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TITLE:

In Vitro Reconstitution of Spatial Cell Contact Patterns with Isolated *Caenorhabditis elegans* Embryo Blastomeres and Adhesive Polystyrene Beads

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SUMMARY:

Tissue complexities of multicellular systems confound the identification of causal relationship between extracellular cues and individual cellular behaviors. Here, we present a method to study the direct link between contact-dependent cues and division axes using *C. elegans* embryo blastomeres and adhesive polystyrene beads.

ABSTRACT:

In multicellular systems, individual cells are surrounded by the various physical and chemical cues coming from neighboring cells and the environment. This tissue complexity confounds the identification of causal link between extrinsic cues and cellular dynamics. A synthetically reconstituted multicellular system overcomes this problem by enabling researchers to test for a specific cue while eliminating others. Here, we present a method to reconstitute cell contact patterns with isolated *Caenorhabditis elegans* blastomere and adhesive polystyrene beads. The procedures involve eggshell removal, blastomere isolation by disrupting cell-cell adhesion, preparation of adhesive polystyrene beads, and reconstitution of cell-cell or cell-bead contact. Finally, we present the application of this method to investigate the orientation of cellular division axes that contributes to the regulation of spatial cellular patterning and cell fate specification in developing embryos. This robust, reproducible, and versatile in vitro method enables the study of direct relationships between spatial cell contact patterns and cellular responses.

INTRODUCTION:

During multicellular development, the cellular behaviors (e.g., division axis) of individual cells are specified by various chemical and physical cues. To understand how individual cell interprets this

information, and how they regulate multicellular assembly as an emergent property is one of the ultimate goals of morphogenesis studies. The model organism *C. elegans* has contributed significantly to the understanding of cellular-level regulation of morphogenesis such as cell polarity[1], cell division patterning[1], cell fate decision[2], and tissue-scale regulations such as neuronal wiring[3] and organogenesis[4,5]. Although there are various genetic tools available, tissue engineering methods are limited.

The most successful tissue engineering method in *C. elegans* study is the classical blastomere isolation[6]; as *C. elegans* embryo is surrounded by an eggshell and a permeability barrier[7], their removal is one of the main procedures of this method. While this blastomere isolation method enables reconstitution of cell-cell contact in a simplified manner, it does not allow for the elimination of unwanted cues; cell contact still poses both mechanical (e.g., adhesion) and chemical cues, thereby limiting our ability to fully analyze the causal relationship between the cue and cellular behavior.

The method presented in this paper uses carboxylate modified polystyrene beads that can covalently bind to any amine-reactive molecules including proteins as ligands. Particularly, we used an amine-reactive form of Rhodamine Red-X as a ligand to make beads both visually trackable and adhesive to the cell. The carboxyl groups of bead surface and primary amine groups of ligand molecule are coupled by water-soluble carbodiimide 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC)[8,9]. Obtained adhesive beads allow for the effects of the mechanical cue on cellular dynamics[10]. We have used this technique to identify mechanical cues required for cell division orientation[10].

PROTOCOL:

1. Preparation of adhesive polystyrene bead

NOTE: This protocol does not require aseptic technique.

1.1. Weigh 10 mg of carboxylate modified polystyrene beads in a 1.5 mL microcentrifuge tube.

1.2. To wash the beads, add 1 mL of 2-(N-morpholino)ethanesulfonic acid (MES) buffer into the tube. Since MES buffer does not contain phosphate and acetate, which can reduce the reactivity of carbodiimide, it is suitable to use in protein coupling reaction. Vortex the tube to mix the beads.

1.3. Spin the tube for 60 s at 2,000 x *g* via a benchtop centrifuge.

1.4. Discard the supernatant by carefully pipetting out the buffer.

1.5. Wash the beads again with 1 mL of MES buffer by following steps 1.2-1.4.

1.6. Add 1 mL of MES buffer containing 10 mg of EDAC into the tube to activate the surface

carboxyl groups. Vortex the tube to mix the beads.

1.7. Rotate and incubate the tube for 15 min at room temperature.

1.8. Spin down the beads for 60 s at 2,000 x *g*.

1.9. Discard the supernatant by carefully pipetting out the buffer.

1.10. To wash the beads, add 1 mL of phosphate buffered saline (PBS) into the tube. Vortex the tube to mix the beads.

1.11. Spin down the tube for 60 s at 2,000 x *g*.

1.12. Discard the supernatant by carefully pipetting out the buffer.

1.13. Wash the beads again with 1 mL of PBS by following steps 1.10-1.12.

1.14. The final concentration of Rhodamine used will depend on the strain being imaged. Prepare 1 mL of 1-, 10-, 100-, and 1000-fold dilution series of Rhodamine Red-X from the 0.65 mM Rhodamine Red-X stock solution.

1.15. Pipette 20 µL of beads into each serial dilution tube.

1.16. Rotate and incubate the tube for 5 min at room temperature.

1.17. Wash the beads twice with 1 mL of PBS by repeating steps 1.10-1.12.

1.18. Add 1 mL of PBS into the tube and store it at 4 °C for up to 6 weeks. Check the fluorescence intensity of the beads under a microscope used for live-imaging. The appropriate concentration of the Rhodamine Red-X succinimidyl ester is dependent on the imaging conditions (**Figure 1**).

NOTE: Beads without Rhodamine Red-X treatment do not adhere to cell. Rhodamine Red-X serves as fluorescence marker as well as adhesive molecule. The electro static interaction between positively charged Rhodamine Red-X and negatively charged plasma membrane is a putative cause of adhesion.

2. Assembly of mouth pipette

2.1. Cut wide (6.35 mm inner diameter) and narrow (3.175 mm inner diameter) Tygon tubes to about 25 cm and 40 cm in length, respectively (**Figure 2**).

2.2. Connect the Tygon tubes with a polytetrafluoroethylene (PTFE) filter (0.2 µm pore size) (**Figure 2**).

2.3. Disassemble a commercially available aspirator tube and attach its capillary holder and mouthpiece to the end of the narrow and wide Tygon tubes, respectively (**Figure 2**).

NOTE: PTFE filter was used to prevent the inhalation of fumes of hypochlorite solution via mouth pipette.

3. Isolation of embryo blastomere

NOTE: Wear gloves and lab coat to avoid cut and contact with the bleaching solution.

3.1. Hold each end of a microcapillary (capacity; 10 µL) with right and left hand.

3.2. Pull the microcapillary towards both ends to apply tension and bring the center of the capillary over a burner to make two hand-pulled capillaries (**Figure 3A**).

3.3. Trim the tips of the hand-pulled capillaries with forceps under the dissecting microscope and attach the pulled capillary into a mouth pipetting apparatus (**Figure 2**). Prepare two types of pipettes. The tip opening sizes for the pipettes should be approximately 2x and 1x the short axis length of *C. elegans* embryos (30 µm) for the embryo transfer and eggshell removal, respectively (**Figure 3B-D**).

3.4. Pipette 45 µL of egg salt solution onto a well of a multiwell slide (**Figure 4A**; bottom).

3.5. Place 5-10 adult *C. elegans* onto a well containing egg salt solution.

3.6. To obtain early *C. elegans* embryos, cut adults into pieces by positioning two needles to the right and left of *C. elegans* body and sliding the needles past each other (**Figure 4A**; upper schematics).

3.7. Pipette 45 µL of hypochlorite solution onto a well next to the well containing egg salt solution (**Figure 4B**).

3.8. Pipette 45 µL of Shelton's growth medium onto the subsequent three wells next to the well containing hypochlorite solution (**Figure 4B**).

3.9. Transfer 1-cell stage and early 2-cell stage embryos into the hypochlorite solution by mouth pipetting with the hand-drawn capillary for embryo transfer (**Figure** 4B).

3.10. Wait for 40–55 s.

3.11. Wash the embryos by transferring the embryos from hypochlorite solution into Shelton's growth medium by mouth pipetting with the hand-drawn capillary for embryo transfer (**Figure 4B**).

3.12. Wash the embryos again by transferring the embryos into a new well of Shelton's growth medium by mouth pipetting with the hand-drawn capillary for embryo transfer (**Figure 4B**).

3.13. Transfer the washed embryos into a new well of Shelton's growth medium by mouth pipetting with the hand-drawn capillary for embryo transfer. Using the hand-drawn capillary for eggshell removal, carefully repeat the pipetting (**Figure 4C**; middle schematics). If the eggshell is successfully removed, the embryonic cells will become more spherical (**Figure 4C**; right).

3.14. Separate the 2-cell stage embryonic blastomeres by gently and continuously pipetting with the hand-drawn capillary for eggshell removal (**Figure 4D**).

4. Reconstitution of contact patterns with blastomere and bead

NOTE: Work under the imaging microscope to avoid dissociation of a cell from a bead and to facilitate the timely image acquisition.

4.1. To observe using an inverted microscope, prepare an imaging chamber as in **Figure 5**.

4.1.1. Place a coverslip onto a coverslip holder (**Figure 5A**).

4.1.2. Tape the edges of the coverslip to stabilize it. The side with tape is the 'back' side (**Figure 5A,B**).

4.1.3. Flip the coverslip holder over to the 'front' side and draw a circle on the coverslip with a hydrophobic pen (**Figure 5C**).

NOTE: Any glass-bottom dish also works, provided the embryos are manipulatable by the mouth pipette.

