Real-Time Force Sensing in the Envelope of Zebrafish Egg during Micropipette Penetration

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Abstract: In biological cell manipulation, manual thrust or penetration of an injection pipette into an egg is currently performed by a skilled operator, relying only on visual feedback information. Massive load of various micro injection of either genes, fluid or cells in the postgenomic era calls a more reliable and automatic micro injection system that can test hundreds of genes or cell types at a single experiment. We initiated to study cellular force sensing in zebrafish eggs as the first step for the development of a more controllable micro injection system by any inexperienced operator. Zebrafish eggs at different developmental stages were collected and an integrated biomanipulation system was employed to measure cellular force during penetrating the egg envelope, the chorion. First of all, the biomanipulation system integrated with cellular force sensing instrument is implemented to measure the penetration force of cell membranes and characterize mechanical properties of zebrafish embryo cells. Furthermore, implementation of cellular force sensing system and calibration are presented. Finally, the cellular force sensing of penetrating cell membranes at each developmental stages was experimentally performed. The results demonstrated that the biomanipulation system with force sensing capability can measure cellular force at real-time while the injection operation is undergoing. The magnitude of the measured force was in the range of several hundreds of uN. The precise real-time measurement should provide the first step forwards for the development of an automatic and reliable injection system of various materials into biological cells.

Keywords: Biomanipulation, Cellular force sensing, PVDF sensor, Zebrafish embryo and Egg envelope.

1. INTRODUCTION

Biological cell manipulation becomes essential in the agricultural industry, and allows individual cell based diagnosis or pharmaceutical test [1]. In spite of great interest on analysis, diagnosis, and manipulation of single biological cells, however, most biomanipulation tasks such as gene or cell injections, intracytoplasmic sperm injection (ICSI) are usually carried out by experienced manual operators relying only on the visual information from the microscope. In case of such embryo cell manipulation tasks in biomedical applications, the operator should spend over a year to perform reliable cell manipulation.

Although egg and embryos are relatively uniform, and well-survive such manual manipulation, different performance occurring in a series of manipulation can cause serious damage by either the manual penetration or abrupt liquid flow. Due to these bad conditions, the cell manipulation tasks require much attention but the success rate is very low even by skillful operators. Recently, the physical characterization and microrobotic manipulation of individual embryo cells become a challenging issue in biomedical applications such as cloning, gene expression analysis, and cell replacement theraphy (CRT). Various cell injection systems have also been developed to provide more controllable manipulation of biological cells, such as incorporation of a piezoelectric actuator [2, 3, 4, 5] in biomedical applications. Sun and Nelson [6] suggested a visually servoed cell manipulation system where the embryo cell and the pipette end-point are identified using the visual information from the optical microscope.

However, researches on minimally invasive micropipette injection based on understanding the mechanical properties of the cellular membranes are rare [7, 8, 9, 10]. Accurate measurement of cellular forces is a requirement for minimally invasive cell injections. Moreover, the cellular force sensing is essential in understanding the biophysical properties of cell injury and membrane modeling studies. Mechanical cell injection with cellular force sensing can also improve success rates and be reproducible with precise control of pipette motion. For example, force sensing techniques of the resistance or rigidity of cellular envelope are very important for accurate and repetitive cell injection tasks, and consequently improves yield rate of embryonic cells after in-vitro fertilization (IVF). Furthermore, understanding elastic properties of the egg envelop in microrobotic cell injection is essential in designing control system for minimally invasive cell injection. Thus, biophysical analysis of the envelope of egg does not provide only a basis for the development of an improved injection system, but also an opportunity to examine physical properties of the egg envelope that underwent chemical modifications during fertilization and hatching for successful embryonic development, thus explaining clinical problems i.e., zona hardening, polyspermy, and implantation failure as in mammals.

In this paper, we present real-time measurement of cellular force in zebrafish eggs during penetrating egg envelope. The paper consists of 5 sections: In Section 2, the biomanipulation system integrated with a cellular force sensing instrument is briefly introduced. The preparation of zebrafish embryo cells for experiments is described in Section 3. In Section 4, experimental results of the cellular force sensing of penetrating cell membranes undergoing fertilization is presented to characterize mechanical properties of zebrafish embryos. Finally, conclusions are given in Section 5.

