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Journal of Biomechanics 38 (2005) 1359–1363

JOURNAL OF BIOMECHANICS

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Technical note

Investigating chorion softening of zebrafish embryos with a microrobotic force sensing system

Deok-Ho Kim[a], Yu Sun[b], Seok Yun[a], Sang Ho Lee[c], Byungkyu Kim[a,*]

[a]*Microsystems Research Center, Korea Institute of Science and Technology (KIST), P.O. Box 131, Cheongryang, Seoul 130-650, Republic of Korea*
[b]*Department of Mechanical and Industrial Engineering, University of Toronto, 5 King's College Road, M5S 3G8, Canada*
[c]*Division of Life Sciences and Biotechnology, Graduate School of Biotechnology, Korea University, Seoul 136-701, Republic of Korea*

Accepted 3 July 2004

Abstract

The zebrafish is a model organism for addressing questions of vertebrate embryo development. In this paper, the softening phenomenon of the chorion envelope of zebrafish embryos at different developmental stages was mechanically quantitated by using a microrobotic force sensing system. The microrobotic system integrates a piezoelectric cellular force sensor to measure the required forces for penetrating the chorion envelope. Magnitude of penetration forces was found to decrease as an embryo develops. The results mechanically quantitate "chorion softening" in zebrafish embryos due to protease activities subtly modifying the chorion structure, providing an understanding of zebrafish embryo development.

Keywords: Zebrafish embryo; Chorion envelope; Force sensing; Microrobotic system; Chorion softening; Protease

1. Introduction

Biological cell manipulation is important for many industries, such as the livestock industry by allowing individual cell-based diagnosis and pharmaceutical tests. Manipulating individual embryos has become a challenging issue in biomedical applications such as cloning, gene expression analysis, and cell replacement therapy (CRT). Despite intense interest in analysis, diagnosis, and manipulation of single biological cells, most biomanipulation tasks are usually conducted by experienced operators relying only on the visual information from a microscope (Kimura and Yanagimachi, 1995).

Many efforts focus on automating biological cell manipulation in order to reduce human labor and increase success rates resulting from improved reproducibility. For example, a cell injection system integrated with piezoelectric actuators (Nakayama et al., 1998; Yanagida et al., 1998; Tan and Ng, 2001) was developed to make the manipulation of biological cells more controllable. Sun and Nelson (2002) developed a visually servoed microrobotic cell manipulation system where embryos and pipettes were automatically identified and controlled.

Accurate force measurements on cell membranes are required for biomanipulation and cell surgery. Moreover, force sensing on cell membranes is essential for understanding the biophysical mechanisms of cell injury and membrane modeling studies. Thus far, there have been limited quantitative biophysical measurements of cell membrane forces on zebrafish embryos.

The zebrafish is receiving increased attention as a model system for vertebrate development because of its similarity in gene structures to the human beings rather than *Drosophila* (Stainier, 2001). The main advantages are its short life cycle of approximately 12 weeks, which makes genetic analysis much easier; and the transparency of the embryos, making the fate of individual cells

*Corresponding author. Tel.: +82-2-958-6730; fax: +82-2-958-6910.
E-mail address: bkim@kist.re.kr (B. Kim).

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doi:10.1016/j.jbiomech.2004.07.014

during development easy to be observed (Lewis et al., 2002). Above all, zebrafish breeds numerous oocytes that are cultured fairly rapidly. The zebrafish oocytes and embryos are approximately 0.7 mm in diameter, with the cytoplasm and nucleus at the animal pole sitting upon a large mass of yolk.

In zebrafish embryos, the chorion envelope undergoes a thinning process called "chorion softening" before the basic body formation is complete and hatched out (Schoots et al., 1983). Upon hatching, the chorion is digested by hatching enzymes, which are proteolytic enzymes secreted from hatching gland cells of the embryo (Inohaya et al., 1999). These hatching enzymes have been partially identified, which are responsible for "chorion softening" (Roberts and White, 1992).

