

Journal of Visualized Experiments

Surgical Size Reduction of Zebrafish for the Study of Embryonic Pattern Scaling --Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59434R1
Full Title:	Surgical Size Reduction of Zebrafish for the Study of Embryonic Pattern Scaling
Keywords:	Scaling, Size Reduction, Zebrafish, Embryology, Patterning, Robustness, Development, Organogenesis
Corresponding Author:	Sean Megason Harvard University Boston, MA UNITED STATES
Corresponding Author's Institution:	Harvard University
Corresponding Author E-Mail:	megason@hms.harvard.edu
Order of Authors:	Kana Ishimatsu, Ph.D. Anna Cha Zach M Collins Sean G Megason
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Boston, MA, USA

TITLE:

Surgical Size Reduction of Zebrafish for the Study of Embryonic Pattern Scaling

AUTHORS AND AFFILIATIONS:

Kana Ishimatsu¹, Anna Cha¹, Zach M Collins¹, Sean G Megason¹

¹Department of Systems Biology, Harvard Medical School, Boston, MA, USA

Corresponding Author:

Sean G Megason (megason@hms.harvard.edu)

Tel: (617)-432-7441

Email Addresses of Co-Authors:

Kana Ishimatsu (kana_ishimatsu@hms.harvard.edu)

Anna Cha (anna_cha@fas.harvard.edu)

Zach M Collins (zuch50@gmail.com)

KEYWORDS:

scaling, size reduction, zebrafish, embryology, patterning, robustness, development, organogenesis

SUMMARY:

Here, we describe a method for reducing the size of zebrafish embryos without disrupting normal developmental processes. This technique enables the study of pattern scaling and developmental robustness against size change.

ABSTRACT:

In the developmental process, embryos exhibit a remarkable ability to match their body pattern to their body size; their body proportion is maintained even in embryos that are larger or smaller, within certain limits. Although this phenomenon of scaling has attracted attention for over a century, understanding the underlying mechanisms has been limited, owing in part to a lack of quantitative description of developmental dynamics in embryos of varied sizes. To overcome this limitation, we developed a new technique to surgically reduce the size of zebrafish embryos, which have great advantages for in vivo live imaging. We demonstrate that after balanced removal of cells and yolk at the blastula stage in separate steps, embryos can quickly recover under the right conditions and develop into smaller but otherwise normal embryos. Since this technique does not require special equipment, it is easily adaptable, and can be used to study a wide range of scaling problems, including robustness of morphogen mediated patterning.

INTRODUCTION:

Scientists have long known that embryos have a remarkable ability to form constant body proportions although embryo size can vary greatly both under natural and experimental conditions¹⁻³. Despite decades of theoretical and experimental studies, this robustness to size variation, termed scaling, and its underlying mechanisms remain unknown in many tissues and

organs. In order to directly capture the dynamics of the developing system, we established a reproducible and simple size reduction technique in zebrafish⁴, which has the great advantage in *in vivo* live imaging⁵.

Zebrafish has served as a model vertebrate animal to study multiple disciplines of biology, including developmental biology. In particular, zebrafish is ideal for *in vivo* live imaging⁶ because 1) development can proceed normally outside the mother and the egg shell, and 2) the embryos are transparent. In addition, the embryos can withstand some temperature and environmental fluctuations, which allows them to be studied in laboratory conditions. Also, in addition to conventional gene expression perturbation by morpholino and mRNA injection^{7,8}, recent advances in CRISPR/Cas9 technology has made reverse genetics in zebrafish highly efficient⁹. Furthermore, many classical techniques in embryology, such as cell transplantation or tissue surgery can be applied^{4,10,11}.

Size reduction techniques were originally developed in amphibian and other non-vertebrate animals¹². For example, in *Xenopus laevis*, another popular vertebrate animal model, bisection along the animal-vegetal axis at blastula stage can produce size-reduced embryos^{12,13}. However, in our hands this one-step approach results in dorsalized or ventralized embryos in zebrafish, presumably because dorsal determinants are distributed unevenly and one cannot know their localization from the morphology of embryos. Here we demonstrate an alternative two-step chopping technique for zebrafish that produces normally developing but smaller embryos. With this technique, cells are first removed from the animal pole, a region of naïve cells lacking in organizer activity. To balance the amount of yolk and cells, which is important for epiboly and subsequent morphogenesis, yolk is then removed. Here, we detail this protocol and provide two examples of size invariance in pattern formation; somite formation and ventral neural tube patterning. Combined with quantitative imaging, we utilized the size reduction technique to examine the how the sizes of somites and neural tube are affected in size reduced embryos.

PROTOCOL:

All fish-related procedures were carried out with the approval of the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

1. Tool and reagent preparation

1.1. Make a wire loop to chop embryos

1.1.1. Take 20 cm of stainless steel wire that is stiff and non-corrosive with a diameter of 40 μm . Loop the wire through into glass capillary (1.0 mm outer diameter, 0.5 mm inner diameter, no filament), making a small loop at the top (loop length is 1.0 mm)

1.1.2. Put a little droplet of clear nail polish onto the tip of the glass capillary between the wire loop to hold it in place. Let dry. Make sure not to get any nail polish onto the loop portion, as it may damage the embryos.

1.1.3. Attach the glass capillary with the loop onto a wooden chopstick (9" bamboo disposable chopsticks broken in half) using lab tape. Leave about 2.5 cm of the glass capillary extending beyond the chopstick, so that the chopstick part of the tool does not dip into the water. Adjust this length to preference.

1.2. Alternatively, make a glass needle to chop embryos

1.2.1. Pinch one end of glass Pasteur pipette with forceps, while holding the other side with a hand. Heat the thin part of the pipette over a spirit lamp or Bunsen burner.

1.2.2. Hand-pull the glass pipette. The ideal pipette as a diameter of approximately 30 μ m and a gentle curve (radius of curvature = approximately 5 mm). Proper diameter and curvature is obtained with practice and some chance.