2. CELLULAR FORCE SENSING SYSTEM

2.1 Biomanipulation System

We have developed a robotic biomanipulation system with total 8-DOF mobility for characterizing mechanical properties

of cell membranes as well as autonomously manipulating individual biological cells. As shown in Fig. 1, it consists of two 3-DOF micromanipulators equipped with injection pipette and holding pipette respectively and a 2-DOF precision positioning system on the vibration isolation table. Fine movement needed for biomanipulation are implemented by a 3-DOF micromanipulator (model: MP–285, SHUTTER Inc.) with a travel of 25 mm and a resolution of 0.04um in each axis, which is integrated with holding pipette, and a 3-DOF micromanipulator (model: InjectMan NI2, Eppendorf) with a workspace of $20 \times 20 \times 18\ mm^3$ and a resolution of 2um, which is integrated with injection pipette. The 2-DOF precision positioning system (model: M-410DG, PI Inc.) has planar motions with a large workspace and a resolution of 8.5 um under the stereomicroscope and changes the field of view of optical vision system. The 8-DOF micromanipulation system with decomposition of manipulation DOF is controlled by PC-based control board. To get visual information on cell behaviors, we used a stereo microscope (model: MZ-12.5, Leica Inc.) with long working distance and mounted multiple CCD cameras. The microscope images are captured by a video frame grabber (model: Matrox Genesis, Matrox Electronic System Ltd.) and displayed and analyzed on the PC. Three CCD cameras are mounted on the top and side of the microscope and give cell images with three different magnification rates.

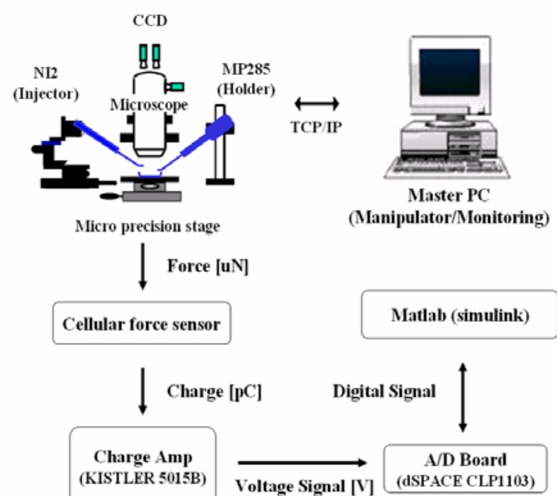

Fig. 1 Biomanipulation system integrated with cellular force sensing instrument.

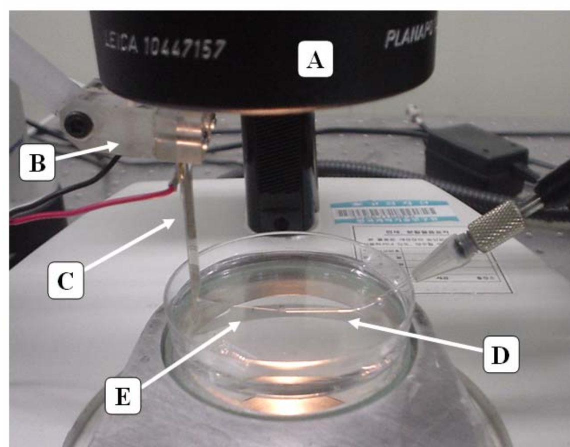

Fig. 2 Cellular force sensing system used in this study.

2.2 PVDF Sensor

We considered the PVDF (polyvinylidene fluoride) piezoelectric polymer film sensor as a force sensor to detect the cell injection force. The PVDF polymer sensor has desirable characteristics as a sensing material: high linearity, wide bandwidth, and high signal to noise (S/N) ratio, which indicates high reliability of the sensor output signal [11, 12, 13]. Fig. 2 shows a cellular force sensing instrument which has been fabricated for characterizing mechanical properties of cell membranes caused by micropipette injection. It has micro glass pipette, capillary holder, and PVDF force sensor. The microinjection pipette was bonded on the tip of the PVDF film (width: 28 um, Model: LDT1-028K of MSI Inc.). While, nickel (Ni) electrodes were deposited in both sides of the PVDF film to get electrical signals. As shown in Fig. 2, the PVDF sensor is clamped on the clamping fixture which is also mounted on the positioning micromanipulator.

The charge generated by the piezoelectric polymer (PVDF) sensor was amplified in the charge amplifier and output was measured through a data acquisition board (model: dSPACE 1103). Then, the output signals from the cellular force sensor were digitalized and filtered for the noise rejecting. In the filtering operation, the DC offset is rejected and the noise due to the AC power source and high frequency noise are eliminated by a low-pass filter (LPF) with the cutoff frequency of 20Hz. Finally, the obtained information of the cellular force sensor signal is displayed in user interface of personal computer.