4.2. Add Shelton's growth medium within the circle drawn by the hydrophobic marker (**Figure 5D**).

4.3. Transfer the isolated blastomere to the imaging chamber.

4.4. Dispense a small volume of the chemically functionalized beads using the hand-drawn capillary for embryo transfer.

4.5. Control the position of the polystyrene beads by blowing into the hand-drawn capillary until the bead attaches to the isolated blastomere.

4.6. Mount a coverslip to avoid evaporation of medium (**Figure 5E**). Perform live imaging.

5. Preparation of important reagents

5.1. Egg Salt Solution (10 mL): Combine 235 µL of 5 M NaCl and 240 µL of 2 M KCl with 9525 µL of dH_2O.

5.2. Hypochlorite Solution (10 mL): Combine 7.5 mL of Clorox (containing approximately 7.5% sodium hypochlorite) with 2.5 mL of 10 N NaOH (final concentration of sodium hypochlorite is approximately 5.625%).

NOTE: Although many published methods have used chitinase to digest chitinous eggshells, we have adopted a method using hypochlorite solution[11]. By avoiding the batch-to-batch variations of chitinase activities, we believe this method is more reproducible and cost-effective approach.

5.3. 0.81 mM Inulin Solution (40 mL)

5.3.1. Add 0.2 g of Inulin to 40 mL of dH_2O.

5.3.2. Autoclave to dissolve.

NOTE: Keeps for 1 month at 4 °C.

5.4. 0.5 M MES Buffer (500 mL)

5.4.1. Dissolve 48.81 g of MES in 400 mL of distilled water (dH_2O).

5.4.2. Adjust pH to 6.0.

5.4.3. Bring the total volume to 500 mL with dH_2O.

5.5. PBS Solution (1 L)

5.5.1. To make 10-fold PBS solution, dissolve 1 package of PBS premix powder in 1 L of dH_2O.

5.5.2. Combine 100 mL of 10-fold PBS solution with 900 mL of dH_2O.

5.6. 5% Polyvinylpyrrolidone (PVP) Solution (4 mL)

5.6.1. Under sterile conditions, dissolve 0.2 g of PVP in 4 mL of Drosophila Schneider's Medium.

NOTE: Always open Schneider's Medium stock in tissue culture (TC) hood. Keep for 1 month at 4 °C.

5.7. 0.65 mM Rhodamine Red-X stock solution (2 mL)

5.7.1. Weigh 1 mg of Rhodamine Red-X.

5.7.2. Add 2 mL of dimethyl sulfoxide (DMSO) to dissolve.

5.7.3. Aliquot and store at -20 ˚C.

5.8. Shelton's Growth Medium (SGM) (10.25 mL)

5.8.1. Under sterile conditions, combine 8 mL of Drosophila Schneider's Medium, 1 mL of Inulin solution, 1 mL of Basal Medium Eagle (BME) Vitamins, 50 µL of Penicillin-Streptomycin, and 100 µL of Lipid concentrate together.

5.8.2. Aliquot 325 µL of SGM into 1.5 mL microtubes.

NOTE: Keep for about 1 month at 4 °C.

5.8.3. On the day of blastomere isolation, add 175 µL of Fetal Bovine Serum (FBS) to the SGM (for total volume of 500 µL).

NOTE: Store FBS at -20 °C and thaw it before usage. FBS does not need to be heat killed.

REPRESENTATIVE RESULTS:

For beads preparation, we determined the optimal amount of Rhodamine Red-X succinimidyl ester for the transgenic strain expressing GFP-myosin II and mCherry-histone (**Figure 1A-D**). We used mCherry tagged histone as a marker of cell cycle progression. Because both Rhodamine Red-X and mCherry will be illuminated by a 561 nm laser, the optimal intensity of Rhodamine Red-X signal is comparable to that of histone to allow simultaneous imaging of cell and bead. For example, the fluorescence signal from the bead treated with 0.005 µg/mL Rhodamine Red-X succinimidyl ester was too weak to visualize the bead (**Figure 1A**). On the other hand, the fluorescence signal from the beads treated with 5 µg/mL Rhodamine Red-X succinimidyl ester was too strong to image mCherry-histone (**Figure 1D**). We determined that 0.5 µg/mL Rhodamine Red-X succinimidyl ester is optimal for this particular transgenic strain (**Figure 1C**).

Blastomere isolation was performed according to the procedures shown in **Figure 4**. The following points should be addressed for successful experiments. 1) The media in the well evaporates over time and becomes viscous; this causes the embryos to stick to the glass capillary and other embryos, so a new well with fresh media needs to be used. 2) In the hypochlorite solution, embryos float to the surface. Therefore, lower the magnification of the microscope and focus on the surface of the droplet to ease with the transferring of embryos. 3) The effectiveness of hypochlorite solution may differ. Hence, duration of embryos spend in the hypochlorite solution should be tested for each new batch of hypochlorite solution made. 4) Place the mouth pipette as close to the embryo as possible to minimize the strength used to blow into the pipette, but also not too close so that the embryo is sucked up as well (this is also true when attaching beads). 5) After eggshell removal, embryos become very delicate. Hence, forces exerted on the mouth pipette need to be slightly lowered to prevent embryos from bursting. 6) Prolonged usage of the mouth pipette may dampen the PTFE filter, and 7) 2-cell stage embryos should only be

separated when the nucleus has been clearly formed (**Figure 4C**, left schematics). Compared to cells in intact embryos (**Figure 4A**; right), cells in embryos without eggshell look rounder (**Figure 4C**; right). Additionally, after blastomere isolation, the shape of blastomeres become spherical (**Figure 4D**; right).

To test the effects of physical contact-dependent cue on cell division orientation, we attached Rhodamine Red-X coated beads to AB blastomeres isolated from the 2-cell stage and performed live-imaging (**Figure 6,7**). The bead without Rhodamine Red-X treatment did not adhere to the blastomeres, suggesting that the Rhodamine Red-X serves as a both fluorescence marker and adhesive molecule. For the analysis of cell division orientation, we have 3D reconstructed the time-lapse image using the "3D project" function of Image J[12] (**Figure 6A**; tilting around Y-axis). To determine the plane containing mitotic spindle (spindle plane), we first identified a plane where two centrosomes were vertically aligned (**Figure 6B**; 110˚): the plane with ± 90˚ angle of this plane is spindle plane (**Figure 6B**; -20˚). Next, we have measured the angle of spindle (spindle orientation) relative to the cell-bead contact interface (contact orientation) after cytokinesis (**Figure 6C**). When both daughter cells were attached to the bead, a line that passes both contact sites was used as a contact orientation.

In our previous study, we have identified an anisotropic cell surface myosin flow during the bead-induced AB cell division orientation[10]. However, it is difficult to measure the intracellular myosin flow as cell moves during oriented division. To perform this, first we have selected the samples with spindle aligned to the imaging plane (xy plane) (**Figure 7**). Second, Z-stacks were projected using maximum projection method (**Figure 7**). Third, cell movements were corrected using the subpixel registration algorithm **Stack reg** plug-in[13] of Image J with **Rigid body** option (**Figure 7B**). By the registration of images, the position of cell was stabled (**Figure 7B**). Finally, by using **Manual tracking** plug-in of Image J, myosin foci were tracked (**Figure 7C**). According to the coordinate information, velocities of myosin along division axis, and axis perpendicular to it were calculated within 50 s after the cytokinesis onset.

FIGURE AND TABLE LEGENDS:

Figure 1. Determination of Rhodamine Red-X concentration. (**A-D**) Embryos expressing GFP-myosin (green) and mCherry-histone (magenta) were placed in proximity to beads bound with different concentrations of Rhodamine Red-X (magenta). The signal intensities of mCherry and Rhodamine Red-X are plotted along the white dotted lines (bottom graphs). The beads and histone signals were clearly detected for beads treated with 0.5 µg/mL Rhodamine Red-X. Scale bars show 10 µm.

Figure 2. Assembly of mouth pipette. Mouth pipette is assembled by connecting narrow (1) and wide (2) Tygon tubes with a PTFE filter (3). At the end of the narrow and wide Tygon tube, capillary holder (4) and mouth piece (5) were attached, respectively. A hand-drawn glass capillary (6) can be inserted in the capillary holder. Scale bar is 50 mm.

Figure 3. Blastomere isolation. (**A**) Hand pulling of glass capillary. (**B**) Hand-drawn glass capillary for embryo transfer. (**C**) Hand-drawn glass capillary for eggshell removal. (**D**) Schematics showing

the appropriate size of capillary opening for the eggshell removal. Arrows indicate embryos. Scale bars show 100 µm.

Figure 4. Blastomere isolation workflow. (**A**) Dissection of adult *C. elegans* in egg salt buffer to obtain embryos. Photographs show 2-cell and 4-cell stage embryos before eggshell removal. (**B**) Hypochlorite treatment and washing. (**C**) Schematics depict the appropriate timing for eggshell removal. Photograph shows a 4-cell stage embryo after eggshell removal. (**D**) Blastomere separation. Photograph shows a separated 2-cell stage embryo. Sizes of the arrows in C and D indicate the relative forces required during pipetting. Scale bars show 50 µm.

Figure 5. Assembly of imaging chamber. (**A**) Attaching coverslip to the coverslip holder. (**B**) Images of a coverslip holder. Coverslip holder before and after assembly is indicated on the left and right, respectively. (**C**) A circle drawn via a hydrophobic pen. (**D**) Addition of Shelton's growth medium and sample within the circle. (**E**) Mounting a coverslip to avoid evaporation. Scale bars show 50 mm.