1.3. Prepare methyl cellulose

1.3.1. Methyl cellulose is used to hold the embryos while chopping. Make 2% methyl cellulose solution in $\frac{1}{3}$ x Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl_2 , and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.2)). Shake methyl cellulose powder in $\frac{1}{3}$ x Ringer's solution at 4 °C overnight.

1.3.2. Optionally, add ~1.5 mL phenol red (0.5% in DPBS stock solution) to 10 mL 2% methyl cellulose solution, until red, to make the solution visible. Shake it until the color becomes uniform.

2. Preparation of Zebrafish Embryos for Surgical Size Reduction

2.1. Collect embryos

2.1.1. Place one or two male and one or two female zebrafish in a mating chamber filled with a lot of water. Separate males and females using a plastic divider. Use the AB line for somite imaging (section 4) and a transgenic reporter line of neural tube patterning (*nkx2.2:mem-gfp*, *dbx1b:gfp*, or *Olig2:dsred*) for neural tube imaging. Leave them in the chamber overnight.

NOTE: The choice of adults is important for obtaining eggs that survive surgery well. Typically, young healthy females produce healthy eggs.

2.1.2. On the next morning, transfer the chamber into shallow water and place the chamber with slight tilt. Remove the divider so the fish can mate.

2.1.3. Collect the eggs by pouring into a tea strainer. Transfer the eggs into a Petri dish with egg water (for 20x egg water, 6 g instant sea salt, 1.5 g CaSO_4 and 1 L H_2O ; use at 1x). For better staging, collect the eggs right after spawning. Place the embryos in a 28.5 °C incubator.

2.1.4. If necessary, inject morpholino, mRNA, etc., at 1–4 cell stages following a common protocol^{7,8}. Inject mRNA for fluorescent membrane label (*mem-mCherry*, *mem-mBFP1*, or *mem-mCitrine*) for neural tube imaging (section 5).

2.2. Dechorionate using pronase

2.2.1. Around the 128-cell to 256-cell stage, transfer healthy embryos to a 35 mm glass dish filled with egg water. Remove as much egg water as possible from the dish.

2.2.2. Add 1 mL of 20 mg/mL pronase. Gently swirl embryos around by moving the plate and gently pipette the solution up and down to help dechoriation using a glass pipette.

2.2.3. When the chorions start to lose elasticity (usually 1–4 min after addition of pronase, depending on the amount of eggs and water), add as much egg water as possible to dilute pronase. Assess elasticity loss by gently touching the chorion with forceps; the chorion should hold the dent without immediately bouncing back. Transfer the eggs to another dish with egg water using a glass pipette (at this point, most of the chorions are not broken).

2.2.3.1. Alternatively, gently pour the embryos from the dish into a large 400 mL glass beaker filled with egg water without exposing embryos to air. Then, let the embryos completely settle. Tilt the beaker to let embryos fall to one side. Collect these embryos using a glass pipette and transfer to a new 35 mm glass dish filled with $\frac{1}{3}\times$ Ringer's solution.

2.2.4. Wait for several minutes until most of the chorions lose elasticity completely. Remove the remaining chorions from embryos by gently pipetting the eggs. Remove damaged embryos and incubate the embryos in a 28.5 °C incubator.

3. Surgical Size Reduction and Recovery

3.1. Prepare one clean 35 mm glass dish. Spread approximately 0.5 mL of 2% methyl cellulose (in $\frac{1}{3}\times$ Ringer's solution, with phenol red to help visualize) near the center of the bottom of the larger dish using a plastic spatula. Thinly and evenly spread the methyl cellulose to a thickness of approximately 0.5 mm.

3.2. Pour approximately 10 mL of $\frac{1}{3}\times$ Ringer's solution to the side of the dish and allow to spread onto the rest of the dish, covering the methyl cellulose.

3.3. Place dechorionated embryos at the 256-cell–1k-cell stage on 2% methyl cellulose. Adjust the orientation of the embryos onto the side to visualize both the cells and the yolk. (**Figure 1**)

3.4. Chop approximately 30%–40% of cells from the blastoderm near the animal pole by cutting perpendicular to the animal-vegetal axis using the wire loop (or the glass needle) (**Figure 1**, top panels). After removing the cells, gently tap the ends together to help the remaining cells stick back. Within minutes, the dead cells should slough off when the embryo starts to heal.

3.5. Make a small wound to the yolk near the vegetal pole instead of “chopping” the yolk (**Figure 1** middle panels). Wound the yolk by nicking the egg membrane with the mounted wire. Yolk will ooze out for few minutes after wounding, and then the wound will heal (**Figure 1** bottom panels).

3.6. When the yolk stops oozing out, move the embryo using a pipet outside of the methylcellulose in the same dish to allow them to better recover.

3.7. Repeat steps 3.4–3.6 for all embryos. Leave the dish stable for 30 min while the embryos recover.

3.8. Transfer the embryos to a new dish with fresh $\frac{1}{3}$ x Ringer’s solution and put them in 28.5 °C incubator to allow them to completely recover.

3.8.1. (Optional) If the stage of interest is the early somite stage, embryos can be incubated at 20 °C after being incubated at 28.5 °C until the shield stage, to adjust timing for experimentation and imaging.

4. Live imaging of zebrafish somitogenesis

4.1. Prepare 100 mL of 1% agarose solution by adding 1 g of agarose to 100 mL of egg water, heating until agarose is fully dissolved. Let cool for 5–10 minutes to a temperature of 62–72 °C.

4.2. Prepare a mount for imaging.

NOTE: This guide was developed for use with an inverted wide-field microscope.

4.2.1. Pour ~15 mL of 1% agarose in to a 100 mm x 15 mm plastic Petri dish.

4.2.2. Gently place a Dorsal Mount V1 mold template⁵ onto the bottom of the Petri dish. Keep the mold on the bottom by using a weight, such as 15 mL tube with water.