2.3 Calibration

For the calibration of the PVDF sensor, the ultra-precision load cell (Model: GSO-10 of Transducer technology Inc., Max. measurement range: 100 mN, resolution: 10 uN) was used.

Fig. 3 shows schematics of experimental measurement setup for calibrating cellular force sensing system. A load cell determined the magnitude of the applied force. The calibration method was a comparison with a reference load cell (model: GSO-10, Transducer techniques; full scale: 100 mN, accuracy: 0.05mN). Fig. 4 shows that the output voltage from PVDF varies linearly with the applied force. The sensitivity of uniaxially oriented PVDF film depends on the direction of measurement. The PVDF is used in thickness mode. The calibration result proves good linearity of the PVDF sensor, where the calibration factor is about 196 uN/V.

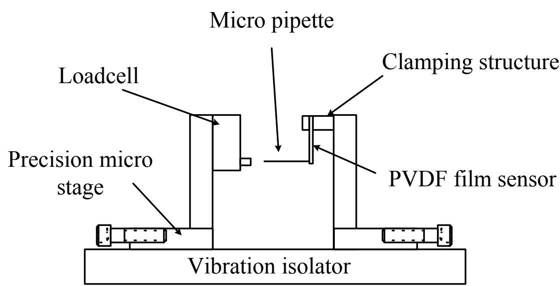

Fig. 3 Schematics of experimental calibration setup.

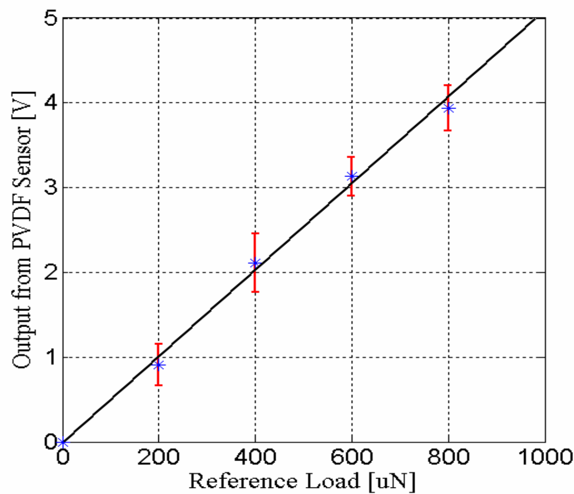

Fig. 4 Calibration result of cellular force sensing instrument.

3. ZEBRAFISH EMBRYO PREPARATION

To study developmental phenomena at cellular and molecular levels in animal model, we choose zebrafish. Recent biological work already showed that a newly initiated genetic approach in zebrafish is making significant contributions to understanding the development of the vertebrate heart, an organ that contains several vertebrate-specific features [14]. The embryo cells used in the experiments are zebrafish eggs, which is collected in accordance with the standard embryo preparation procedures. The zebrafish belongs to the vertebrate family. The zebrafish is receiving increasing attention as a model for vertebrate development [15]. Its two great advantages are its short life cycle of approximately 12 weeks, which makes genetic analysis so much easier; the transparency of the embryo, so that the fate of individual cells during development can be observed. The zebrafish egg is about 0.7 mm in diameter, with the cytoplasm and nucleus at the animal pole sitting upon a large mass of yolk.

Fig 5 shows structure of zebrafish egg envelop and the plasma membranes. The egg coat is called the chorion where our force sensing was detected experimentally. Underneath the envelope various chemical substances are filled upon fertilization, forming extracellular space, called the perivitelline space (PVS). All experiments were performed on zebrafish egg cells from 2 to 6 h after fertilization. The zebrafish embryo cell were placed on a petridish and observed under a stereomicroscope during the penetrating egg envelope.

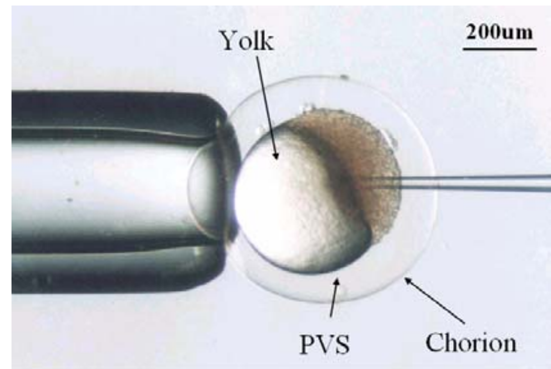

Fig. 5 Structure of zebrafish egg envelope, the plasma membranes, and the perivitelline space (PVS).