Figure 6. Analysis of cell division orientation. (**A**) A diagram of cell division orientation analysis. (**B**) Determination of spindle plane. Left images show an example of a sample. 3D reconstructed 4-D movies were rotated around Y-axis to determine the plane wherein two centrosomes align vertically (right image; right upper schematics). In this example, spindle plane is ± 90˚ of 110˚ (middle image; right bottom schematics). (**C**) Measurement of spindle orientation relative to the cell-bead contact. Using images of the spindle plane, spindle orientation after cytokinesis was determined based on angle between lines connecting two centrosomes (orange dotted lines in the right schematics) and cell contact (blue dotted lines). When both daughter cells were attached to the beads, cell-bead contact orientation was the line that passes both contact sites. Green is myosin and centrosome, magenta is histone and beads. Scale bars are 10 µm. Times are minutes and seconds.

Figure 7. Analysis of myosin flow. (**A**) A diagram of myosin flow analysis. (**B**) Correction of cell division orientation. To quantify intracellular myosin flow dynamics, the rotation of dividing cell was corrected using the Image J plugin Stack reg (**Rigid body** option). Upper and bottom images are before and after the Stack reg processing. Right most images show temporal color code of time series. (**C**) Tracking of myosin foci. Using the images processed by Stack reg, individual myosin foci movements were tracked by Image J **Manual tracking** plugin. Division axis and the axis perpendicular to it were defined as x and y, respectively (right schematics). Myosin flow velocities in x and y axis (Vx and Vy) were measured according to the coordinate information. Scale bars are 10 µm. Times are minutes and seconds.

DISCUSSION:

Reconstitution of simplified cell contact patterns will let researchers to test the roles of specific cell contact patterns in different aspects of morphogenesis. We have used this technique to show that cell division axis is controlled by the physical contact with adhesive beads[10]. As division axis specification is crucial for multicellular development by contributing to morphogenesis[14], stem cell division[15,16], and tissue homeostasis[15,16], this method should be able to illuminate a new

mechanism of animal tissue formation.

In addition to cell division orientation, this method has a potential to be a platform to test the relationship between mechanical cues and cell fate. Previous studies reported that mechanical cue controls cell fate specification. Human mesenchymal stem cells differentiate into neuron, adipocyte, skeletal muscle cell, and osteoblast when cultured in substrate with different stiffness[17]. In response to substrate stiffness, the transcriptional regulators Yes-associated protein (YAP) and TAZ (transcriptional co-activator with PDZ-binding motif) will translocate to nucleus, to regulate cell fate specification[18]. The method presented in this paper should also be useful to test the role of mechanical cues on cell fate specification, by culturing cell long-term in contact with adhesive beads.

This method has limitations in recapitulating certain mechanical cues observed in vivo. In *C. elegans*, chitinous eggshell and permeability barrier serve as a physical constrain. Although the removal of eggshell does not affect normal development, removal of both eggshell and permeability barrier results in abnormal pattern formation[19]. In the absence of eggshell and permeability barrier, cell shape becomes more spherical, suggesting that the pressure between cells and cell-eggshell plays a critical role in cell deformation and patterning. Indeed, cell-cell squeezing forces has been proposed to regulate cell division orientation[20]. Without having physical constrain and putative pressure among tissues, we expect that this method also cannot recapitulate in vivo cell contact area. As cell contact area is known to affect Notch signaling[21], this limitation needs to be further considered when certain mechanical or chemical cue does not work using this in vitro method.

We have so far tested the cell division orientation of isolated AB, P1, EMS, P2, ABa, ABp cells (cells up to 6 cell stages) in our hands[10], but the method can be applied to later development as far as individual cell can be isolated. Recent single cell sequencing study has used pronase digestion of worm body to isolate larval cell[22]. Therefore, potentially one can use pronase treatment to isolate later stage embryonic cells and larval cells.

In this study, we showed the example of interaction between mechanical cue and cell division orientation, but cell-cell communication is also mediated by chemical cues such as Wnt[23], Notch[24], adhesion coupled receptors[25] and so on. Importantly, the beads preparation method presented here can couple the beads with any proteins, thereby allowing the reconstitution of chemical cues. Previous studies have also used Sepharose bead[26] and protein-A magnetic bead[27] coated with Wnt protein to demonstrate Wnt-dependent developmental processes. However, these beads are not commercially available in various sizes compared to carboxylate modified polystyrene beads. Hence, future research can use the presented method as a platform to test the role of both chemical and physical cues in more tunable manners. Taken together, this method is suited for researchers, who are studying cellular behaviors and responses that result from spatial cell contact patterns and wish to eliminate other cellular cues.

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DISCLOSURES:

The authors declare no conflict of interests.

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Figure 1

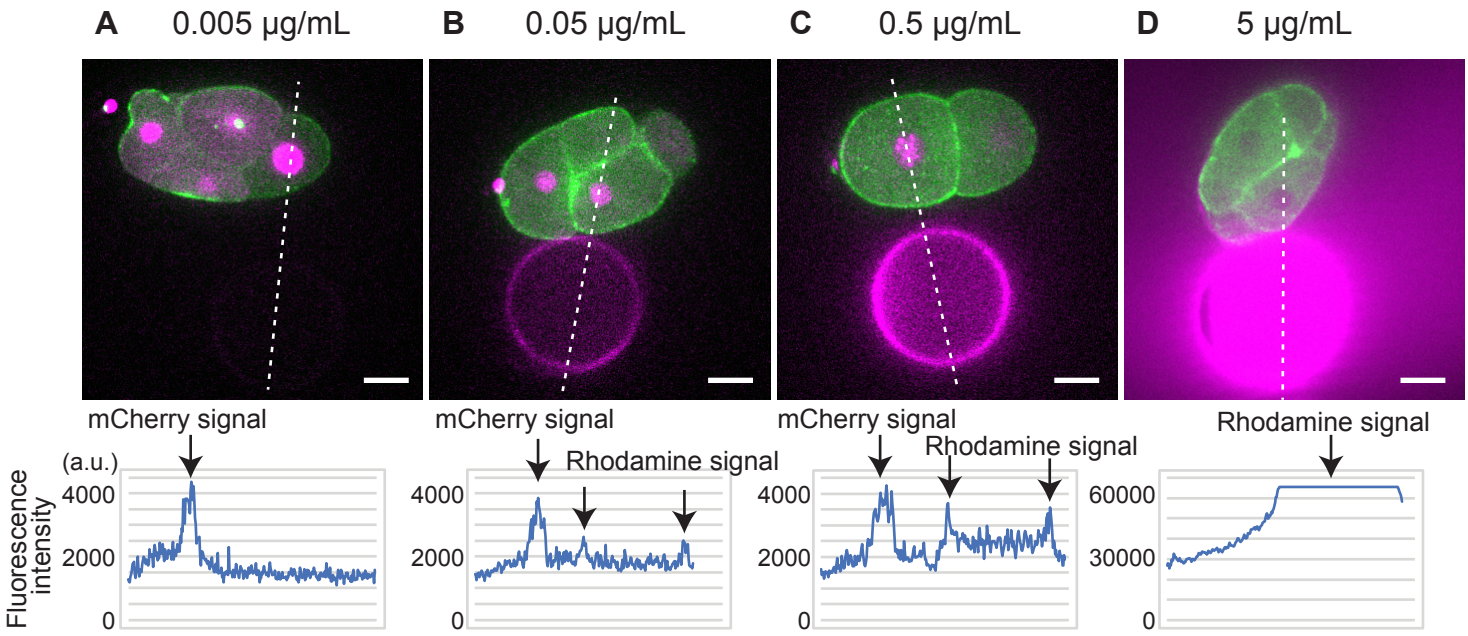

Figure 1. Determination of Rhodamine Red-X concentration.
(A-D) Embryos expressing GFP-myosin (green) and mCherry-histone (magenta) were placed in proximity to beads bound with different concentrations of Rhodamine Red-X (magenta). The signal intensities of mCherry and Rhodamine Red-X are plotted along the white dotted lines (bottom graphs). The beads and histone signals were clearly detected for beads treated with 0.5 µg/mL Rhodamine Red-X.Scale bars show 10 µm.

Figure 2

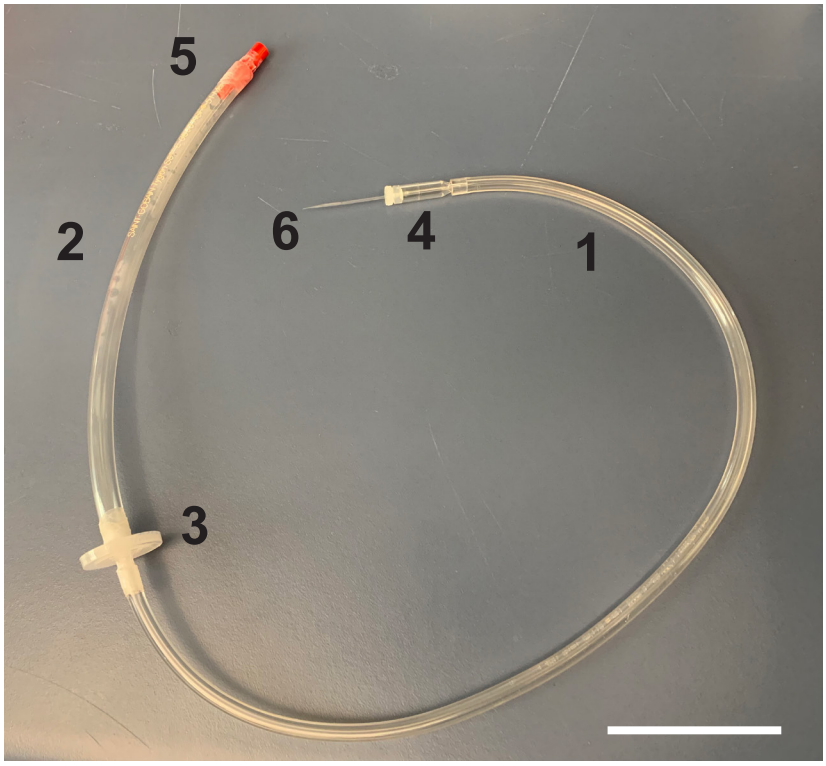

Figure 2. Assembly of mouth pipette
Mouth pipette is assembled by connecting narrow (1) and wide (2) tygon tubes with a PTFE filter (3). At the end of the narrow and wide tygon tube, capillary holder (4) and mouth piece (5) were attached, respectively. A hand-drawn glass capillary (6) can be inserted in the capillary holder. Scale bar is 50 mm.