NOTE: This is so the embryos are as close to the bottom of the Petri dish as possible, when using an inverted microscope. Although the embryos are mounted laterally for somite imaging, here Dorsal Mount V1⁵ is used since it holds the embryos better at early somite stages.

4.2.3. Let cool until agarose is fully solidified. Pour in ~30 mL of egg water with 0.01% tricaine, and carefully remove mold by gently prying off with forceps.

4.3. Mount embryos for somite imaging

4.3.1. Prepare 1% Low Melting Agarose in $\frac{1}{3}$ x Ringer’s solution (or egg water) and keep it at 42 °C. Wait until the temperature comes down to 42 °C.

4.3.2. Under a dissection microscope, place one embryo per well. Pipette approximately 1 μ L of Low Melting Agarose in the well to finely adjust the well size to the size of individual embryos that vary in size, especially between those with and without size reduction.

4.3.3. Quickly orient the embryos before the Low Melting Agarose is solidified so the embryos face completely laterally to the dish. After mounting embryos, gently place the cover slip in the agarose mount to hold the embryos in place. The orientation is particularly important for the long-term somite imaging because the embryos have to be exactly laterally mounted for all the somite boundaries to be clearly imaged at later stages.

4.3.4. Submerge away from the mounting area and manipulate a 25 mm x 25 mm cover glass such that it is 45 degrees offset from the orientation of the square indent made by the mold. Slide the coverslip over the mold in this orientation until each corner is resting on a different side of the mold.

NOTE: Although this protocol is for an inverted microscope, placing a cover glass on top of the mold is still important so that the embryos do not move while transferring to the microscope.

4.4. Imaging somite formation process

4.4.1. Prewarm the microscope to 28 °C in an incubator built with foamcore boards and a cabinet heater.

4.4.2. Place the Petri dish with the mounted embryos onto the microscope stage. Find the embryo mounted in the top-left well of the agarose mount with lower magnification lens, then switch the objective to 10x.

4.4.3. Set up image acquisition.

NOTE: This instruction is for bright field.

4.4.4. Find conditions for the light power, exposure time, and condenser so that the somite boundary can be clearly seen. Using z-stack settings set the lowest and highest desired imaging plane. Set the total time and time interval. Find and register the xy positions of all the embryos mounted so multiple embryos can be timelapse imaged at once.

4.5. Measure the lengths of PSM and somites using Fiji¹⁶.

5. Imaging of neural tube patterning

5.1. Prepare 100 mL of 1% agarose solution by adding 1 g of agarose to 100 mL of egg water, heating until agarose is fully dissolved. Let cool for 5–10 minutes to 62–72 °C. Adjust tricaine to 0.01%.

5.2. Prepare a dorsal mount for imaging.

NOTE: This guide is developed for use with an upright fluorescence microscope. For inverted microscopes coverslip bottom dishes are necessary and mounting orientation in step 3 is flipped.

5.2.1. Pour in ~15 mL of 1% agarose in to a 100 mm x 15 mm plastic Petri dish. Gently float a Dorsal Mount V1 mold on the surface of the agarose to avoid introducing air bubbles. Let cool until agarose is fully solidified.

5.2.2. Carefully remove the mold with forceps or a razor blade. Fill the dish with egg water with 0.01% tricaine and cover until use.

5.3. Mount embryos for neural tube imaging.

5.3.1. Allow transgenic or injected fluorescent size-reduced and control embryos to develop until the 20–25 somite stage (roughly 18–22 h after fertilization). Embryos expressing a fluorescent membrane label (*mem-mCherry*, *mem-mBFP1*, or *mem-mCitrine*) and a transgenic reporter of neural tube patterning (*nkx2.2:mem-gfp*, *dbx1b:gfp*, or *Olig2:dsred*) are used for imaging.

5.3.2. Replace egg water medium in prepared dorsal mount with 0.01% tricaine working solution. Gently pipette in embryos to be imaged in to wells of the dorsal mount.

5.3.3. Manipulate embryos into the correct orientation such that the head is facing forward in the mount and the tail is pointing towards the rear with the dorsal portion facing towards the water surface.

5.3.4. Orient the embryo such that the tail is lying flat and not pointed downward towards the bottom of the dish. It is sometimes helpful to rest the posterior portion of the tail on the side ledge of the mounting well to prevent the tail from sinking and inadvertently imaging the hindbrain. Repeat for each embryo. Make certain with size reduced embryos that they do not fall to deeply into the wells to be imaged.

5.3.5. Submerge away from the mounting area and manipulate a 25 mm x 25 mm cover glass such that it is 45 degrees offset from the orientation of the square indent made by the mold. Slide the coverslip over the mold in this orientation until each corner is resting of a different side of the mold.

5.3.6. Slowly rotate the coverglass until it gently falls on to the mounting area. Check to be sure embryos are still mounted in the correct positions and orientations. If too much movement is caused by the falling of the coverglass, remove gently and repeat mounting from step C.

5.4. Image in 3-D the developing spinal cord.

5.4.1. Gently transport the dish containing mounted embryos to the confocal microscope. For long-term live imaging, be sure to use an incubated microscope. Otherwise, low temperatures are tolerable.

5.4.2. Under brightfield illumination, navigate to the embryo to image and center the field of view on the preferred anterior-posterior position. To enable a detailed comparison between embryos, this position on each embryo must be consistent.

5.4.3. Set the imaging parameters to excite and capture signal from the fluorescent proteins being imaged. Parameters can be tuned by eye to create a desired image on the initial sample. To enable measurement consistency, apply these settings to all embryos in the dataset. If many z-stacks are required optimize settings for speed.

