A novel micropipette approach to studying the mechanical forces and their effects on Zebrafish embryo development

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Abstract

Embryos undergo special morphogenesis, forming stereotyped embryonic structures, such as morula, blastula, gastrula, etc., across all different vertebrate species, an indication of a conserved mechanism for embryogenesis. Generation, maintenance and dynamics of these embryonic structures involve coordinated mechanical forces. However, due to lack of available methods, little is known about how these forces are generated and what roles they play during embryonic development and morphogenesis. Here we described a micropipette culture system, in which live embryos were loaded into a micropipette to study the effects of the micropipette-generated constraining forces on the embryo development during in vitro culture. It is interesting to find that the constraining forces disrupted the normal embryo development while the unperturbed control embryos were not affected. It is noted that the embryos under constraining forces failed to maintain the rhythmic rotational movements and subsequent hatching out from the zona pellucida. Therefore, early embryos are sensitive to the disruption of the mechanical forces, which can cause detrimental effects on normal embryo development.

Introduction

Fertilized eggs undergo fast stereotypic mitotic divisions (called egg cleavage), forming 2-cell, 4-cell, morulae, blastocyst, etc., which eventually develop into a fully grown individual [1,2]. Embryonic development is a very dynamic process, involving many mechanical forces working in a coordinated fashion to generate and maintain a certain morphology. In the case of zebrafish, the fast dividing and growing embryo undergoes dramatic morphological changes from a blastula embryo to an elongated larva with visible eyes, heart, and other organs. Apparently, every morphological change involves mechanical forces [3,4]. There have been reports showing that simply by changing the tensions of the physical surface where cells grow can change the cell fates, forming cells and tissues with distinct functions [5,6]. It is important to understand how mechanical forces contribute to the above described processes of embryonic development, what consequences it may have if these forces are disrupted. It is known that human embryos are vulnerable and prone to abnormalities during various stages of development, leading to arrest of development, miscarriages, or birth defects in new born children [7]. There are many factors, such as genetics, infected diseases, environmental factors, etc., which may contribute to the formation of defective embryos. However, little is known about how the mechanical forces control and regulate embryo development.

The biggest challenge to studying mechanical forces during embryonic development is lack of proper tools and methods to measure and manipulate the forces to determine the roles of mechanical forces during embryo development. Here we described a simple method to evaluate

the effects of mechanical forces on embryo development using zebrafish as a research model. The purpose of this project is to experimentally test and determine how external mechanical forces affect embryo development. Hopefully, the knowledge gained from animal research can be applied to humans and help understand the roles of mechanical forces in embryo development, which may provide hints to prevent birth defects from a force perspective.

Materials and Methods

1. Fabrication of micropipettes for embryo culture

Glass pipettes were purchased from Fisher Scientific. To make micropipettes, a glass Pasteur pipette was pulled using two hands over a flame of a gas burner, making it thinner. Rotate the pipette on the flame until the middle of the pipette starts to melt and then pull in opposite directions, making the middle of the pipette thin enough to be able to hold the zebrafish embryo (with an inner diameter about 1-1.2 mm). The reason for choosing this diameter to make micropipette is that the diameter of the zebrafish fertilized eggs is around 0.7 mm [1], so the micropipette allows single embryos to be loaded one by one into the micropipette without crowding together. Use a grind wheel to cut the micropipette (by scrolling around the pipette) at the right diameter with a length about 30 mm, so multiple embryos can be loaded in a single micropipette. The prepared micropipettes used for the experiments are as shown in Figure 1.

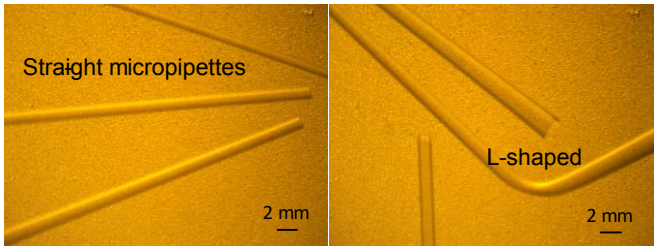

Figure 1. Hand-made micropipettes used for embryo culture.

2. Source of fertilized zebrafish eggs

The fertilized eggs were purchased from Carolina Biological Supply Company. The eggs were shipped by overnight express in a temperature-controlled package handled by the company. After receiving the package, the eggs were washed with fresh prepared E3 zebrafish embryo culture medium, which is routinely used for zebrafish embryos handling and culture, with a simple composition of 34.8 g NaCl, 1.6 g KCl, 5.8 g $CaCl_2 \cdot 2H_2O$, 9.78 g $MgCl_2 \cdot 6H_2O$ as described in Cold Spring Harbor Protocols [8]. Only embryos with normal morphology (smooth zona pellucida, regular round shape) were selected and used for experiments.

