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# 통합 자동화 세포 조작을 위한 마이크로 로보틱 서스펜디드 셀 인젝션 시스템

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# Micro Robotic Suspended Cell Injection System for Automatic Batch Bio-manipulation

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생물학적인 셀 인젝션 기술은 유전자 주입, 시험관 배양, 인공수정 및 신약개발 분야에서 광범위하게 사용되어 오고 있는 기술이다. 생물공학에서 다루는 셀 인젝션 기술은 크게 착생세포 인젝션과 서스펜디드 셀 인젝션으로 구분할 수 있다. 최근 상용화 장비로 출시되고 있는 것들은 착생세포에 대한 자동 인젝션 시스템이 대부분을 차지하고 있다. 반면, 서스펜디드 셀 인젝션 시스템의 경우는 비교적 최근들어 자동화 장비 및 방법론의 개발에 대한 논의가 이루어지고 있는 실정으로 실제 수많은 연구자들의 노력에 힘입어 서스펜디드 셀을 대상으로 한 통합 자동화 셀 인젝션 시스템의 개발이 가속화되고 있기는 하지만 이에 대한 만족할 만한 성과는 아직 이루어 지지 않고 있는 실정이다. 본 논문은 서스펜디드 셀을 대상으로 한 인젝션 시스템 개발에 관한 것으로 특히 셀 홀딩 시스템과 최적의 인젝션 피펫 궤적을 결정하기 위한 시스템 개발에 관한 것이다. 본 논문에서 다루어지는 서스펜디드 셀은 통합 자동화를 위하여 특별히 고안된 셀홀딩 시스템에 의하여 배열의 형태로 고정되며, 셀 인젝션 시스템은 엠브리오와 인젝션 피펫을 이미지 프로세싱 기술에 의하여 인식하고 피펫의 인젝션 궤적을 결정하는 것을 포함하고 있다.

**Keywords** : Injection, Biotechnology, Automation, Bio-manipulation, Suspended cell

## 1. INTRODUCTION

The ability to analyze individual cells rather than averaged properties over a population is a major step towards understanding the fundamental elements of biological systems. Studies on single cells are key components in the development of highly selective cell-based sensors, the identification of genes, bacterial synthesis of specific DNA and certain approaches to gene therapies. Treatments for severe male infertility and the production of transgenic organisms require that individual cells are isolated and individually injected. These recent advances in microbiology, as well as other sig-

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nificant research efforts, such as cloning, demonstrate that increasingly complex micromanipulation strategies for manipulating individual biological cells are greatly in demand.

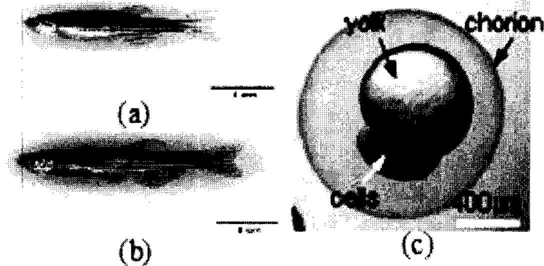
Several technologies exist for introducing foreign materials into a cell to analyze, such as viral vectors (Walther, 2000), electroporation (Rols, 2006), gene gun (Lin, 2000), ultrasonics (Sundaram, 2004), and Micro Electromechanical System (MEMS)-based injection (Hashmi, 1995; Chun, 1999). Compared to these techniques, microinjection with a single glass micropipette remains the most effective in terms of cell damage, cell viability and cell waste, as well as the effectiveness of delivering macromolecules, specificity and freedom from concerns about phenotype alteration. So since its invention during the first half of the last century, this technology has been widely applied in gene injection (Kuncova, 2004), in-vitro fertilization (Sun, 2002; Yu, 2001; Kim, 1997), intracytoplasmic sperm injection (Yanagida, 1998; Tan, 2002) and drug development (Nakayama, 1998). However, in order to enable fast, precise and robust screening for molecular targets, the manual injection must be replaced with a fully automated operation.

Automatic micromanipulation of single cell has been the focus of many research projects and commercial development for several years (Yamamoto, 2001; Arai, 1997; Kapoor, 2003; Mattos, 2007; Brufau, 2005). The main ideas of these test-bed or commercial systems is to transfer the manual injection procedures, either for adherent cells or suspended cells, into a fully automated flow. More recently, some novel techniques such as injection volume control (Zhang, 2005), high precision injector pipette motion control, cell sorting technology (Chen, 2004; Chen, 2005; Furlong, 2001), micro injection force sensor and sophisticated cell holder etc., have been studied in-depth.