Measuring the penetration forces of the zebrafish embryo chorion provides an opportunity to examine physical and mechanical property changes of the chorion envelope that undergoes chemical modifications during fertilization and embryonic development. In this paper, we report force measurement results on the chorion envelope of zebrafish embryos at various developmental stages. In Section 2, the microrobotic biomanipulation system that is capable of measuring forces on cell membranes is introduced. The preparation process of zebrafish embryos for the experiments is also described in Section 2. In Section 3, measurement results of chorion penetration forces are presented to mechanically quantitate "chorion softening" of zebrafish embryos.

2. Materials and methods

2.1. Micromanipulation system

We have developed a microrobotic biomanipulation system with a total of 8-DOF mobility for quantitating mechanical properties of cell membranes. It consists of two 3-DOF micromanipulators equipped with an injection pipette and a holding pipette, respectively, and a 2-DOF precision positioning system on the vibration isolation table (Fig. 1). Fine motion required for biomanipulation is provided by the 3-DOF micromanipulator (MP-285, SUTTER Inc.) with a travel of 25 mm and a resolution of 0.04 μm along each axis, which is integrated with a holding pipette. The 3-DOF micromanipulator (InjectMan NI2, Eppendorf) with a workspace of $20 \times 20 \times 18\,\text{mm}^3$ and a resolution of 2 μm is integrated with an injection pipette. The 2-DOF precision positioning system (M-410DG, PI Inc.) produces planar motion with a large workspace and a resolution of 8.5 μm under a stereomicroscope and changes the field of view of the optical vision system. The micromanipulation system is controlled by a PC-based control board. To obtain visual information

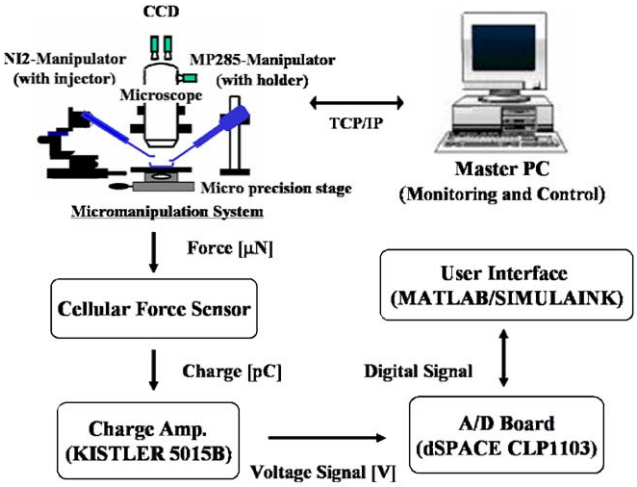


Fig. 1. Microrobotic biomanipulation system integrated with the cellular force sensing instrument.

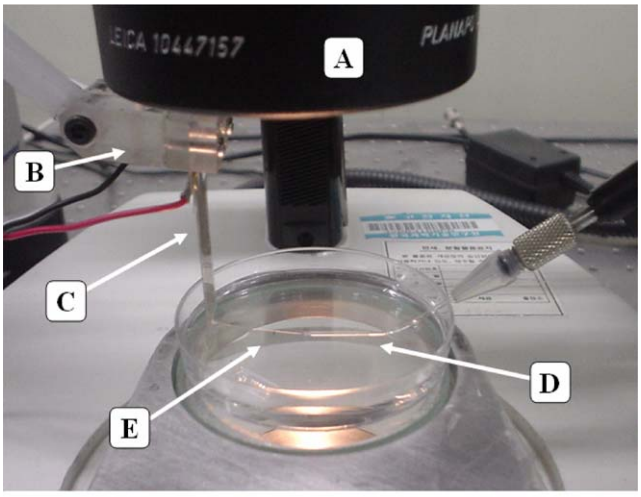
on cell membrane deformations, we used a stereo microscope (MZ-12.5, Leica Inc.) with long working distance objectives. The microscope images were captured by a video frame grabber (Matrox Genesis, Matrox Electronic System Ltd.), displayed and analyzed on the host PC.