5.4.4. Using z-stack settings, set the lowest and highest desired imaging plane. For optimal image quality in the z-stack, set the z-resolution to the highest that is optimal for the aperture setting. Good images can be generated with 1 μm z-spacing.

5.5. Analyze imaging data by any preferred method.

NOTE: In this case, analysis was performed with custom MATLAB scripts that enable simple segmentation of the neural portions of an image and quantification of imaging signal.

REPRESENTATIVE RESULTS:

Yolk volume reduction is important for normal morphology

As recently described in Almuedo-Castillo et al.¹⁷, size reduction of embryos can be achieved without reducing yolk volume. To compare with and without yolk volume reduction, we performed both two-step chopping (both blastula and yolk) and blastula-only chopping (**Figure 2** and **Supplemental Movie 1**). Two-step chopped embryos showed seemingly normal overall morphology compared to the control (dechoriation only) embryos, other than size difference, throughout the developmental stages (see top and middle panels in **Figure 2**). On the other hand, blastula-only chopped embryos showed a peculiar morphology, especially at earlier stages. During epiboly, the embryos had a constricted and indented appearance (See bottom panel for 70% epiboly in **Figure 2**). At the following somite stage, midline structures were found to be flattened (i.e. DV length is relatively shorter than ML length) at many axial levels (See bottom panels for 8 and 20 somite in **Figure 2**). At later stages, the body structures adjacent to the yolk, such as the mid- and hindbrain, and the first ~10 somites, still showed a relatively flattened shape, possibly due to increased tension from the relatively larger yolk.

Somite size reduction in size reduced embryos

Somites are segmental structures that appear transiently during embryogenesis and give rise to vertebrae and skeletal muscle. From presomitic mesoderm (PSM), somites are formed one by one from the anterior to posterior direction in a periodic manner (e.g. 25 min for zebrafish, 2 h for mice) (**Figure 3A**). We performed time lapse imaging of somite formation both for control and chopped embryos and measured the size of most newly formed somites (**Figure 3B**). In both

control and chopped embryos, the sizes of somites that were formed at later stages were found to be smaller compared to the ones from earlier stages. Also, throughout the somite formation stages, chopped embryos had smaller somites than the ones in control embryos (**Figure 3C**).

Neural tube heights are reduced following size reduction

To see the effect of embryo size reduction on neural tube size, we performed our two-step chopping technique on *mem-mCherry* injected embryos and imaged their spinal cords at 20hpf using our confocal imaging system (**Figure 4A,B**). In this dataset, neural tube heights were reduced following size reduction by $12.4\% \pm 3.2\%$, as measured manually using custom image analysis code (**Figure 4C**). Taken together, these data show that size reduction reduces neural tube height. This technique can be used to measure the effects of size reduction on neural patterning.

FIGURE AND TABLE LEGENDS

Figure 1: Size reduction technique. Approximately 30%–40% of the cells were cut from the animal pole (top panels). The membrane surrounding the yolk was carefully wounded so that the yolk oozed out (middle panels). For the following few minutes, yolk oozed out and then the wounds on both blastoderm and yolk healed up (bottom panels). Scale bar, 200 μm .

Figure 2: Comparison between two methods of size reduction. Control embryos (top panels, top embryos for 24 hpf and 30 hpf), size reduced embryos with two-step chopping (blastula and yolk, middle panels, middle embryos for 24 hpf and 30 hpf) and size reduced embryos with blastula-only chopping (bottom panels, bottom embryos for 24 hpf and 30 hpf) are compared along developmental stages. Note that in blastula-only chopped embryos, the blastoderm volume is much smaller compared to the yolk (at 70% epiboly). As a result, the embryo has a disproportionately flattened shape at somite stages (i.e., DV axis is relatively shorter compared to AP axis in blastula-only chopped embryos, compared to the control or a two-step chopped one). Scale bar, 200 μm .

Figure 3: Size reduction reduces the length of somites. (A) Schematic illustration of somite formation. (B) Bright field images of control and chopped embryos over time. Yellow arrowheads indicate the most newly formed somite at each somite stage. (C) Somite length (in anterior-posterior axis) measurements over time for both control and chopped embryos. Error bars represent standard deviation.

Figure 4: Size reduction reduces the height of the neural tube. (A-B) Example images of normal sized (A) and size reduced (B) *tg(ptch2:kaede)* embryos which were injected at the single cell stage with *mem-mCherry* mRNA. Scale bar, 20 μm . (C) Neural tube heights extracted from manual segmentation of the neural tube in each z-stack. Statistically significant differences are observed in average neural height when values are compared using an unpaired t-test ($p = 0.0397$).

Supplemental Movie 1: Comparison between two-step chopping versus blastula-only chopping. Top row: control embryos, middle row: size reduced embryos with two step chopping,

bottom row: size reduced embryos with blastula only chopping. Movies were taken every 3 min for 12 h. Scale bar, 1 mm.

DISCUSSION:

Historically, among vertebrate animals, size reduction has been mainly performed using amphibian embryos, by bisecting the embryos along animal-vegetal axis at a blastula stage¹². However, there are mainly two differences between frog and zebrafish embryos when we bisect embryos. First, at the stage when zebrafish embryos become tolerant of bisecting (blastula stage), the organizer is located in a restricted area of blastula margin¹⁸⁻²¹. Because one cannot tell the position of the organizer from the morphology of embryos, randomly cutting the embryo along the animal-vegetal axis produces dorsalized or ventralized embryos. Second, unlike frog embryos, zebrafish embryos go through a process called epiboly, where cells move towards the vegetal pole around a separated yolk until it is completely surrounded by cells. If only a portion of blastoderm is removed, fewer cells remain to engulf a yolk of relatively larger volume, and as a result, morphology appears affected after epiboly. Therefore, we employ two-step chopping in which we chop blastulae near the animal pole, to avoid cutting off the organizer, and wound the yolk membrane, to make the yolk size proportional to the blastula.