4. EXPERIMENTAL STUDIES

4.1 Experimental Conditions

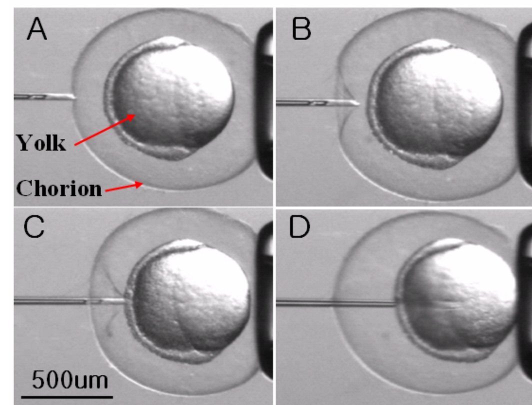

Fig. 6 Microinjection procedure of a zebrafish embryo cell.

Fig. 6 shows the whole microinjection procedure for the zebrafish embryo cell. (A) A glass pipette contacts the chorion, where the pipette is controlled by 3-DOF micromanipulator under a visual feedback. (B) When a point of pipette thrusts the chorion, the injection force begins to be proportionally increased. (C) The pipette thrusts into the outer membrane of yolk. (D) Finally, the pipette penetrates chorion. Most of the biological cell manipulation tasks including nucleus removal, nucleus transfer, and DNA injection follow this manipulation procedure.

The first barrier in the penetration into the egg cytoplasm is an extracellular envelope (the chorion) of the egg overlying the plasma membrane. The properties of the chorion is modifying during fertilization and peri-hatching stage.

In Fig. 7, the output voltage signal from the PVDF cellular force sensor that was used to measure the force of the probes as penetrating through the membrane of a zebrafish embryo cell. As indicated, force sensing was sharply changed during a controlled penetration of the micropipette at constant speed. About 600uN of force was recorded in the chorion penetration. Then the force sensing was dropped to the basal level, thus suggesting that no measurable force present in the PVS under the speed condition used in this experiment. Therefore, we used this condition in subsequent experiments.

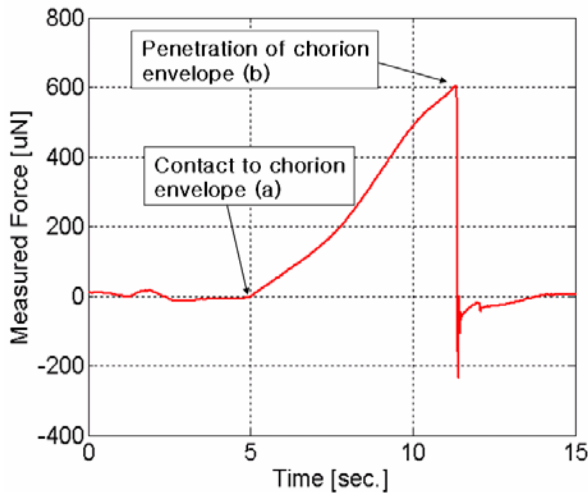

Fig. 7 The output signal of cellular force sensor when zebrafish embryo cell is thrust by injection pipette: (a) The starting point of penetrating chorion. (b) The ending point of penetrating chorion.

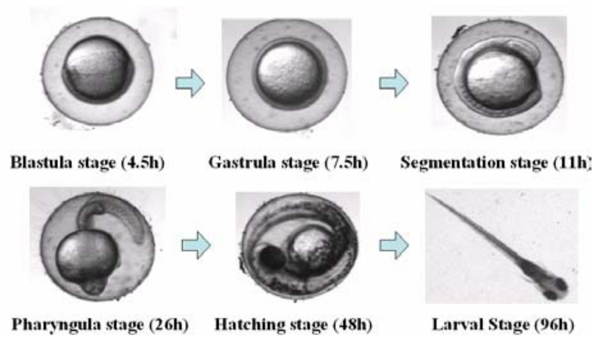

Fig.8 Early embryonic development in zebrafish.

Fig. 8 shows embryonic development stages of the zebrafish egg cell. Typical developmental stages are presented with time after fertilization. As shown, each stage is clearly discriminated due to the transparent cell structure that is largely composed of yolk and chorion and the diameter is about 700 um.