2.2. Force sensing instrument

Polyvinylidene fluoride (PVDF) piezoelectric polymer films were adopted to construct a force sensor to detect forces on cell membranes. The PVDF force sensor has desirable characteristics as a sensing material such as a high linearity, wide bandwidth, and high signal-to-noise (S/N) ratio, which provides high reliability of the sensor output signal (Benech et al., 1996; Marat-Mendes et al., 1999; Dargahi et al., 2000). The PVDF force sensor was integrated with a glass injection pipette and a capillary holder, together forming the cell membrane force sensing instrument (Fig. 2). The injection pipette was bonded to the tip of the PVDF film (width: 28 μm, Model: LDT1-028K of MSI Inc.). Nickel (Ni) electrodes were deposited on both sides of the PVDF film to obtain electrical signals. The PVDF sensor was clamped on the clamping fixture which was mounted on the positioning micromanipulator.

Charges generated by the PVDF force sensor were amplified by a charge amplifier, and output was sampled through a data acquisition board (dSPACE 1103). The output signals from the PVDF force sensor were digitalized and filtered for noise rejecting. In the filtering operations, the DC offset was rejected, and noises such as from the AC power source were attenuated by a low-pass filter (LPF) with a cutoff frequency of 20 Hz.

In order to calibrate the PVDF force sensor, a precision load cell (GSO-10, Transducer techniques, full



A. Stereo Microscope
B. Clamping Fixture
C. PVDF Cellular Force Sensor
D. Holding Pipette
E. Micro Injection Pipette

Fig. 2. Cellular force sensing system setup for the study.

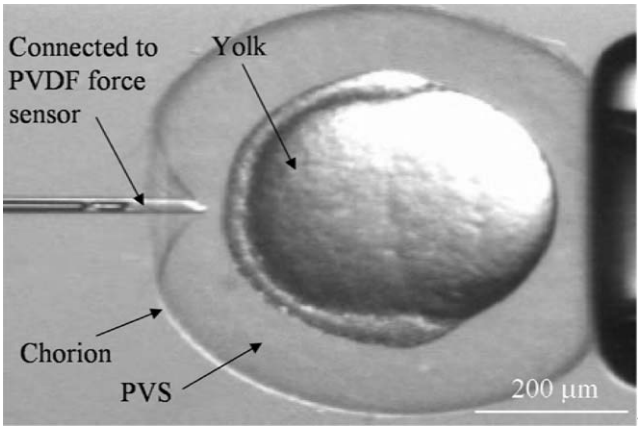


Fig. 3. Structure of zebrafish embryos: chorion envelope, the perivitelline space (PVS) and the yolk.

scale: 100 mN, resolution: 0.05 mN) was used. The output voltage from the PVDF force sensor varies linearly with applied forces. The calibration results prove a good linearity of the PVDF sensor (<5%) and a sensitivity of 196 μN/V.

2.3. Zebrafish embryo preparation

The outer membrane of a zebrafish embryo is the chorion envelope, under which are various complexes filled upon fertilization forming the pervitelline space (PVS) (Fig. 3). Major developmental processes occur during the first three days after fertilization. Embryos at different developmental stages were accurately collected by examining morphological features of each live embryo under the stereomicroscope according to the standard stages of embryonic development of the zebrafish (Kimmel et al., 1995). For force measurement experiments, 30 embryos were collected with 10 at the blastula stage, 10 at the gastrula stage, and 10 at the pharyngula stage (Fig. 4).

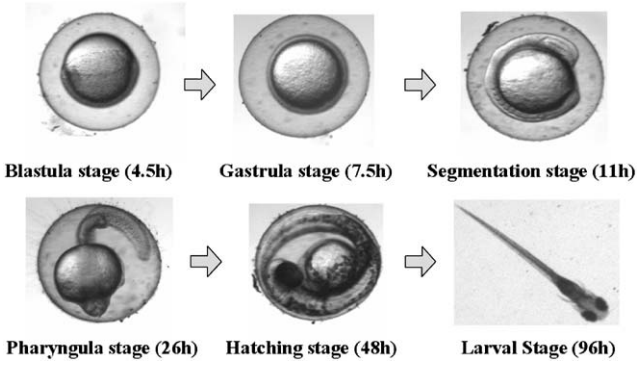


Fig. 4. Zebrafish embryonic developmental stages.