3. Loading of embryos into micropipettes

The selected embryos were aspirated into the prepared micropipette using a mouth-controlled pipette connected to the micropipette with a rubber tube. By gently sucking to generate a negative pressure, the embryos were aspirated into the micropipette one by one inside the micropipette as shown in Figure 2. The embryos outside the micropipettes were used as a control to compare the embryo development under the micropipette-exerting constraining forces vs free of external forces.

4. Culture and observation of embryo development

Multiple micropipettes each loaded with embryos were put in a 60 mm petri dish (Fisher Scientific) containing E3 medium. For comparison, control embryos were put outside the micropipettes as shown in Figure 2. Over 11 embryos were successfully loaded into the micropipettes, which were cultured with control embryos in the same culture dish.

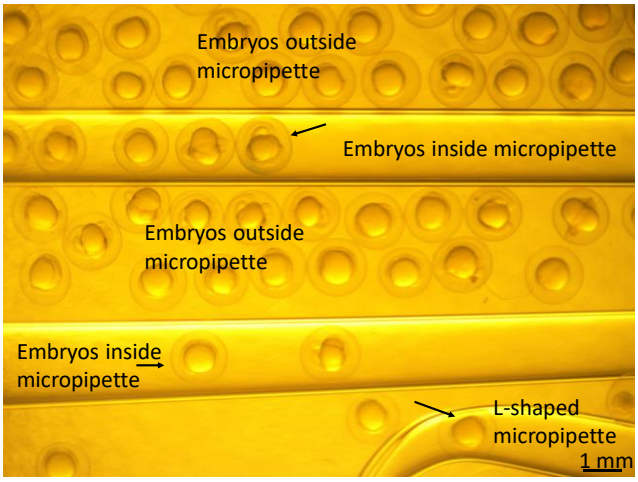

Figure 2. A taken picture showing the embryos successfully loaded into the micropipettes (the arrow-pointed) and the control embryos outside the micropipettes. The scale bar=1 mm

To observe and monitor the embryo development, an AmScope stereo microscope with digital imaging capabilities（7X-45X Trinocular Stereo Zoom Embryonic Microscope with a 14MP USB3.0 Camera（all AmScope products） was used to record movies, with a 2-minute interval. The temperature was maintained at 30°C during the process of movie recording. All the recorded movies were analyzed and compared between mechanical force-perturbed and non-perturbed control embryos.

Results

1. Normal embryo development to larval stage in the in vitro culture system

First, to make sure whether our in vitro culture system can support normal embryo development and be used to evaluate the effects of perturbation of mechanical force on embryo development, the received fertilized eggs were first cultured in the 60 mm petri dish containing E3 medium without any perturbation. Ninety percent of the fertilized eggs (n=34) developed to larval stage (Figure 3A) and 70% of them successfully hatched out from zona pellucida (Figure 3B-E). The hatched fish showed robust heart beating and free-swimming activities (Supplemental data, video 1, 2). The

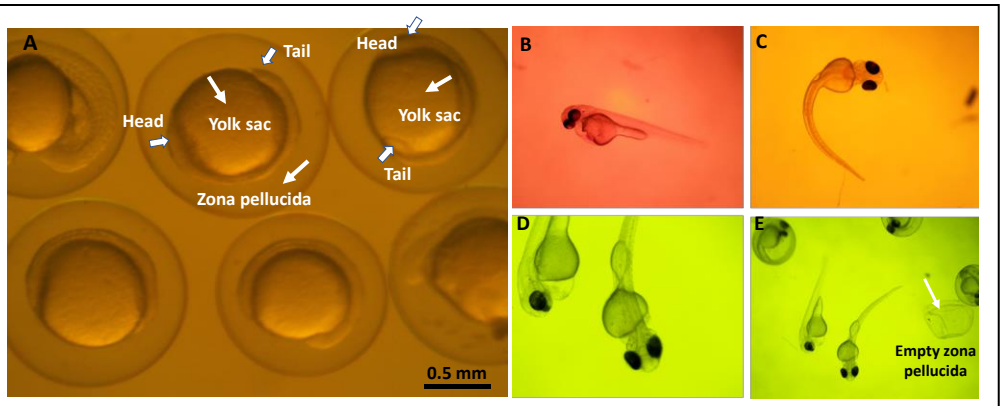

Figure 3. Development of unperturbed embryos cultured in vitro. A. 6 hours of culture. Note the elongated embryo with a clear head-tail polarity establishment. B-E. Hatched embryos. Note the hatched fish with well-developed eyes and the left empty zona pellucida.

experiments were repeated twice and the consistent high percentage of the cultured embryos developing to larval stage gave us sufficient confidence that the culture conditions are suitable for zebrafish embryo development and can be used to evaluate the effects of mechanical constraining forces on the embryo development during culture in the micropipettes.