During gastrula period blastula embryos form three germ layers at about 7.5 h post-fertilization. When embryos develop further, thus forming morphology similar to early fry. These embryos are called pharyngula at about 26h post-fertilization, showing distinct movement within the chorion. The embryos are hatching at about 48h post-fertilization by dissolving the chorion envelope by secreting protease enzyme by the embryo itself. During protease secretion, the chorion becomes thinner, thus eventually making a hole that may enlarged by dynamic movement of the developing fry.

4.2 Experimental Results

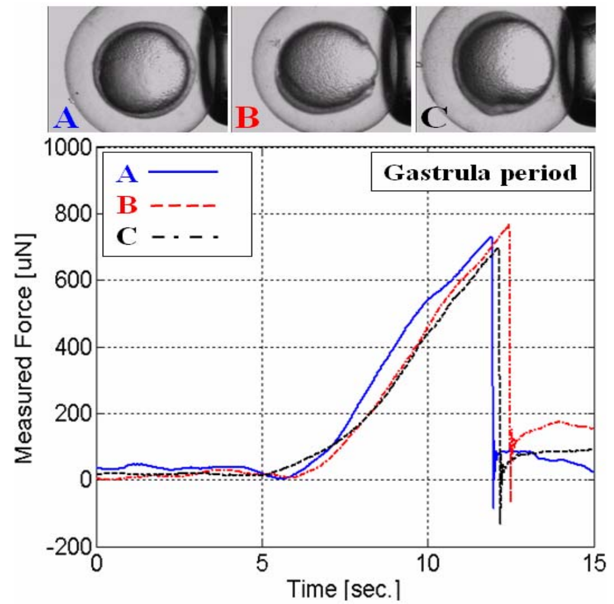

Fig. 9 Cellular force sensing of micropitte penetrating into the chorion envelope in gastrula embryos.

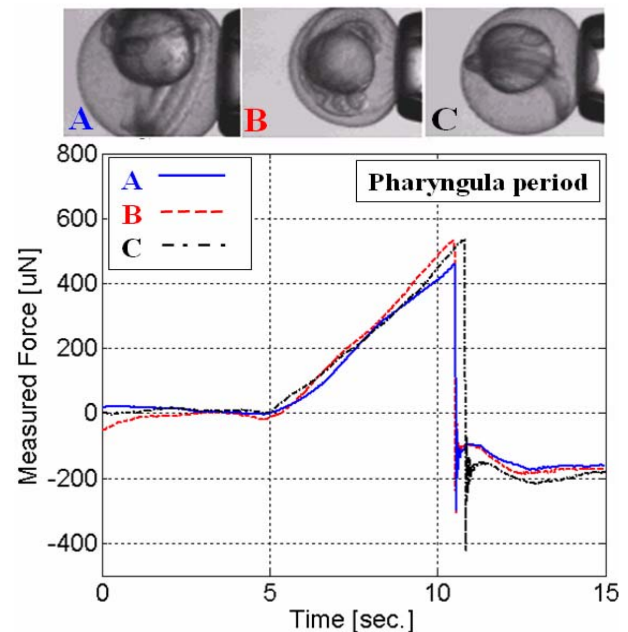

Fig. 10 Cellular force sensing of penetrating into the chorion envelope during pharyngula period.

The signal output in Fig. 9 and Fig. 10 show that the status of the zebrafish embryo cell can be distinguished by the output signal of cellular force sensor. All of embryo cell were stage of the gastrula period of approximately 7h and pharyngula period of approximately 26h after fertilization. In this experiment, the micro manipulator was used to move the injection pipette to approach the zebrafish embryo cell at 120um/sec. Once the injection pipette touched the zebrafish embryo cell, the sensing signal begins to be detected, and increases with regular slope.

Fig. 9 shows cellular force sensing of penetrating cell

membrane during gastrula period. Similar stage of embryos demonstrated relatively a similar force sensing in the three representative embryos as indicated, ranging from 700 to 800 uN.

Fig. 10 shows cellular force sensing of penetrating cell membrane during pharyngula period. As noticed in Fig 10, about 500uN force sensing was recorded in pharyngula embryos. The different values of force sensing among the data in Fig. 9 and Fig. 10 may indicate less force is required for the micropipette penetration in later stages of embryos. In fact, as described above protease secretion is occurring and this lead thinning of the chorion for the preparation of hatching during pharygula and pre-hatching stages. A similar thinning of the zona pellucida in mammalian blastocyst just before implantation is well described. This is an exciting finding that thinning of the extracellular coat can be biophysically measured.