3. Results and discussion

Force measurements were conducted at room temperature of 22–24 °C . The end tip of the micropipette is 11 μm in diameter. The chorion envelopes were loaded at a speed of 120 μm/s. In the experiments, a glass pipette attached to the PVDF force sensor first approached and contacted the chorion envelope. The pipette then penetrated the chorion envelope, when forces began to increase.

In the experiments, 10 embryos at the gastrula stage and 10 embryos at the pharyngula stage were tested. The embryos were 7.5 h after fertilization for the gastrula stage and 26 h after fertilization for the pharyngula stage. The output voltage signal from the PVDF cellular force sensor was sampled during chorion loading. Three data sets out of 10 tested embryos at the pharyngula stage and the gastrula stage are shown (Figs. 5 and 6). When the pipette continuously loaded the chorion, forces began to increase proportionally. Average penetration forces were determined to be 738 μN with a standard deviation of 35.1 μN (1δ) for embryos at the gastrula stage (Fig. 5) and 523 μN with a standard deviation of 37.9 μN (1δ) at the pharyngula stage (Fig. 6). The force signal dropped to the basal level upon penetrating chorion envelopes, suggesting that no detectable force was present in the PVS under the experimental conditions. The experimental data demonstrate that the required forces for penetrating the zebrafish chorion envelopes at the gastrula stage are 1.41 times higher than those at the pharyngula stage.

The lower penetration forces at the pharyngula stage than those at the gastrula stage can be explained by proteolytic activities induced by hatching enzymes (protease secretion) (Roberts and White, 1992; Inohaya et al., 1999; Denker, 2000). The previous biological

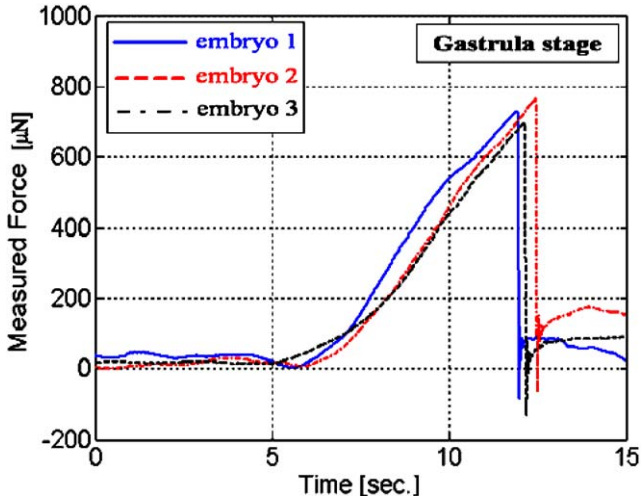


Fig. 5. Force sensing results on 3 zebrafish embryos at the gastrula stage.

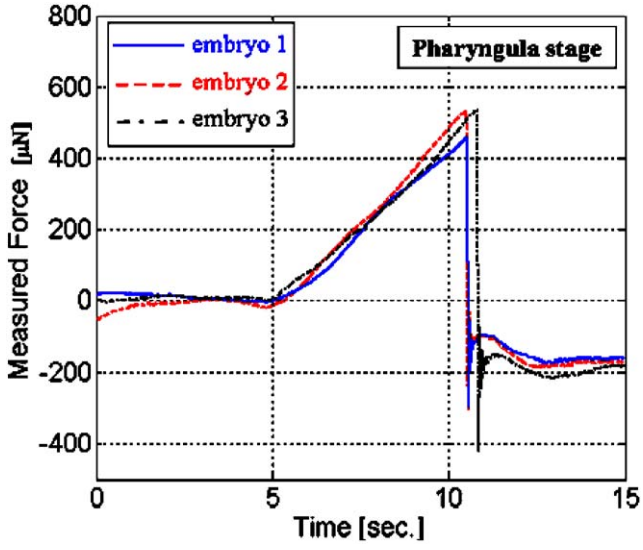


Fig. 6. Force sensing results on 3 zebrafish embryos at the pharyngula stage.