Figure 3

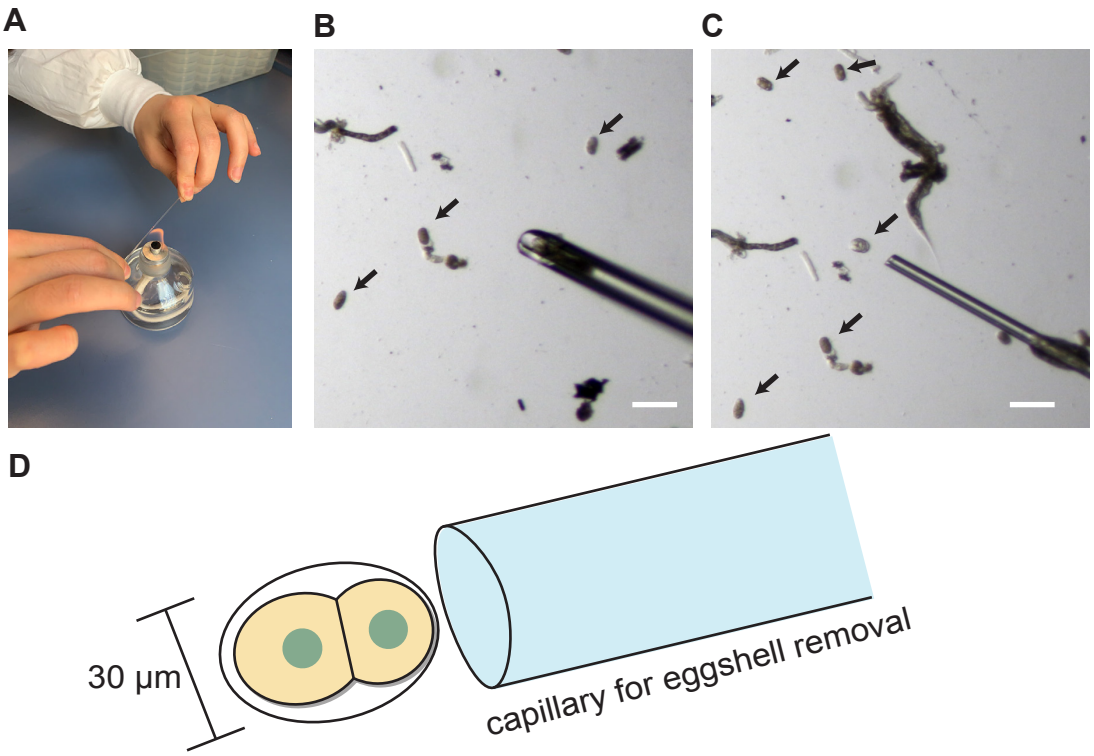

Figure 3. Blastomere isolation
(A) Hand pulling of glass capillary. (B) Hand-drawn glass capillary for embryo transfer. (C) Hand-drawn glass capillary for eggshell removal. (D) Schematics showing the appropriate size of capillary opening for the eggshell removal. Arrows indicate embryos. Scale bars show 100 µm.

Figure 4

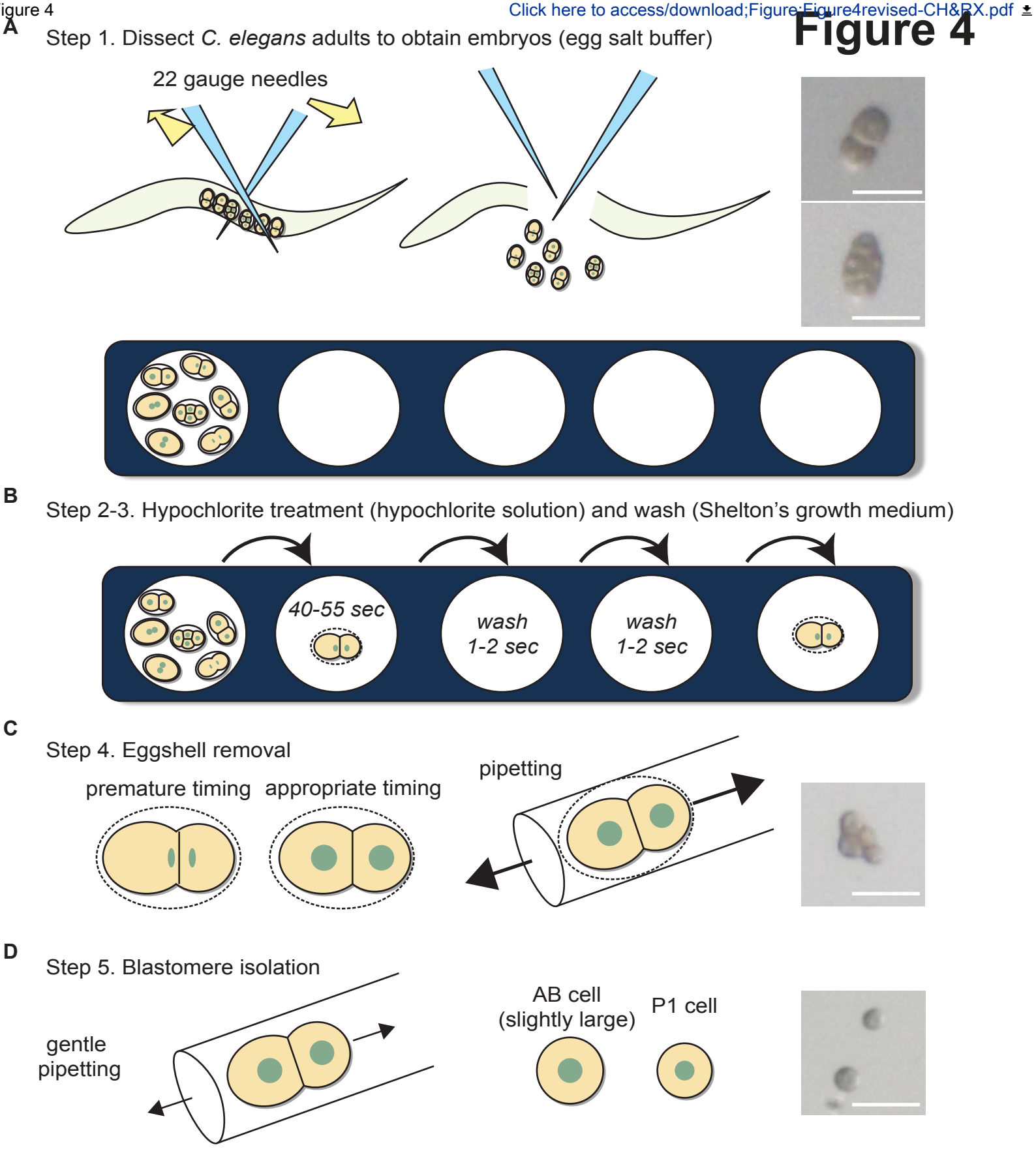

Figure 4. Blastomere isolation work flow
(A) Dissection of adult *C. elegans* in egg salt buffer to obtain embryos. Photographs show 2-cell and 4-cell stage embryos before eggshell removal. (B) Hypochlorite treatment and washing. (C) Schematics depict the appropriate timing for eggshell removal. Photograph shows a 4-cell stage embryo after eggshell removal. (Step 5.) Blastomere separation. Photograph shows a separated 2-cell stage embryo. Sizes of the arrows in C and D indicate the relative forces required during pipetting. Scale bars show 50 μm.