2. Effect of physical constraining forces on the embryo development

A total of 11 embryos were loaded into the straight-shaped micropipettes and 21 embryos were positioned outside the pipettes and cultured in the same petri dish under the same culture conditions as a control (In order to minimize the experimental errors between different experiments and to obtain an accurate comparison between the loaded and the control embryos). It was found that the embryos that were experiencing constraining forces from the micropipettes slowed down and eventually stopped development after 10 hours culture, while all the nonperturbed control embryos developed to the larval stage with clear head-tail formation (Figure 4). Consistently, all the embryos (n=9) cultured in the L-shaped micropipettes (also in the same petri dish) stopped development. Despite extension of culture time to 16 hours, the embryos inside the micropipettes could not be as developed as the control embryos (Figure 5), suggesting that the embryo development was completely suppressed, not just delayed.

The experiments demonstrated unambiguously that both even or uneven lateral forces coming from the micropipette walls all suppressed the embryo development.

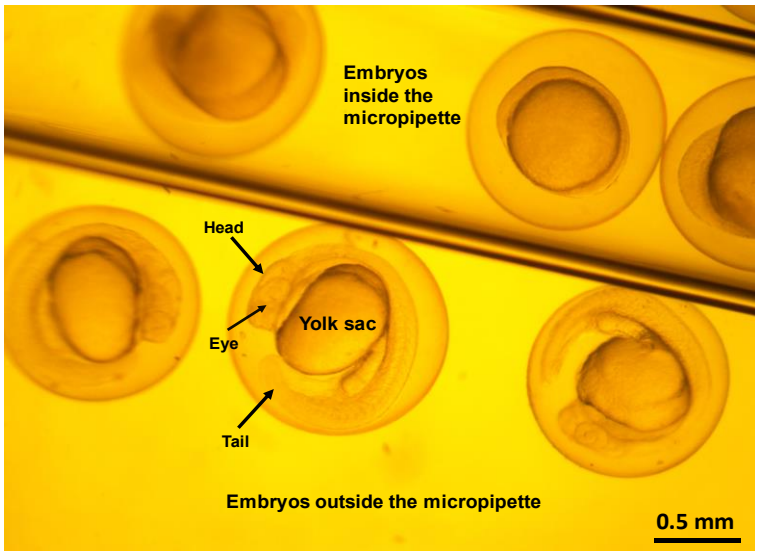

Figure 4. Comparison of the development of the embryos inside and outside the micropipettes after 10 hours culture. Note the advanced development of the control embryos outside the micropipette and the arrested development of the embryos inside the micropipettes.

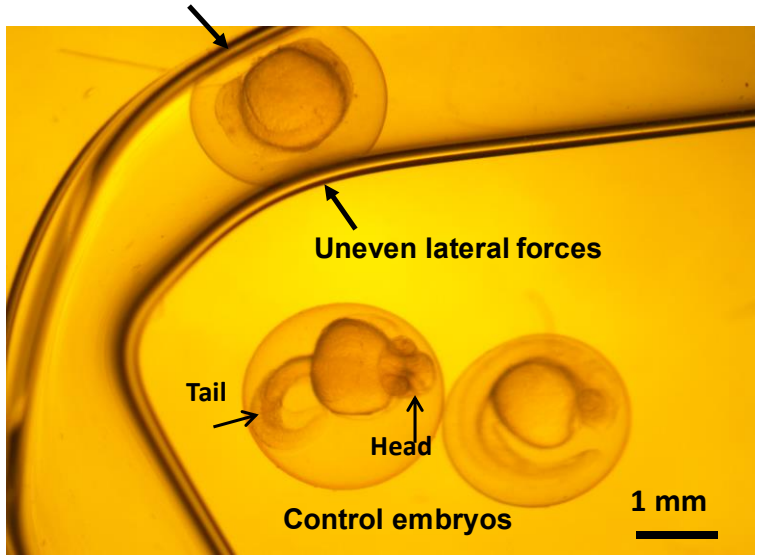

Figure 5. Comparison of embryo development after 16 hours culture in the L-shaped micropipette with the control embryos. Note the significant differences.