In addition to two-step chopping, we found the medium in which the size reduction surgery is performed is critical for recovery of embryos following the surgery. Among several media we tried (Egg water, Egg water + Albumin, Danieau buffer, L15, L15 + FBS, 1/3x Ringer, 1x Ringer), only 1/3x Ringer and 1x Ringer yielded high survival rates; in other media, embryos failed to recover from the wounds.

An important troubleshooting tip for low survival rate is to use healthy embryos from healthy and young parental fish. We noted that even when the control non-size reduced embryos show almost 100% survival rate, when size reduced, embryos from older fish tend to show lower survival rate. Also, note that the survival rate tends to decrease when the size reduction is combined with additional perturbations, such as morpholino injection.

The simplicity of the size reduction technique described here allows researchers to apply this technique without specialized equipment or intensive training. Further, since the size reduced embryos remain smaller until later stages of development (once they start eating, their size seems to catch up with the control fish), this technique can be applied to study scaling of many tissues and organs. Therefore, this technique makes it possible to combine size reduction and quantitative in vivo live imaging to study scaling and size control of various systems.

ACKNOWLEDGMENTS:

The work was supported by the PRESTO program of the Japan Science and Technology Agency and a National Institutes of Health grant (R01GM107733).

DISCLOSURES:

The authors declare no competing or financial interests.

REFERENCES:

1. Cooke, J. Scale of body pattern adjusts to available cell number in amphibian embryos. *Nature*. **290**, 775-778 (1981).
2. Driesch, H. Entwicklungsmechanische Studien: I. Der Werthe der beiden ersten Furchungszellen in der Echinogdermenentwicklung. Experimentelle Erzeugung von Theil- und Doppelbildungen. *Zeitschrift fur wissenschaftliche Zoologie*. (1892).
3. Morgan, T. H. Half embryos and whole embryos from one of the first two blastomeres. *Anatomischer Anzeiger*. **10**, 623-638 (1895).
4. Ishimatsu, K. et al. Size-reduced embryos reveal a gradient scaling-based mechanism for zebrafish somite formation. *Development*. **145**, pii: dev161257 (2018).
5. Megason, S. G. In toto imaging of embryogenesis with confocal time-lapse microscopy. *Methods in Molecular Biology*. **546**, 317-332 (2009).
6. Graeden, E., Sive, H. Live imaging of the zebrafish embryonic brain by confocal microscopy. *Journal of Visualized Experiments*. (26), e1217 (2009).
7. Rosen, J. N., Sweeney, M. F., Mably, J. D. Microinjection of zebrafish embryos to analyze gene function. *Journal of Visualized Experiments*. (25), e1115 (2009).
8. Yuan, S., Sun, Z. Microinjection of mRNA and morpholino antisense oligonucleotides in zebrafish embryos. *Journal of Visualized Experiments*. (27), e1113 (2009).
9. Sorlien, E. L., Witucki, M. A., Ogas, J. Efficient Production and Identification of CRISPR/Cas9-generated Gene Knockouts in the Model System *Danio rerio*. *Journal of Visualized Experiments*. (138), e56969 (2018).
10. Kemp, H. A., Carmany-Rampey, A., Moens, C. Generating chimeric zebrafish embryos by transplantation. *Journal of Visualized Experiments*. (29), e1394 (2009).
11. Mizuno, T., Shinya, M., Takeda, H. Cell and tissue transplantation in zebrafish embryos. *Methods in Molecular Biology*. **127**, 15-28 (1999).
12. Cooke, J. Control of somite number during morphogenesis of a vertebrate, *Xenopus laevis*. *Nature*. **254**, 196-199 (1975).
13. Inomata, H., Shibata, T., Haraguchi, T., Sasai, Y. Scaling of dorsal-ventral patterning by embryo size-dependent degradation of Spemann's organizer signals. *Cell*. **153**, 1296-1311 (2013).
14. Gomez, C. et al. Control of segment number in vertebrate embryos. *Nature*. **454**, 335-339 (2008).
15. Lauschke, V. M., Tsiairis, C. D., Francois, P., Aulehla, A. Scaling of embryonic patterning based on phase-gradient encoding. *Nature*. **493**, 101-105 (2013).
16. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods*. **9**, 676-682 (2012).
17. Almuedo-Castillo, M. et al. Scale-invariant patterning by size-dependent inhibition of Nodal signalling. *Nature Cell Biology*. **20**, 1032-1042 (2018).
18. Koos, D. S., Ho, R. K. The *nieuwkoid* gene characterizes and mediates a *Nieuwkoop*-center-like activity in the zebrafish. *Current Biology*. **8**, 1199-1206 (1998).
19. Yamanaka, Y. et al. A novel homeobox gene, *dharma*, can induce the organizer in a non-cell-autonomous manner. *Genes and Development*. **12**, 2345-2353 (1998).
20. Jesuthasan, S., Stahle, U. Dynamic microtubules and specification of the zebrafish embryonic axis. *Current Biology*. **7**, 31-42 (1997).