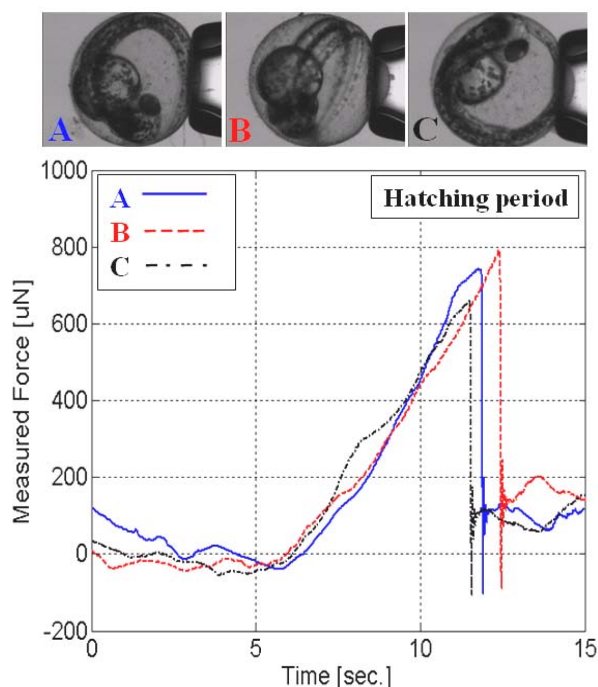

Fig. 11 Cellular force sensing of penetrating into the chorion envelope during hatching period.

Fig. 11 shows cellular force sensing of penetrating chorion envelope during hatching period. Due to dynamic movement of the embryos, it was not easy to measure the force in peri-hatching embryos. However, low force sensing is still detected as indicated in the Fig 11. This experiment should be further performed using drug that immobilize the embryo itself, but without any effect on the chorion.

Fig. 12 shows cellular force sensing of penetrating chorion envelope in lysed condition during gastrula period. It was also interesting to observe a similar force recording in lyses embryos in the line that the protease secretion leads to less force sensing in later stages of embryos as discussed earlier.

As shown in Fig 12, about 500uN was recorded in lysed embryos that release cellular hydrolases including proteases when the embryonic cell membranes are disrupted. These embryos have analogy to pre-hatching ones in the sense that the chorions of both embryos are affected by protease activities by normal protease secretion and abrupt release of protease from dying cells, respectively.

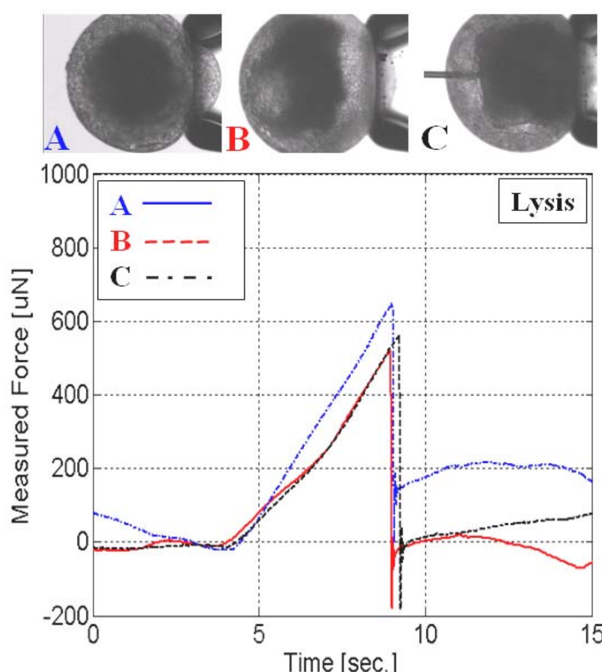

Fig. 12 Cellular force sensing of penetrating into chorion envelope in lyses condition.

5. CONCLUSIONS

This paper describes cellular force sensing in zebrafish eggs during penetrating egg envelope. In order to measure the penetration force of cell membranes and characterize mechanical properties of a zebrafish embryo, the biomanipulation system integrated with cellular force sensing instrument was developed. Furthermore, implementation of cellular force sensing system and calibration were presented. Finally, the cellular force sensing of penetrating egg envelope during each developmental stages was experimentally performed. The results demonstrated that the biomanipulation system with force sensing capability can measure cellular force at real-time while the injection operation is undergoing. The magnitude of the measured force needed for penetrating egg envelope was in the range of several hundreds of uN. The precise real-time measurement should provide the first step forwards for the development of the automatic and reliable injection system of various materials into biological cells.

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