3. Disruption of the rhythmic embryo rotational movements by the constraining forces

To test the effect of the constraining forces on later stage embryos, the received early stage embryos were first cultured in E3 medium for overnight, with all embryos showing vigorous rotational movements. The rotational movements had a special pattern, with tail periodically beating which pushed the embryo moving forward, cycling inside the zona pellucida as indicated in Figure 6 (and supplemental data, video 3, 4). Seventeen embryos that were exhibiting the periodic rotational movements were loaded into the micropipettes, and the rest 27 embryos (also showing the rotational movements) were put outside the micropipettes and cultured together in the same petri dish as a control. All the embryos were cultured in the same petri dish for 10.5 hours to compare the differences in the rotational movements and embryo development. It was found that during the 1st 3.5 hours culture, there was no significant difference between the embryos inside and outside micropipettes in terms of frequency and embryo morphology (Student's t test shows no significant difference, $P>0.05$).

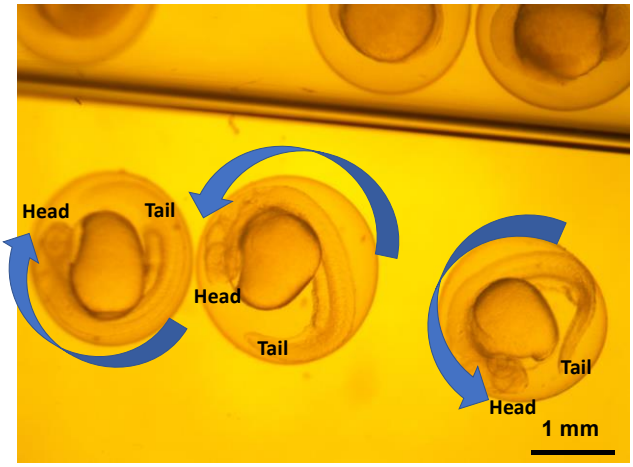

Figure 6. Periodic rotational movements of embryo inside the zona pellucida. Note the direction of the rotations of the embryos with head leading and tail beating to generate pushing forces.

However, during the second 1st 3.5 hours culture, the frequency of rotational movements significantly reduced (table 1, Student's t test shows significant difference, $P<0.01$). In the 3rd 3.5 hours culture time, all the embryos inside the micropipettes stopped the rotational movements while the controls embryos still showed robust rotational movements (Table 1). Obviously, the lateral constraining forces slowed down and eventually suppressed the embryo rotational movements. After longer culture time, the control embryos continued to show the periodic rotational movements and eventually hatched out from the zona pellucida (n=18/27). None of the embryos that had stopped the rotational movements inside the micropipettes hatched out of the zona pellucida, suggesting a positive role of rotational movement in hatching out of the zona pellucida.

Table 1. Quantification of embryo rotational movements during culture

Groups	1st 3.5 hours rotation numbers	2nd 3.5 hours rotation numbers	3rd 3.5 hours rotation numbers
Embryos inside micropipettes(n=17)	42±3	11±2	0
Embryos outside micropipettes(n=27)	45±5	38±3	31±3

Note the comparison of the average numbers of embryo rotations between the embryos inside and outside the micropipette during the 10.5-hour culture.

Discussion

This is the first time micropipettes were used to study the effects of mechanical forces on embryo development and the preliminary results are exciting and encouraging. The results show that the developing zebrafish embryos are very sensitive to the perturbation of the mechanical forces. Even gentle pressure or squeezing by laterally constraining of the embryos can produce profound effects, slowing down and eventually suppressing the embryonic development. The observed disruption of embryo development can not be contributed to the embryo contacting or touching the glass micropipette which may be toxic to the embryos, because some of the control embryos outside the micropipettes were also in direct contact (on one side) to the glass micropipette and they all developed well to hatching stage. Therefore, just the contact with the micropipette without any constraining forces does not disrupt the normal embryo development.

The embryos cultured inside the micropipettes were only experiencing the lateral constraining forces from the micropipette wall, generating a belt of constraining forces on the embryo (Figure 7). Because the two longitudinal sides of the micropipette are open and are free for culture medium coming in and going out, the embryos do not experience any mechanical forces from longitudinal directions. Therefore, it can be concluded that the observed suppressive effects of micropipettes on the embryo development are due to long lasting constraining forces on the embryos. Further experiments are needed to determine the effects of the lateral constraining forces at different temporal scales, and whether release of the constraining forces by removing the embryos out of the micropipette can rescue the temporarily suppressed embryo development.