482 21. Schier, A. F., Talbot, W. S. The zebrafish organizer. *Current Opinion in Genetics and*
483 *Development*. **8**, 464-471 (1998).
484
485

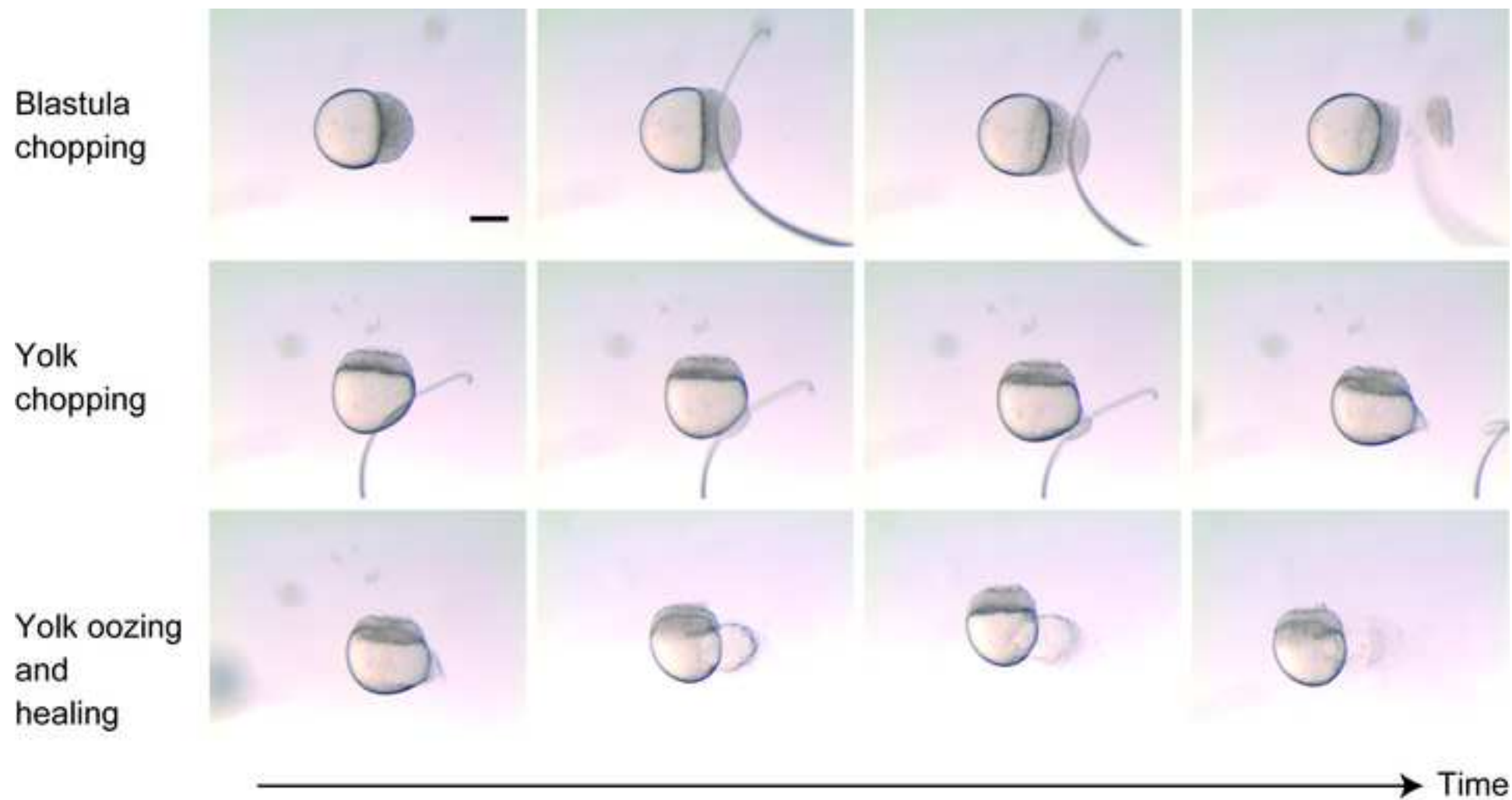


Figure 2

[Click here to access/download;Figure;Fig. 2.tif](#)