Figure 5

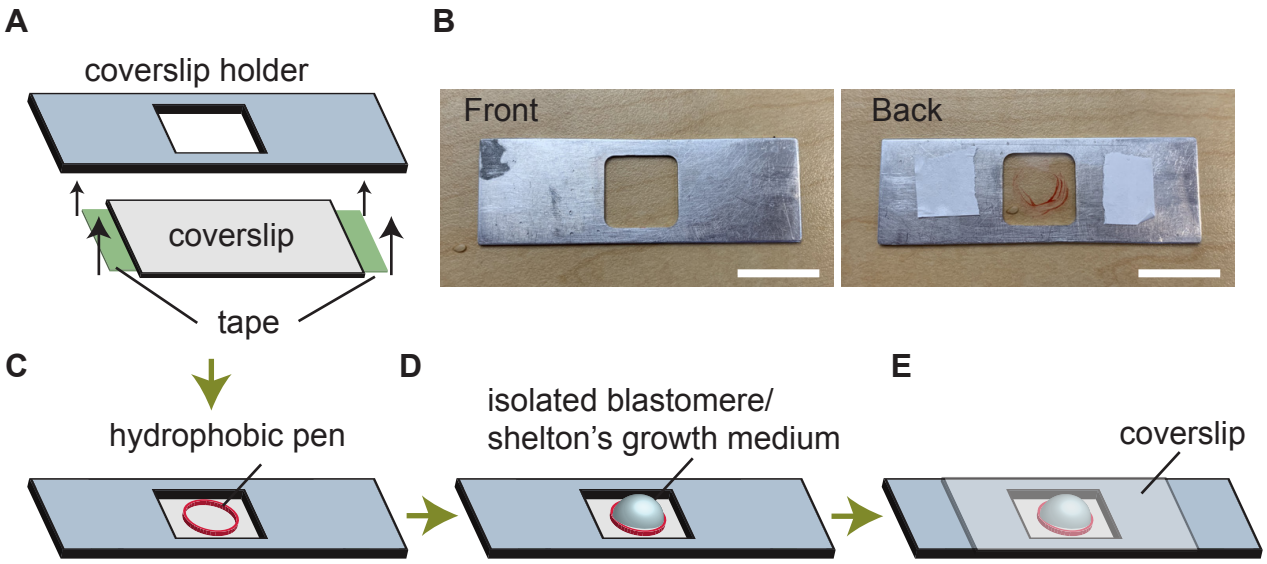

Figure 5. Assembly of imaging chamber
(A) Attaching coverslip to the coverslip holder. (B) Images of a coverslip holder. Coverslip holder before and after assembly is indicated on the left and right, respectively. (C) A circle drawn via a hydrophobic pen. (D) Addition of Shelton's growth medium and sample within the circle. (E) Mounting a coverslip to avoid evaporation. Scale bars show 50 mm.

Figure 6

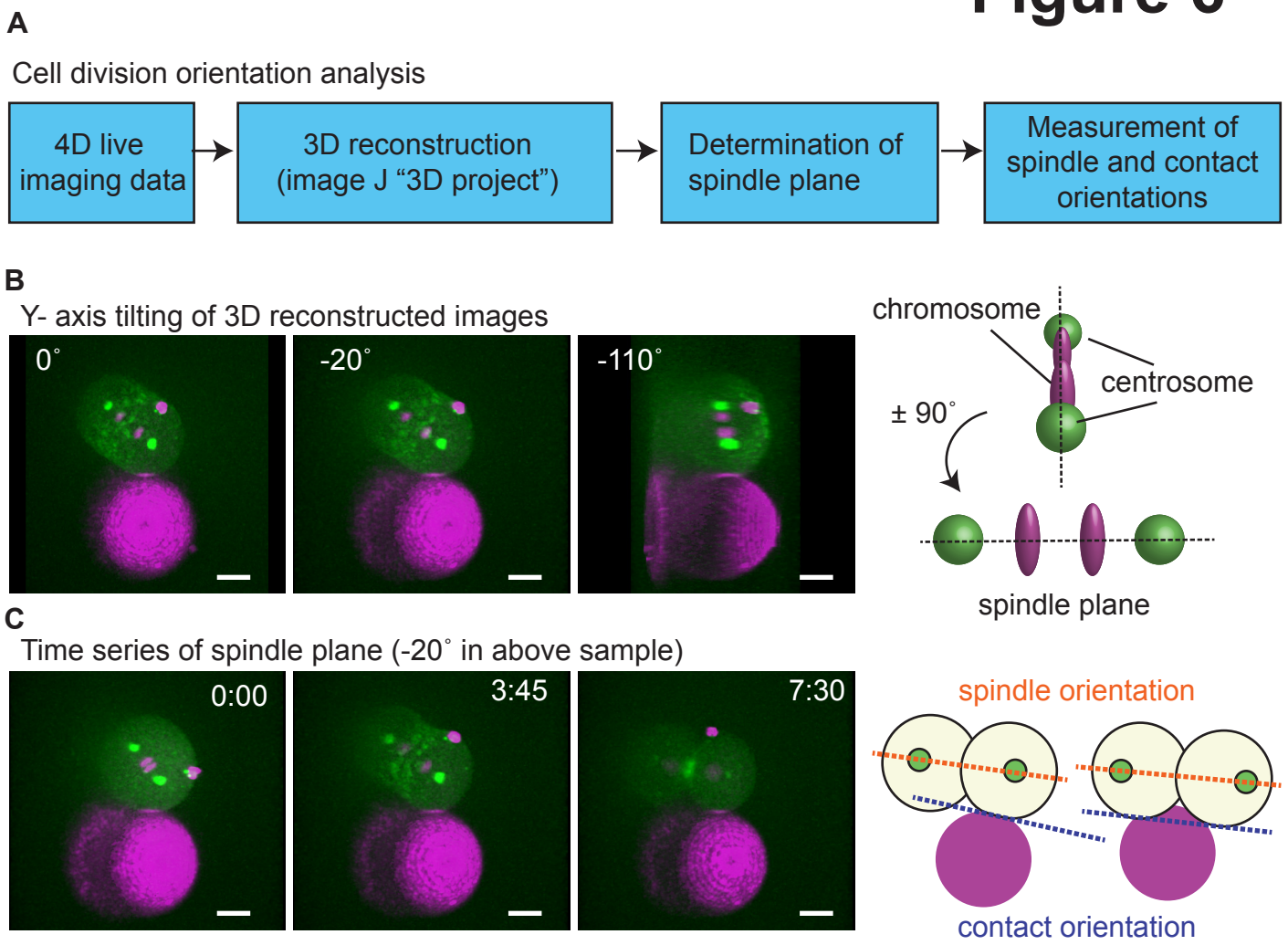

Figure 6. Analysis of cell division orientation
(A) A diagram of cell division orientation analysis. (B) Determination of spindle plane. Left images show an example of a sample. 3D reconstructed 4-D movies were rotated around Y-axis to determine the plane wherein two centrosomes align vertically (right image; right upper schematics). In this example, spindle plane is ± 90˚ of 110˚ (middle image; right bottom schematics). (C) Measurement of spindle orientation relative to the cell-bead contact. Using the images of the spindle plane, spindle orientation after cytokinesis was determined based on angle between lines connecting two centrosomes (orange dotted lines in the right schematics) and cell contact (blue dotted lines). When both daughter cells were attached to the beads, cell-bead contact orientation was the line that passes both contact sites. Scale bars are 10 μm. Times are minutes and seconds.