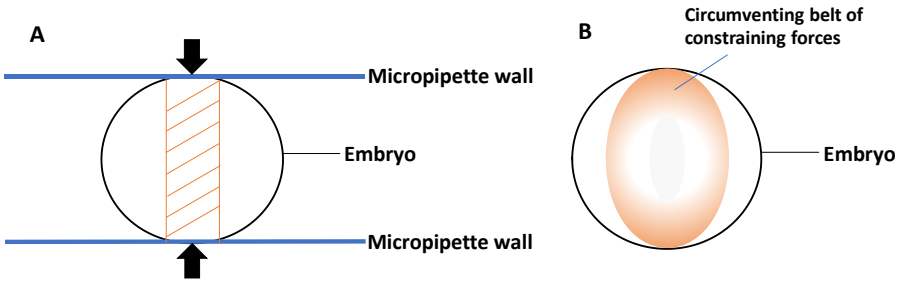

Figure 7. A diagram depicting external mechanical forces exerting on an embryo inside a micropipette. A. An embryo inside a micropipette. The two black arrows indicate constraining forces and the orange bar represents the belt of constraining forces exerting on the embryo. B. Another representation of the figure showing the circumventing belt of forces shown in orange color.

It is interesting to notice the special patterns of rhythmic, long lasting rotational movements of zebrafish embryos during development inside the zona pellucida. It seems that the rotational movements originate from the tail beating which pushes the embryo moving forward with the head leading. Because the developing embryo is confined inside the zona pellucida, the tail beating-driven forward movements are manifested as circling movements, e.g. rotations. It is not known why the tail exhibits the periodic beating movements at such early stage of embryo development. It seems that the muscle cells in the tail are self-exiting cells and contracting rhythmically just like heart muscles. This pattern of tail movements may play important roles for fish swimming after hatching out. But why this type of movements starts so early at embryonic stage when the embryo is still developing inside the zona pellucida? A most likely explanation is that the rhythmic tail movements may be beneficial for the wellbeing and healthy development for the embryos. This possibility is validated by our experiments since disruption of the rhythmic

tail movements by constraining the embryo in the narrow micropipette stopped the further development of embryo and failure to hatch out of the zona pellucida. It would be interesting to test in our future experiments whether release of the constrained embryos from the micropipettes would rescue the rhythmic rotations of the embryos and hatching out of the zona pellucida.

In the future experiments, we will consider designing methods to measure and determine how much forces are required to disrupt the normal embryo development. Though it is still technically challenging, recent reports have shown it is possible to measure the forces in the living tissues and embryos [9-11].

References

1. Gilbert, S. Developmental Biology, 6th edition, Oxford University Press, 2000.
2. Hasley, A., Chavez, S., Danilchik, M., Wühr, M., Pelegri, F. (2017). Vertebrate Embryonic Cleavage Pattern Determination. Adv Exp Med Biol. 953:117-171.
3. Mammoto, T., Ingber, D. E. (2010). Mechanical control of tissue and organ development. *Development*, **137**(9): 1407–1420.
4. Davidson, L. A. (2011). Embryo mechanics: balancing force production with elastic resistance during morphogenesis. *Curr Top Dev Biol*. **95**:215-241.
5. Vining, K. H., Mooney, D. J. (2017). Mechanical forces direct stem cell behaviour in development and regeneration. *Nature Reviews Molecular Cell Biology*, **18**: 728–742.
6. Li, J., Wang, Z., Chu, Q., Jiang, K., Li, J., Tang, N. (2018). The Strength of Mechanical Forces Determines the Differentiation of Alveolar Epithelial Cells. *Dev Cell*. 44(3):297-312.
7. What causes birth defects? NIH Report, https://www.nichd.nih.gov/health/topics/birthdefects/conditioninfo/pages/causes.aspx
8. E3 medium (for zebrafish embryos), Cold Spring Harbor Protocols, Cold Spring Harbor Laboratory Press, 2011.
9. Campàs, O., Mammoto, T., Hasso, S., Sperling, R.A., O'Connell, D., Bischof, A. G., Maas, R., Weitz, D. A., Mahadevan, L., Ingber, D. E. (2014). Quantifying cell-generated mechanical forces within living embryonic tissues. *Nat Methods*. 11(2):183-9.
10. Nelson, C. M. (2017). From static to animated: Measuring mechanical forces in tissues. *J Cell Biol*. 216(1): 29–30.
11. Grashoff, C., Hoffman, B. D., Brenner, M. D., Zhou, R., Parsons, M., Yang, M. T., McLean, M. A., Sligar, S. G., Chen, C. S., Ha, T., Schwartz, M. A. (2010). Measuring

mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*. 466(7303):263-6.