., Fischer, R.S., Christensen, R., Santella, A., York, A.G., Winter, P.W., Watterman, C.M., Bao, Z., Colón-Ramos, D.A., McAuliffe, M., Shroff, H., "Spatially isotropic four-dimensional imaging with dual-view plane illumination microscopy," *Nat. Biotechnol.* 31, 1032-1038 (2013)
- [12] Huisken, J., Stainier, D.Y., "Selective plane illumination microscopy techniques in developmental biology," *Development* 136(12), 1963-1975 (2009).
- [13] Kumar, A., Wu, Y., Christensen, R., Chandris, P., Gandler, W., McCreedy, E., Bokinsky, A., Colón-Ramos, D.A., Bao, Z., McAuliffe, M., Rondeau, G., and Shroff, H., "Dual-view plane illumination microscopy for rapid and spatially isotropic imaging," *Nat. Protoc.* 9, 2555-2573 (2014).