This study developed the autonomous suspended cell injection system (AIS) for the purpose of injecting gene materials into batch zebrafish embryos to create transgenic organisms, as well as for other applications. The AIS mainly consists of three modules executive module, sensory module and control module. A specially designed suspended cell holding device is utilized by holding the zebrafish embryos in a cell array, while a rotary plate permits the suspended cells to be transported, one by one, into the injection site field of view. Through the cooperation of hardware and software, this autonomous suspended cell injection system can solve the suspended cell injection problem in a similar manner to the batch adherent cell manipulation and provides high rates, optimum reproducibility and accurate cell injection performance.

## 2. Injection Task

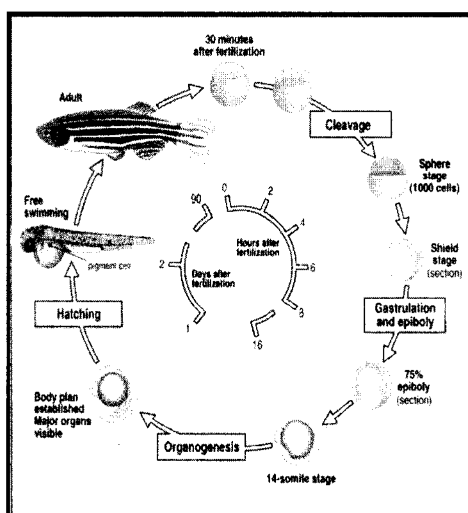
The experimental target cells used for the newly designed suspended cell injection system are zebrafish embryos, which were collected in accordance with the standard embryo preparation procedures. Zebrafish have been a model organism for developmental biologists for more than 20 years, and now zebrafish have also become a model for understanding cancer, neurobiology, infection and immunity, aging, congenital heart defects, tissue regeneration and genetic diseases of the muscle (Deiters, 2006). Most recently, zebrafish have been employed as a tool in the drug discovery proofs (Daters, 2006).




<Figure 1> (a) Female zebrafish ; (b) Male zebrafish ; (c) Structure of zebrafish egg

The zebrafish belongs to the vertebrate family and many of its genes are similar to those of humans. It is receiving increasing attention as a model for vertebrate development. The two main advantages of this animal species are its short life cycle (approximately 12 weeks), which makes genetic analysis much easier, and the transparency of the embryo so that the fate of individual cells during development can be observed. The zebrafish embryos are about 600～700㎛ or 1.15～1.25㎜ including chorion in diameter, with the cytoplasm and nucleus at the animal pole sitting upon a large mass of yolk. <Figure 1> (a) and <Figure 1> (b) show a female zebrafish and male zebrafish. <Figure 1> (c) shows the structure of a zebrafish egg envelope and plasma membranes. The egg coat is called the chorion. Underneath the envelope, various chemical substances are released upon fertilization, forming extra-cellular space, called the perivitelline space (PVS).

The zebrafish embryo grows rapidly. Two days after fertilization, the tiny fish, still attached to the remains of its yolk, hatches out of the egg. A zebrafish embryo development cycles is mainly composed of the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching periods. <Figure 2> shows a biological cycle for a zebrafish (Kimmel, 1995). Molecular and genetic analyses of zebrafish embryo genesis depend on the injection of foreign materials into early zebrafish embryos (Daiters, 2006). DNA injection is used to generate transgenic zebrafish lines, and mRNA injection is used to over-express gene-products in zebrafish embryos. Reverse genetic or loss-of-gene-function studies require the injection of antisense morpholino-modified oligonucleotides (morpholinos or MOs) to specifically inhibit RNA splicing and/or translation in vivo (Daiters, 2006). The gene materials should always be injected in a special position in the zebra fish embryos, such as the cell part or into the yolk during the early developmental stage (within six hours after fertilization). Despite their relatively large size, zebra fish embryos have a delicate structure and can be easily damaged, which makes automated, high-throughput injection difficult. Furthermore, for testing cellular responses to molecular targets and to obtain statistically significant data, the injection of thousands of cells needs to be conducted within a short time window (e.g., within one and one half hours after fertilization, before the 16-cell stage for zebra fish embryo injection). A high rate and accurate automatic cell injection system is needed.