Figure 7

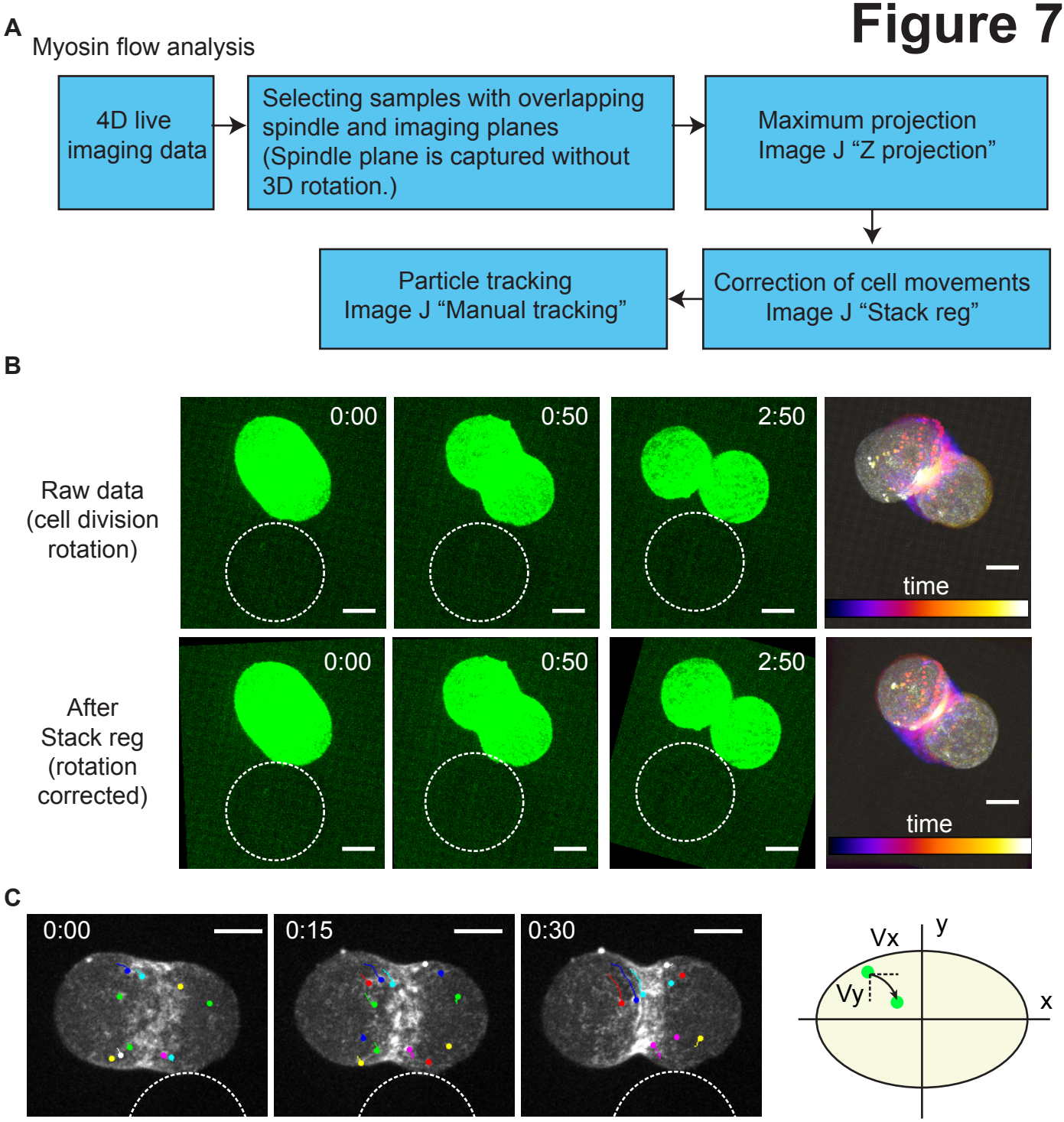

Figure 7. Analysis of myosin flow

(A) A diagram of myosin flow analysis. (B) Correction of cell division orientation. To quantify intracellular myosin flow dynamics, the rotation of dividing cell was corrected using the Image J plugin Stack reg ("rigid body" option). Upper and bottom images are before and after the Stack reg processing. Right most images show temporal color code of time series. (C) Tracking of myosin foci. Using the images processed by Stack reg, individual myosin foci movements were tracked by Image J "Manual tracking" plugin. Division axis and the axis perpendicular to it were defined as x and y, respectively (right schematics). Myosin flow velocities in x and y axis (Vx and Vy) were measured according to the coordinate information. Scale bars are 10 μm. Times are minutes and seconds.

Name of Material/ Equipment

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
Aspirator Tube Assembly
Caenorhabditis elegans strain: N2, wild-type

Caenorhabditis elegans strain: KSG5, genotype: zuIs45; itIs37
Calibrated Mircopipets, 10 µL
Carboxylate-modified polystyrene beads (30 µm diameter)

CD Lipid Concentrate

Clorox

Coverslip holder

Dissecting microscope: Zeiss Stemi 508 with M stand. Source of light is built-in LED. Magnification of eye piece is 10X.

Fetal Bovine Serum, Qualified One Shot, Canada origin

General Use and Precision Glide Hypodermic Needles, 25 gauge
Inulin
MEM Vitamin Solution (100x)
MES (Fine White Crystals)
Multitest Slide 10 Well
PBS, Phosphate Buffered Saline, 10 x Powder
Penicillin-Streptomycin (10,000 U/mL)
Polyvinylpyrrolidone
Potassium Chloride
Rhodamine Red-X, Succinimidyl Ester, 5-isomer

Schneider's Drosophila Sterile Medium

Sodium Chloride
Sodium Hydroxide Solution, 10 N
Spinning disk confocal microscope: Yokogawa CSU-X1, Zeiss Axiovert inverted scope, Quant EM 512 camera, 63X NA 1.4 Plan apochromat objective lens. System was controlled by Slidebook 6.0.
Syringe Filters, PTFE, Non-Sterile
Tygon S3 Laboratory Tubing,, Formulation E-3603, Inner diameter 3.175 mm
Tygon S3 Laboratory Tubing,, Formulation E-3603, Inner diameter 6.35 mm

Company	Catalog Number
Alfa Aesar	AAA1080703
Drummond	21-180-13
Caenorhabditis Genetics Center	N2
in house	KSG5
Drummond	21-180-13
KISKER Biotech	PPS-30.0COOHP
Life Technologies	11905031
Clorox	N. A.
In house	N.A.
Carl Zeiss	Stemi 508
Gibco	A3160701
BD	14-826AA
Alfa Aesar	AAA1842509
Gibco	11120052
Fisher BioReagents	BP300-100
MP Biomedicals	ICN6041805
Fisher BioReagents	BP665-1
Gibco	15140148
Fisher BioReagents	BP431-100
Bioshop	POC888
Molecular Probes	R6160
Gibco	21720024
Bioshop	SOD001
Fisher Chemical	SS255-1
Intelligent Imaging Innovation	N.A.
Basix	13100115
Saint Gobain Performance Plastics	89403-862
Saint Gobain Performance Plastics	89403-854

Comments/Description
For the bead preparation
For the blastomere isolation.
Strain used in this study
Strain used in this study
For the blastomere isolation
For the bead preparation
For the blastomere isolation. Work in the tissue culture hood.
For the blastomere isolation. Open a new bottle when the hypochlorite treatment does not work well.
For the blastomere isolation.
For the blastomere isolation.
For the blastomere isolation. Work in the tissue culture hood.
For the blastomere isolation
For the blastomere isolation
For the blastomere isolation.
For the bead preparation
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For the bead preparation
For the blastomere isolation.
For the blastomere isolation
For the blastomere isolation
For the bead preparation
For the blastomere isolation. Work in the tissue culture hood.
For the blastomere isolation
For the blastomere isolation
For live-imaging
For the blastomere isolation.
For the blastomere isolation.
For the blastomere isolation.

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Author(s):	Christina Rou Hsu, Rain Xiong, Kenji Sugioka

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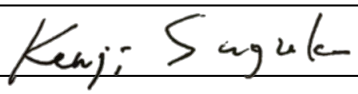
Signature:	
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Firstly, we would like to emphasize that we appreciate the helpful comments provided by the reviewers and editor. We have extensively revised our manuscript to correct the points raised during review process. The followings are point by point responses to reviewers' comments (original comments are colored in blue).

Reviewer 1:

Minor Concerns:

1) Controls are not really mentioned. Perhaps cells without beads or with beads that are not treated could be a control.

We have now included the results using the uncoated beads to show Rhodamine Red-X is working as an adhesive material.

P. 3, Lines 119-122

"Note: Beads without Rhodamine Red-X treatment do not adhere to cell. Rhodamine Red-X serves as fluorescence marker as well as adhesive molecule. The electro static interaction between positively charged Rhodamine Red-X and negatively charged plasma membrane is a putative cause of adhesion."

P. 8, Lines 321-323

"The bead without Rhodamine Red-X treatment did not adhere to the blastomeres, suggesting that the Rhodamine Red-X serves as a both fluorescence marker and adhesive molecule."

2) Some things to consider in the list of reagents:

List all solutions with the final concentrations of each reagent.

The capillaries are not listed (include the size). The worm strain used should be mentioned.

We have added the final concentrations for each reagent (P. 5-7, Lines 218-285). Size of capillary was not specified in the product sheet. Thus, we only provided its capacity in the text (Page 4, line 140) and a material table (excel file). We have added worm strains used in a material table (excel file).

3) The authors could mention that this method could be used to look at differences in cell fate if cells are left to develop longer.

According to the suggestion, we have included the following sentences in the Discussion.

P. 10, Line 409-417

"In addition to cell division orientation, this method has a potential to be a platform to test the relationship between mechanical cues and cell fate. Previous studies reported that mechanical cue controls cell fate specification. Human mesenchymal stem cells differentiate into neuron, adipocyte, skeletal muscle cell, and osteoblast when cultured in substrate with different stiffness[17]. In response to substrate stiffness, the transcriptional regulators Yes-associated protein (YAP) and TAZ (transcriptional co-activator with PDZ-binding motif) will translocate to nucleus, to regulate cell fate specification[18]. The method presented in this paper should also be

useful to test the role of mechanical cues on cell fate specification, by culturing cell long-term in contact with adhesive beads."

Reviewer 2:

p3 lines 61-66. It is better to improve the clarify of the text, especially the identity of adhesive molecules and ligand molecules in this context.

We have edited the text to clarify our description as follows (modified part was underlined).

P. 2, Line 60-67.

"The method presented in this paper uses carboxylate modified polystyrene beads that can covalently bind to <u>any amine-reactive molecules including</u> proteins <u>as ligands</u>. <u>Particularly, we used an amine-reactive form of Rhodamine Red-X as a ligand to make beads both visually trackable and adhesive to the cell.</u> The carboxyl groups of bead surface and primary amine groups of ligand molecule are coupled by water-soluble carbodiimide EDAC [1-ethyl-3-(dimethylaminopropyl) carbodiimide][8,9]. <u>Obtained adhesive beads</u> allow for the <u>effects</u> of the mechanical cue <u>on cellular dynamics</u>[10]."

The authors may want to add more background information of cell-cell communications via mechanical cues (i.e. physical constrain) and chemical cues (i.e. adhesion receptor-mediated intracellular signalling) to highlight potential application of this method.

We have extensively added the discussion to consider different mechanical cues, chemical cues, and highlight the potential application of this method. Please see Discussion section.

p4 line 128- p5 line 171. Readers may want to check specification of a dissecting scope (lens, magnification, source of light, etc) to perform this blastomere isolation experiment.

We have added the technical specification of the dissecting scope in the material list (excel).

Figure 1 needs to distinguish signals of mCherry::Histon and Rhodamine-X-labeled beads. However, mCherry::Histon signal is barely seen in these images. Readers may want to see quantitative comparison of these signals to understand how the optimal amount of Rhodamine-X (0.5 ug/mL) was defined. In addition, I do not understand why addition of beads treated with 5 ug/mL Rhodamine-X (after 3 rounds of wash) caused very high background signal even in the non-beads region.

We have replaced the figure so that the mCherry-histone signal is more visible. We have also added the line plots showing signal intensities of mCherry histone and Rhodamine Red-X coated beads as suggested. In the original figure, we have set the brightness of signal intensity similarly between different concentrations such that signal intensities look overly saturated in the field of view at the 0.5 µg/mL Rhodamine Red-X. Now we have reduced the brightness of the same image to better show the positions of bead and cell.