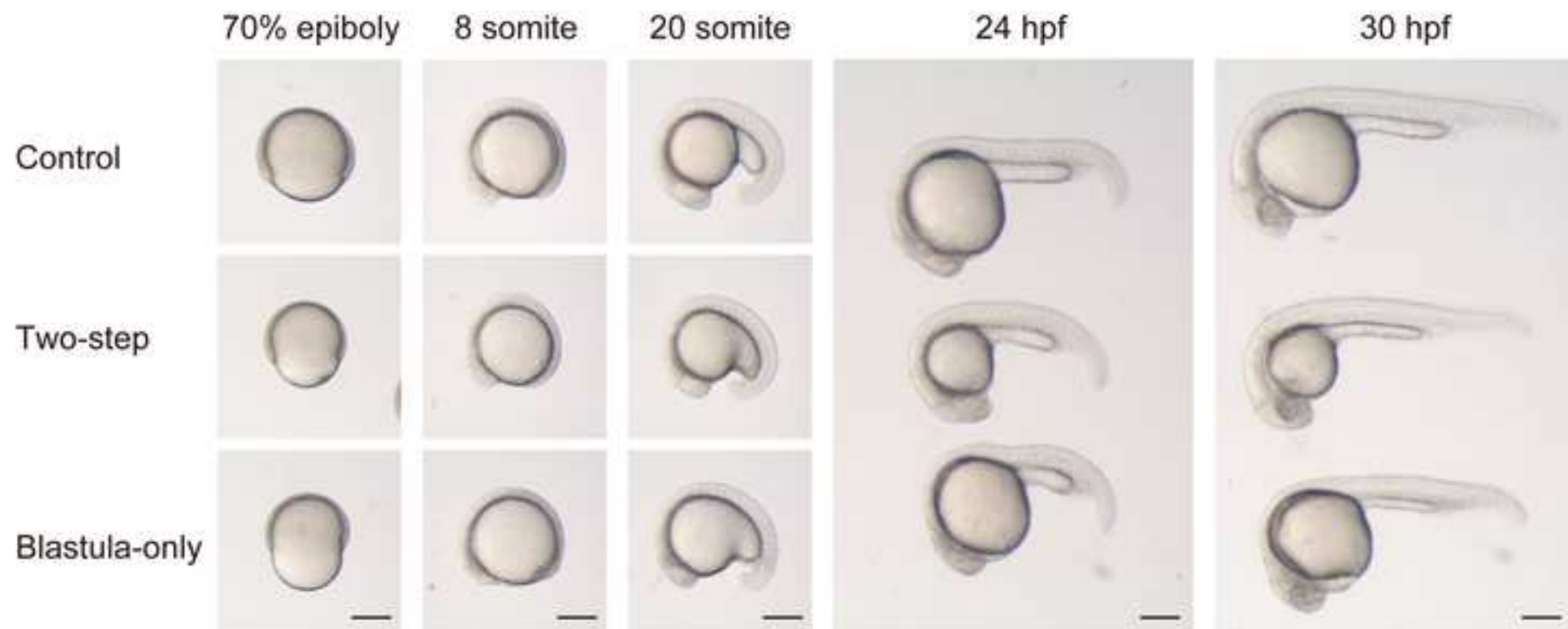
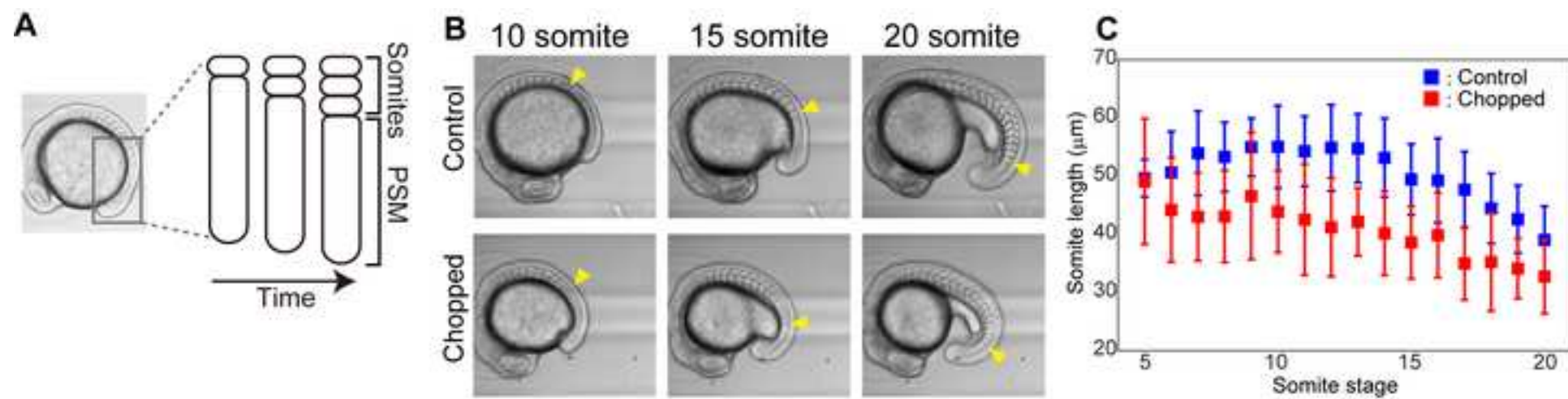
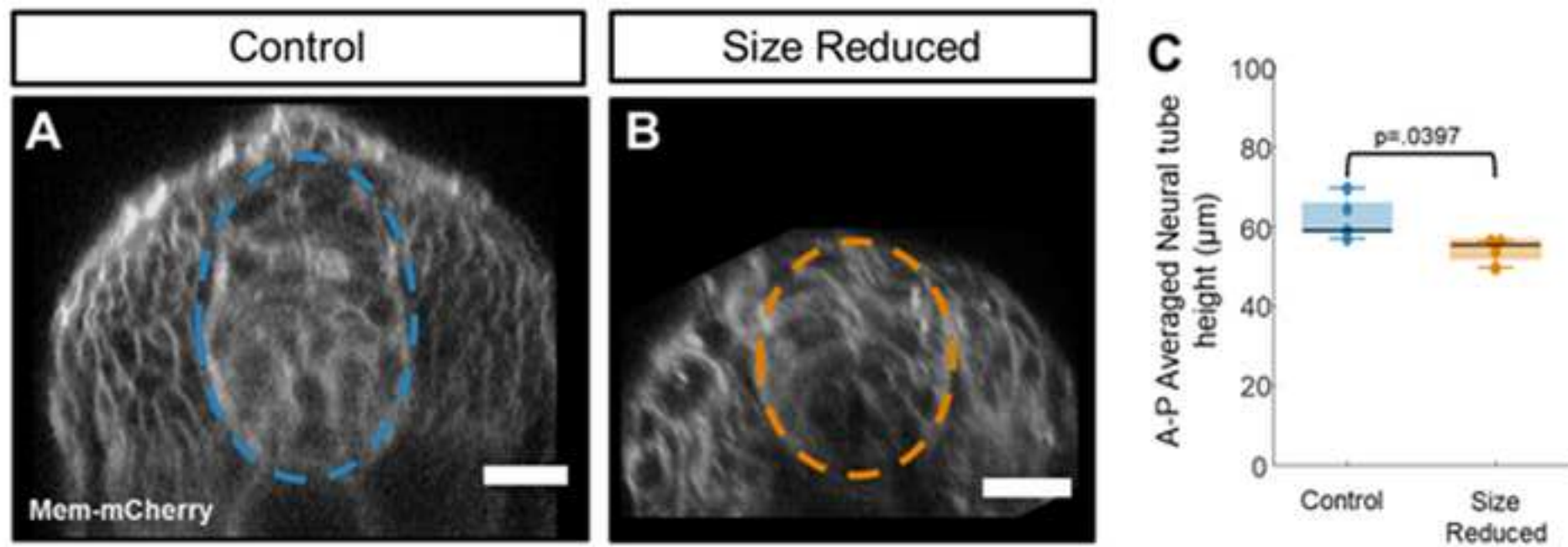
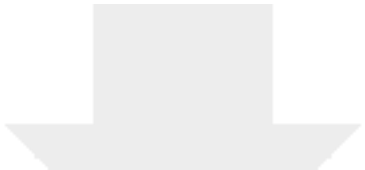