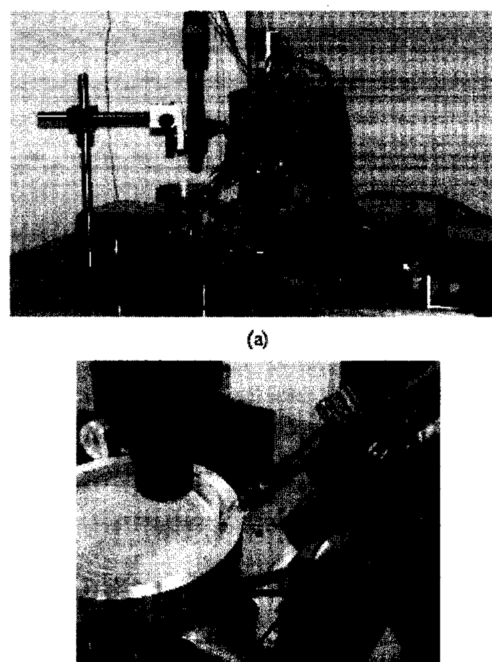



<Figure 2> Biological cycle for zebra fish (Kimmel, 1995)

In this study, all experiments were performed on zebrafish eggs within six hours after fertilization. The zebrafish embryos were placed on a specially designed cell holding device and observed under the microscope during the pipette penetrating through the embryos chorion and into the yolk. Combining some image processing techniques, the embryo structures can be identified more clearly and the effect of the gene injection could be improved, in accordance with earlier works by Sun (Wang, 2007a; 2007b).

## 3. Automatic Suspended Cell Injection System Design

<Figure 3> (a) illustrates the automatic suspended cell injection system developed in the laboratory for this research, and <Figure 3> (b) is a close-up view of the injection area. This system is designed to simulate automatic cell injection of large batches of suspended cells (such as fish embryos) in biological engineering processes. To achieve this purpose, the system is designed for the use of cells arrays instead of individual holding cells. A cell holding device, designed specifically for this purpose, is fixed on the actuated rotary plate, permitting batches of cells to be transported one by one, into the field of view of the microscope, for injection.

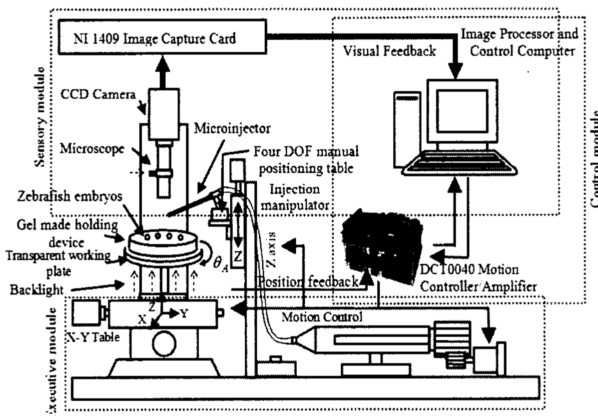



<Figure 3> Photographs of the new laboratory test-bed suspended cell injection system, (a) whole system setup and (b) close-up

The injection process is achieved with the injector pipette tilted out of the focal plane of the microscope, as shown in <Figure 3> (b). It is essential to carry out the insertion with the micro-pipette held at an angle of attack with respect to the horizontal plane in which the cells are held. If this was not the case, the injection pipette would simply brush the cells out of the holding devices, failing to pierce the cells at all.

<Figure 4> illustrates a schematic of the system, consisting of an executive module, a sensory module and a control module. These modules compose an automatic cell injection loop.

Vibration must be well controlled for embryo injection. Vibration not only causes difficulty in visually tracking features but could also produce permanent, even fatal, harm to the injection location and surrounding areas. To minimize vibration, all units, except the host computer and vacuum units, of the embryo system are mounted on a floating table.