studies employed chemicals to test the time period for dissolving the chorion, which chemically describes hardness or softness of the chorion (Schoots et al., 1983; Roberts and White, 1992; Inohaya et al., 1999). A notable finding here is that the modification of chorion mechanical properties around the time of hatching (the pharyngula stage vs. the gastrula stage) was revealed by the measured penetration force differences.

Experiments were also conducted on lysed zebrafish embryos. Ten lysed embryos were prepared by heating embryos at the blastula stage in a petri dish at a temperature of 40 °C for 20 min and subsequently placing them at room temperature of 22–24 °C for 2 h. Blastula embryos are more uniform in morphology compared to embryos at the gastrula stage when asynchronous development becomes evident. In order

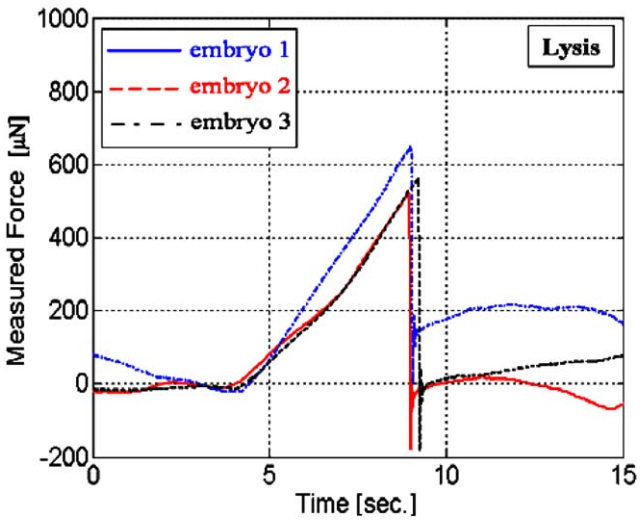


Fig. 7. Force sensing results on 3 lysed zebrafish embryos.

to make the protease secretion effect of lysed embryos more obvious, embryos were lysed at the blastula stage.

An average penetration force of 499 μN with a standard deviation of 100 μN (1δ) was measured in the lysed embryos that release cellular hydrolases including proteases when the embryonic cells were disrupted by heating. Three data sets out of 10 tested embryos (lysed) are shown (Fig. 7). Similar to the penetration force for pharyngula stage, the penetration force for lysed embryos was also found lower than the penetration force required at the gastrula stage. The lysed embryos have analogy to the pharyngula ones in the sense that both the chorion envelopes are affected by protease activities, with lysed embryos affected by abrupt release of protease from dying cells and pharyngula embryos affected by normal protease secretion (Inohaya et al., 1999; Denker, 2000).

In summary, this paper describes force sensing results on chorion envelopes of zebrafish embryos. In order to measure the chorion penetration forces, a microrobotic system with force sensing capabilities was developed. Cellular force sensing results on the chorion of the zebrafish embryos at various developmental stages demonstrate that lower penetration forces are required to penetrate the chorion of embryos at the pharyngula stage (523 μN) than at the gastrula stage (738 μN). Furthermore, it was also found that required penetration forces are lower for lysed embryos (499 μN) than those for embryos at the gastrula stage (738 μN). These results mechanically quantitate "chorion softening" in zebrafish embryos, which is due to protease activities subtly modifying the chorion structure.

Acknowledgements

This work was supported by the 21st Century's Frontier R&D Projects, under the Contract no. MS-02-324-01,

sponsored by the Ministry of Science and Technology, Korea. We thank C. Hwang for special contribution in obtaining the zebrafish embryos and H. Kang for the collaboration in developing the early experimental setup.

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