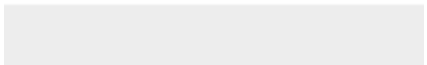

Figure 3







Click here to access/download
Video or Animated Figure
Supplemental movie.avi



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
60 mm PYREX Petri dish	CORNING	3160-60	
Agarose	affymetrix	75817	For making a mount for live imaging
Agarose, low gelling temperature			
Type VII-A	SIGMA-ALDRICH	A0701-25G	
CaCl ₂	EMD	CX0130-1	For 1/3 Ringer's solution
CaSO ₄			For egg water
Cover slip (25 mm x 25 mm, Thickness 1)	CORNING	2845-25	
Disposable Spatula	VWR	80081-188	
Foam board	ELMER'S	951300	For microscope incubator
Forcep (No 55)	FST	11255-20	
Glass pipette	VWR	14673-043	
	SIGMA Life		
HEPES	Science	H4034	For 1/3 Ringer's solution
	INCUBATOR		
INCUKIT XL for Cabinet Incubators	Warehouse.com		For microscope incubator
Instant sea salt	Instant Ocean	138510	For egg water
KCl	SIGMA-ALDRICH	P4504	For 1/3 Ringer's solution
Methyl cellulose	SIGMA-ALDRICH	M0387-100G	
NaCl	SIGMA-ALDRICH	S7653	For 1/3 Ringer's solution
Petri dish	Falcon	351029	For making a mount for live imaging
	SIGMA Life		
Phenol red	Science	P0290	
	BEL-ART		
Pipette pump	PRODUCTS	F37898	
	EMD Millipore		
Pronase	Corp	53702-250KU	

Tricaine-S (MS222) WESTERN
CHEMICAL INC NC0135573
Ultra thin bright annealed 316L dia. Sandra

The wire we used was obtained ~20 years ago and we could

d not find exactly the same one. This product has the same material and diameter as the one we use.

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Surgical Size Reduction of Zebrafish for the Study of Embryonic Pattern Scaling

Author(s):

Kana Ishimatsu, Anna Cha, Zach M Collins, Sean G Megason

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Sean G Megason	
Department:	Department of Systems Biology	
Institution:	Harvard Medical School	
Title:	Associate Professor	
Signature:		Date: 11-23-18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We are not using figures from previous publication.

3. Figure 4: Please define the scale bars in the figure legend.

We have defined the scale bars in the figure legend for Figure 4B, C and D.

4. Supplemental Movie: Please include a scale bar in the movie and define the scale in the legend. Please also include a title in the legend.

We have included a scale bar in the movie and defined the scale in the legend.

5. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We have sorted the items in the Table of Material in alphabetical order.

6. Please provide an email address for each author.

We have provided an email address for each author in the title page.

7. Please include a space between all numerical values and their corresponding units: 30 μm , 5 mm, 15 mL, 37 $^{\circ}\text{C}$, 60 s; etc.

We have included a space as suggested.

8. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have included an ethics statement before the numbered protocol.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the protocol so any personal pronouns do not appear.

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions

should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised the manuscript so “could be,” “should be,” and “would be” do not appear in the protocol.

11. Protocol: Please refrain from using indentations.

We have fixed the indentations in the manuscript.

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined the shorter protocol steps as suggested.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted the essential steps of the protocol.

16. Please do not abbreviate journal titles.

We have fixed the citations so there is no abbreviation in journal titles.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, Ishimatsu et al., reported a new method for generation of size-reduced zebrafish embryos, which could be used for examination of the pattern-scaling ability during development of vertebrate. To achieve healthy and sized reduced zebrafish embryo, the authors sequentially removed blast cells and yolk, and confirmed that pattern scaling was occurred in somites and in the ventral part of developing spinal cord in manipulated embryos. Also, the authors optimized culture medium to improve the viability of embryos after surgery.

For the study of pattern scaling of vertebrate development, size reduction technique was previously developed in amphibian, which is not easy for application to genetic approach and for live-imaging of events progressed in the lateral side of embryos. However, this new method will make it possible to apply the genetics and live-imaging techniques to the study of pattern scaling of vertebrate development. Therefore, I would strongly recommend this new technique for publication in JoVE.

Major Concerns:

No major concern.

Minor Concerns:

Fish strain name should be described in 2.1.1 because genetic background possibly affect the efficiency of survival after surgery.

We have described fish strain in 2.1.1.

Line126: No sentence in 1.3.3

We have removed 1.3.3.

Line207: woundingand →wounding and

We have corrected this typo.

Reviewer #2:

Manuscript Summary:

This manuscript details a protocol for reducing embryo size while preserving patterning to allow for scaling effect studies during patterning. Previous methods resulted in significant embryo lethality due to patterning and gastrulation defects caused in part by an abnormal cell to yolk ratio. The authors present a two-step method to first reduce the number of cells and then to reduce yolk volume. This new method can still result in significant numbers of abnormal embryos, but the authors share pointers for optimizing the yield of smaller and normally patterned embryos. Overall the protocol is clearly written.

Major Concerns:

No major concerns

Minor Concerns:

1) The section on somite scaling somewhat detracts from the protocol. This could be

addressed by either removing the corresponding section and figure or setting it up better. As written it is not clear why there would be a scaling delay during segmentation since the PSM is not specified at the time that the embryo size is reduced, therefore, one might expect that without an additional manipulation the smaller embryo would have a smaller PSM to begin with, and thus should segment according to its smaller size from the beginning. Additional detail is needed here to set this up.

We have simplified the manuscript and the figures so the main claim of somite scaling is clearer and the delay gets less attention, as this is not the main point of this paper.

2) The manuscript should be checked for consistency in nomenclature usage. For example, the names of genes, RNAs and transgenes should be italicized. This is followed in some places but not in others.

We have made the nomenclature usage consistent.