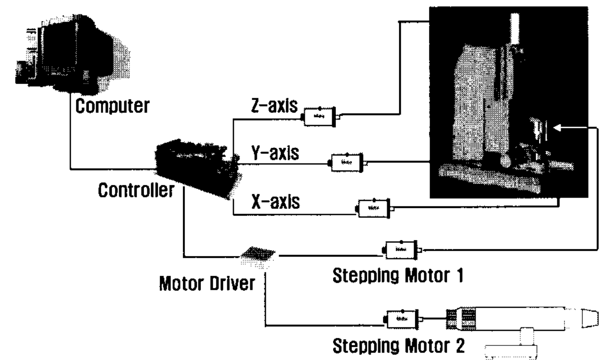



<Figure 4> Schematic of the cell injection system

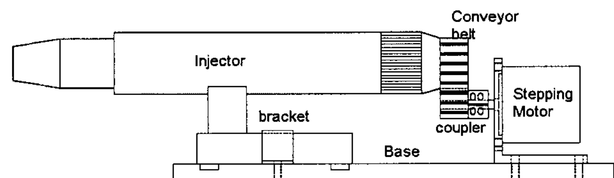
## 3.1 Executive Module

The executive module consists of an $X-Y-\theta$ positioning table and the injection manipulator mounted on the $Z$-axis, as shown in <Figure 5>. The cells to be injected are placed in a cell holder, to be described in detail subsequently, which is fixed to the rotary table, mounted on the $X-Y-\theta$ positioning table with three degrees of freedom (DOF). The center of the cell holder is coincident with the center of the rotation axis. Coordinated motion of the axes of the $X-Y-\theta$ table and the $Z$-axis injector is required to perform the cell injection task, as the pipette is held at an angle with respect to the plane of the cell holder. The $X-Y$ stage has a workspace of $60mm \times 60mm$, with a positioning accuracy of $0.3175\mu m$ in each axial direction. The $Z$-axis stage has a workspace of $50mm$, and a positional accuracy of $0.3175\mu m$. The injector pipette is oriented at a fixed angle $\phi$ with respect to the $X-Y$ plane. All $X$, $Y$, and $Z$ axes are driven by DC brushless motors with an embedded encoder to measure the position information. The injecting pose of the pipette can be precisely adjusted by the four DOF ($X-Y-\phi-\beta$) manual positioning table mounted on the $Z$-axis injection manipulator with a work space of ($13mm \times 13mm \times \pm 20^\circ \times 45^\circ$). The rotation axis $\theta$ is driven by a high precision stepping motor with an rotation accuracy of 0.045°.

A manual hydraulic CellTram vario (Eppendorf) micro-injector is driven by a stepper motor through a conveyor belt, permitting a minimum volume resolution of $2nl$ to be delivered into the cell, as shown in <Figure 6>.




<Figure 5> Configuration of executive module




<Figure 6> Configuration of a stepping motor driven micro-injector

## 3.2 Sensory Module

The sensory module mainly contains a vision system that includes four parts: optical microscope, CCD camera, PCI image capture and processing card, and an image processing computer. The Mitutoyo optical microscope has a working distance of $2cm$ to supply enough space for micromanipulation. The PULNIX CCD camera and the NI 1409 image

capture card have a high image capture frequency of 60$Hz$ to observe the cell injection process. The image process and micro-visual servoing control are performed on the host computer.

Biological cells are fairly transparent, and bright field illumination does not reveal differences in the brightness of the structural details, i.e., no contrast is apparent. The lighting system is improved to be suitable for observing transparent biotechnology objects. The working plate was made of glass and very thin chip LED back lights (model LDL-TP-27X27-SW) to provide illumination of the working area from behind, thus creating a silhouette image.

### 3.3 Control Module

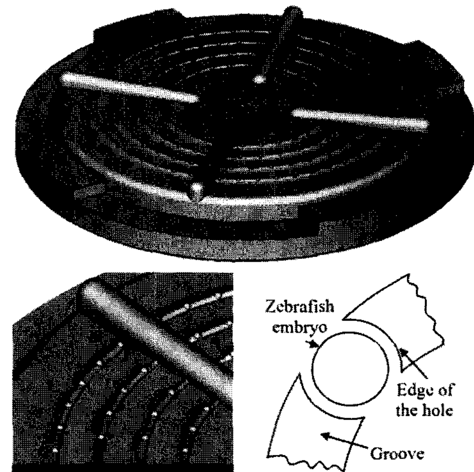
The control module consists of a host computer (P4 2.0 GHz) and a DCT0040 motion control/drive system provided by DynaCity Technology (HK) Ltd. The DynaCity motion control/drive system DCT0040 contains one control board and two driver boards. Each driver board can drive two motors (DC brushless or AC servo motor). The control module includes a DSP(Digital Signal Processing), a non-volatile FLASH memory, DCDC circuit and a circuit for RS232 and RS485 communications. Motion command processing, trajectory generation, position control and current control are performed by DSP on the control board. The stepping motor on the rotation axis $\theta$ is also driven by the DCT0040 through a specially designed stepping motor driver. This motion control system is relatively independent and does not occupy much of the control computer's running time.

The control module is a core module of the developed automated microinjection system. Specially designed software is running in the main computer to receive instructions from an operator, perform given tasks and finally show the results to the operator.