Figure 3D-I can be reorganized to separate each step. Otherwise Figure 3D explains too much method sections in p5 lines 139-171.

We have separated Figure 3 into two new figures Figure 3 and Figure 4 to better explain blastomere separation in a step-by-step fashion.

White arrows in Figure 4B are confusing to see the division orientation, especially in the left embryo. The authors may want to show three-dimensional axes to highlight the division orientation relative to the contacts with beads.
To avoid the confusion, we have made new figures Figure 6 and 7 to better explain how we measure the division axis and myosin flow.

Figures need to be labeled with scale bars (Figures 1, 3, 4). Figure 4B needs a description of the time-stamp (min:sec).
We have added scale bars and time stamps to all figures.

Reviewer 3:
1. In PROTOCOL, Step 1: It may be better if the authors could suggest how to prepare beads without adhesive function, as a negative control.
We have now included the results using the uncoated beads to show Rhodamine Red-X is indeed working as an adhesive material.

P. 3, Lines 119-122

"Note: Beads without Rhodamine Red-X treatment do not adhere to cell. Rhodamine Red-X serves as fluorescence marker as well as adhesive molecule. The electro static interaction between positively charged Rhodamine Red-X and negatively charged plasma membrane is a putative cause of adhesion."

P. 8, Lines 321-323
"The bead without Rhodamine Red-X treatment did not adhere to the blastomeres, suggesting that the Rhodamine Red-X serves as a both fluorescence marker and adhesive molecule."

2. In PROTOCOL, Step 2: I would like to know why PTFE filter is needed, instead of using a commercially available aspirator tube.
We have added the description to explain why we used the PTFE filter as follows.

P. 4, Lines 135-136
"Note: PTFE filter was used to prevent the inhalation of fumes of hypochlorite solution via mouth pipette."

3. Line 173: This should be Step 4, instead of Step 3. (Similar with Line 195, which should be Step 5).
Thank you for pointing this out. We have made corrections.

4. Line 189: I think attaching the bead to the blastomere at a precise position is a key of this entire protocol. However, the description seems too simple ("Control the position … by blowing …"). Can the authors provide more detail on this process (any tips)? Or is this process so easy?

This is an easy procedure among the protocols. We have added additional tips as follows.

P. 7-8, Line 308-311
“4) Place the mouth pipette as close to the embryo as possible to minimize the strength used to blow into the pipette, but also not too close so that the embryo is sucked up as well (this is also true when attaching beads).”

5. Figure 3 B&C: I recommend the authors to add arrows or circles to clarify which is the embryos.
We have added the arrows to the Figure 3B and 3C.

6. Figure 3 E-I: The quality of the images is poor. Can the authors show more in-focus images?
Unfortunately, this is the limitation of our dissecting microscope. C. elegans embryos are 50 µm and 30 µm in long and short axes, respectively, such that the visualization with a lower magnification optical system is technically difficult. We believe that showing actual images obtained with the dissecting scope are useful, albeit low resolution, for the readers to identify the condition of manipulated embryos during their experiments.

7. Figure 4 A: It was hard to understand the configuration of the sample. How about providing a cross-section schematic?
We have replaced the old Figure 4A with new Figure 5 to explain the assembly of imaging chamber.

Reviewer #4:
Manuscript Summary:
This article describes the methods for cell isolation and reconstitution of physical contacts using beads. While the Method builds on prior work in C. elegans for blastomere isolation, the use of small beads to mimic cell-cell contacts, and to thus distinguish physical from chemical cues, is novel. The detailed account of this protocol will be very useful to researchers studying a number of developmental processes. Overall, I think the protocol is clear and easy to follow and has enough detail, except for a few key areas outlined below under "major concerns". These parts of the manuscript should be clarified before publication. I also list some other typos/issues for clarity in the "minor" section.

Major Concerns:
1) Line 61-62, and corresponding parts of the protocol: It is not clear as written whether the beads are activated in a way that allows them to bind to "adhesive molecules and other proteins" on the cell vs one would (or could add) a specific adhesive molecule. Indeed, the end of the paragraph sounds like you are coating the bead with an adhesive molecule, but I do not see that in the protocol. In the original research article by the senior author, it is stated the beads are binding directly to the cells, so I assume the same is true here. In that case:
The wording needs to be clarified to be clear this treatment makes the beads able to attach to cells with no further treatment.

At the same time, it would also be of interest to know if one could also then incubate the beads with a recombinant protein to deliver additional cues. I know that Goldstein has used Wnt coated glass beads for examples, but very large beads were used in contrast to the current method that utilizes more relevant "cell size" bead. This is later mentioned in the Discussion section- but if the authors think/know their technique could be adapted in such way…it would be great if something could be added to the protocol. For ex is this particular treatment of beads compatible with incubating with recombinant protein? And regardless in the Discussion it would help to include a reference to this idea, rather than just stating the potential (e.g. referencing Goldstein's Wnt bead paper).

We have edited the text to clarify our description as follows (modified part was underlined).

P. 2, Line 60-67.
"The method presented in this paper uses carboxylate modified polystyrene beads that can covalently bind to <u>any amine-reactive molecules including</u> proteins <u>as ligands</u>. <u>Particularly, we used an amine-reactive form of Rhodamine Red-X as a ligand to make beads both visually trackable and adhesive to the cell.</u> The carboxyl groups of bead surface and primary amine groups of ligand molecule are coupled by water-soluble carbodiimide EDAC [1-ethyl-3-(dimethylaminopropyl) carbodiimide][8,9]. <u>Obtained adhesive beads</u> allow for the <u>effects</u> of the mechanical cue <u>on cellular dynamics</u>[10]."

In the Developmental Cell paper, we did not determine the mechanism by which the beads acquired the adhesive function. Here, we have used the chemically activated beads without Rhodamine Red-X and found that Rhodamine Red-X served as an adhesive molecule. This is probably due to the electro static interaction between positively charged Rhodamine and negatively charged plasma membrane. Thus, we have added following description in the result section.

P. 3, Lines 119-122

"Note: Beads without Rhodamine Red-X treatment do not adhere to cell. Rhodamine Red-X serves as fluorescence marker as well as adhesive molecule. The electro static interaction between positively charged Rhodamine Red-X and negatively charged plasma membrane is a putative cause of adhesion."

P. 8, Lines 321-323
"The bead without Rhodamine Red-X treatment did not adhere to the blastomeres, suggesting that the Rhodamine Red-X serves as a both fluorescence marker and adhesive molecule."

Lastly, we have added the discussion of potential chemical coupling of beads with protein with citing Bob Goldstein's Wnt bead paper as described below. Indeed, the beads can be coupled with any proteins or amine-reactive molecules.

P.10, Line 437-445.

“In this study, we showed the example of interaction between mechanical cue and cell division orientation, but cell-cell communication is also mediated by chemical cues such as Wnt[22], Notch[23], adhesion coupled receptors[24] and so on. Importantly, the beads preparation method presented here can couple the beads with any proteins, thereby allowing the reconstitution of chemical cues. Previous studies have also used Sepharose bead[25] and protein-A magnetic bead[26] coated with Wnt protein to demonstrate Wnt-dependent developmental processes. However, these beads are not commercially available in various sizes compared to carboxylate modified polystyrene beads. Hence, future research can use the presented method as a platform to test the role of both chemical and physical cues in more tunable manners.”

2) Line 102 - the dilution for the Rhodamine is confusing as written- e.g by " x 1000 dilutions" do you mean a 1000-fold dilution of the stock?
We have collected x 1000 dilution to 1000-fold dilution.

Also, in the Reagents section the stock concentration was not specified - that should be added for clarity there instead of here.
As suggested, we have added the concentration of stock solution and final concentration.

Then in the protocol under #14, based on the Results…it would be clearer I believe to state here something like "The final concentration of Rhodamine used will depend on the strain being imaged. Start with a 1/1000 dilution in PBS, but adjust if needed (see Results).
Thanks for pointing this out. We have modified our text as follows.

Page 3, Lines 104-117.
“1.14 The final concentration of Rhodamine used will depend on the strain being imaged. Prepare 1 ml of 1-, 10-, 100-, and 1000-fold dilution series of Rhodamine Red-X from the 0.65 mM Rhodamine Red-X stock solution.

1.15 Pipette 20 µL of beads into each serial dilution tube.

1.16 Rotate and incubate the tube for 5 min.

1.17 Wash the beads twice with 1 ml of PBS by repeating steps 1.10, 1.11, and 1.12.

1.18 Add 1 ml of PBS into the tube and store it at 4°C for up to 6 weeks. Check the fluorescence intensity of the beads under a microscope used for live-imaging. The appropriate concentration of the Rhodamine Red-X succinimidyl ester is dependent on the imaging conditions (Figure 1).”

3) Protocol Section 2-
In comparison to the earlier Method cited (ref 6), there is no use of chitinase to remove the eggshell. So first, I wanted to double check that this is really the case. If so, I actually believe it is worth noting this somewhere. Perhaps even in the paragraph of lines 61-66 where the authors are stating what they are presenting with this new protocol. Those of us who have tried to

remove the eggshell know how problematic chitinase can be, so I believe it is worth stressing if it is not really required for successful cell isolation!

Our protocol does not use chitinase at all. We have adapted this protocol from James Priess's lab. We agree that this protocol is cost effective and we can get more consistent results. Thus, we have modified our text as follows to emphasize this point.

P. 6, Lines 229-232.

"Note: Although many published methods have used chitinase to digest chitinous eggshells, we have adopted a method using hypochlorite solution[11]. By avoiding the batch-to-batch variations of chitinase activities, we believe this method is more reproducible and cost-effective approach."

4) Figure. 4: I did not understand the slide/chamber set up at all - this needs much more explanation. Since this is a key part of the protocol, should the details be added to section 3 of the Protocol?

We have replaced the old Figure 4A with a new Figure 5 to better explain the assembly of imaging chamber and we have added the suggested note as follows.

P. 5, Lines 192-200.

"4.1 To observe using an inverted microscope, prepare an imaging chamber as in Figure 5.

4.1.1 Place a coverslip onto a coverslip holder (Fig. 5A).

4.1.2 Tape the edges of the coverslip to stabilize it. The side with tape is the 'back' side (Fig. 5A, B).

4.1.3 Flip the coverslip holder over to the 'front' side and draw a circle on the coverslip with a hydrophobic pen (Fig. 5C). "

5) The notes on how long solutions keep are very helpful. I would like to see similar notes added for the activated beads part of the protocol (where it states to store them at 4°).

We have added the suggested note in the section 5 (P. 5, Line 220-287.
) and as follows.

P. 3, Line 114-117.

"1.18 Add 1 ml of PBS into the tube and store it at 4°C for up to 6 weeks. Check the fluorescence intensity of the beads under a microscope used for live-imaging. The appropriate concentration of the Rhodamine Red-X succinimidyl ester is dependent on the imaging conditions (Figure 1)."

Minor Concerns:

Line 74- Should MES be defined here? (I think first use of abbreviation)
We have added the definition.

Line 243 - Should BME be defined?
We have added the definition.

Line 251 - Does the FBS need to be heat killed?
This protocol does not require the heating of FBS. We have added the following description in the text.

P. 7, Line 286.
"FBS does not need to be heat killed."

Figure 3: Add a scale bar for reference please.
We have added scale bars to all figures.

Discussion:
First sentence should be geared towards potential use, not "us", so for example reword as below (also suggestions for second sentence).
Reconstitution of spatial cell contact patterns will let researchers test the roles of specific cell contact patterns in morphogenesis. We have used this technique of isolated bead-blastomere association to show that cell division axis is controlled by physical contact (ref).
We have modified our text as follows.

Page 10, Lines 402-407.
"Reconstitution of simplified cell contact patterns will let researchers to test the roles of specific cell contact patterns in different aspects of morphogenesis. We have used this technique to show that cell division axis is controlled by the physical contact with adhesive beads[10]. As division axis specification is crucial for multicellular development by contributing to morphogenesis[14], stem cell division[15,16], and tissue homeostasis[15,16], this method should be able to illuminate a new mechanism of animal tissue formation."

There are several instances of misplaced plurals, or "the" that the copy editors should double check.
Thank you for pointing this out. We have corrected our revised manuscript.

Reviewer #5:
Manuscript Summary:
Physical and chemical cues play essential functions for various biological processes such as polarity establishment and maintenance, spindle orientation, cell fate decision, and morphogenesis. However, how these intricate processes are spatiotemporally regulated by the physical influence of cell to cell interaction or by physicochemical modulation is still poorly understood. Recently, Sugioka and Bowerman developed an excellent system to study mitotic spindle orientation using carboxylate modified polystyrene bead. In this method paper entitled

'in vitro reconstitution of spatial cell contact patterns with isolated C. elegans embryo blastomeres and adhesive polystyrene beads' Sugioka and colleagues are neatly explaining the method to study the link between contact-dependent physical cues and division axes using early C. elegans blastomeres.

The manuscript by Hsu et al., is interesting, and the authors have well explained the method starting from the preparation of adhesive polystyrene bead to reconstituting the physical contact patterns between embryos and the bead. I believe this method will be beneficial for the investigators working in several different areas and utilising worm embryos as a model system.

Major Concerns:
No major concerns

Minor Concerns:
I have no major concerns. However, I have only one minor suggestion to the reviewers.
In line 44-45, I would like the reviewers to elaborate the part where they discuss various physical and chemical cues specify 'cellular behaviours of individual cells'. I believe expending this section by giving examples, including references, will help the readers to understand the context better. Also, in the discussion section, the authors can further mention the application of their methods in embryogenesis beyond division axis specification.

Thank you for the suggestions. We have added more descriptions in discussion to elaborate the multicellular cues and future potential of this protocol.

Reviewer #6:
Hsu et al., described a method for in vitro reconstitution of spatial cell contact patterns with isolated Caenorhabditis elegans embryo blastomeres and adhesive polystyrene beads. Successful implementation of the method is very important for definitive investigation of how extracellular clues affect cellular functions, including cell division axis, asynchrony in division and cell fate specification in vitro, which is difficult to measure in vivo.

My major comments are listed below.
1. Biologically relevant cell contact may function transiently and reversibly, i.e., the contact duration and contact area may have specific meanings in vivo. Given that the polystyrene beads can covalently bind to adhesive molecules and other proteins, this will limit the capability of controlling the contact duration and area. The author should elaborate the limitation with their system.
2. A relevant question is that the isolated blastomeres lose their original shape imposed by egg shell and neighboring cells. A deformed cell shape may also affect contact duration and area.
3. This method can only be applicable to early embryonic blastomeres for which one can recognize the cell identity. After a few round of cell divisions, loss of track on the cell identity will significantly compromise the value of the system.

We agree that these are all very important points that we have overlooked during the preparation of this manuscript. We have elaborated the limitation of our protocol regarding each point as described below.

My specific comments are listed below.
1. PRESSURE. In vivo, cells are under great pressure from their neighbors as well as from the eggshell. Removal of eggshell will change the shape of blastomere and probably result in cellular dysfunction or even death.

2. DEFORMATION. The cells are polyhedral in real embryo due to external forces, and have reproducibly specific shape among individuals (at least at the 4-cell stage). However, the isolated ones are round because of elimination of mechanical cues (cell-cell and cell-eggshell force) (Figure 3/4). Whether and to what extent this will affect cellular function needs to be discussed.

We have added the following discussion for the points 1 and 2.

P. 10, Line 419-425.
"This method has limitations in recapitulating certain mechanical cues observed *in vivo*. In *C. elegans*, chitinous eggshell and permeability barrier serve as a physical constrain. Although the removal of eggshell does not affect normal development, removal of both eggshell and permeability barrier results in abnormal pattern formation[19]. In the absence of eggshell and permeability barrier, cell shape becomes more spherical, suggesting that the pressure between cells and cell-eggshell plays a critical role in cell deformation and patterning. Indeed, cell-cell squeezing forces has been proposed to regulate cell division orientation[20]."

3. AREA. Contact area may be an important regulatory factor for physical and chemical cues. However, it seem it could not be controlled in this method. As shown in Figure 4B, the cell-bead contact area is pretty small.
We have added the following discussion of limitation of our protocol.

P. 10, Line 425-429.
"Without having physical constrain and putative pressure among tissues, we expect that this method also cannot recapitulate *in vivo* cell contact area. As cell contact area is known to affect Notch signaling[20], this limitation needs to be further considered when certain mechanical or chemical cue does not work using this *in vitro* method."

4. DURATION. How far can this method be applied? This paper mentions ABa and ABp cells (4-Cell Stage). As cells in other lineages (e.g. E,MS,C,P3,D,P4) may have different responsive or regulatory behaviors, can they also be isolated and identified?
We have added the following discussion of limitation of our protocol.

P. 10, Line 431-435.

“We have so far tested the cell division orientation of isolated AB, P1, EMS, P2, ABa, ABp cells (cells up to 6 cell stages) in our hands[10], but the method can be applied to later development as far as individual cell can be isolated. Recent single cell sequencing study has used pronase digestion of worm body to isolate larval cell[21]. Therefore, potentially one can use pronase treatment to isolate later stage embryonic cells and larval cells. ”

5. VISUALIZATION. The velocity vector field of Myosin Flow (Figure 4B), which was well quantified and visualized in Kenji's previous paper, which should be discussed here as well, in particular the algorithm and program for visualization. (Journal of Visualized Experiments)
We have added the method to quantify the myosin flow velocities from time lapse movies in Figure 7 and in the text as follows.

P. 8, Line 332-341.
“In our previous study, we have identified an anisotropic cell surface myosin flow during the bead-induced AB cell division orientation[10]. However, it is difficult to measure the intracellular myosin flow as cell moves during oriented division. To perform this, first we have selected the samples with spindle aligned to the imaging plane (xy plane) (Fig. 7). Second, Z-stacks were projected using maximum projection method (Fig. 7). Third, cell movements were corrected using the subpixel registration alogrithm “Stack reg” plug-in[13] of Image J with “rigid body” option (Fig. 7B). By the registration of images, the position of cell was stabled (Fig. 7B). Finally, by using “Manual tracking” plug-in of Image J, myosin foci were tracked (Fig. 7C). According to the coordinate information, velocities of myosin along division axis, and axis perpendicular to it were calculated within 50 s after the cytokinesis onset.”

6. ORIENTATION. "As reported before, the contact with the adhesive beads orient cell division in parallel to the plane of the contact". In Figure 4B, for the cell on the right, its division axis is not parallel to the contact plane; the intersection angle is about 30°.
We now replaced the figure to the 3D reconstructed images so that the relationship between the cell division angle and cell-cell contact plane are more accurately visualized (Figure 6).