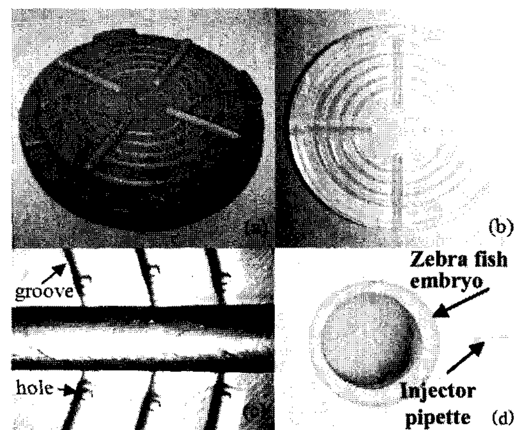
The whole system is executed according to the following procedures: The images are captured by the micro-vision system and sent to the image processing computer. The images will be processed in the controller to detect targets (zebrafish embryos) and tool (injector pipette) position. Then motion control algorithm will process the motion planning of the micromanipulation system. After the motion instructions are sent to the motion control/drive system (DCT0040), the co-operation of the transportation belt and the $Z$-axis injector is performed to complete the bio-manipulation tasks.

## 4. Specially Designed Suspended Cell Holding Device

Combining the merits of the two different types of cell holders, an improved suspended cell holding device was proposed, shown in <Figure 7>. A hole-array is embedded in circular profile grooves which are centered about the geometric center of the holder. This design has three advantages. First, the grooves make positioning the cells easier.




<Figure 7> CAD prototype of model for improved cell holding device

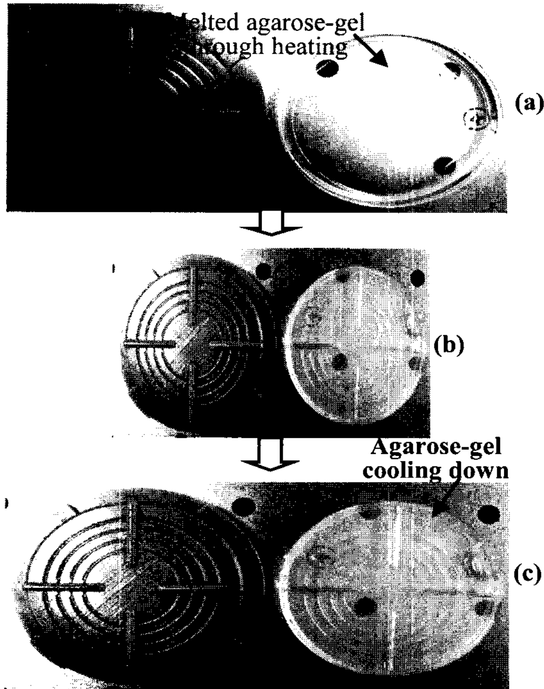



<Figure 8> Improved cell holding device, (a) mould ; (b) agarose-gel made cell holder ; (c) cell holding device under the microscope ; and (d) zebrafish embryo placing in cell holder

Second, the hole-array can immobilize the cells well in equal space and the cells will not move around and can be injected from all directions. Third, some fluid will be left

in the grooves during the injection period and the image quality can be improved, as shown in <Figure 8> (c) and <Figure 8> (d).

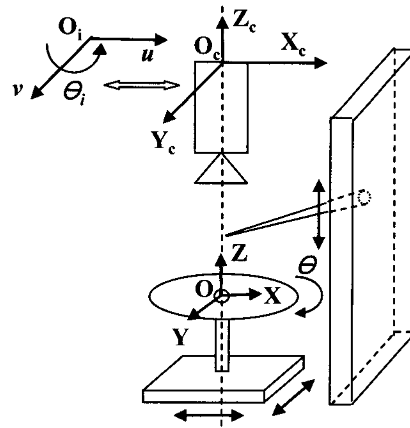
The cell holding device is fabricated from low-melting point agarose gel, a material commonly used in biological research. With soft, hydrous and transparent characteristics, agarose gel is well suited for fabricating the proposed cell holder. To make this cell holding device, the gel placed in Petri dish is made from 3.5% agarose by dissolving and heating it in a microwave oven. Next, a mould is pressed onto the gel surface and removed after the agarose-gel has cooled. Procedures to make this cell holding device are shown in <Figure 9>.




<Figure 9> Procedures to make cell holding device ; (a) dissolving and heating low-melting point agarose gel in a microwave oven ; (b) pressing the mould on the gel surface ; and (c) removing the mould after the agarose-gel has cooled

## 5. System Modeling of Micro-robotic Cell Injection System

<Figure 10> illustrates the configuration of the new cell injection system. It defines $O$-$XYZ$ as the coordinate frame whose origin $O$ is located at the center of the working plate. A microscope that is composed of a visual servoing system is mounted on a work table, as shown in <Figure 10>. The optical axis of the microscope coincides with the $Z$-axis. It then defines $O_c - X_cY_cZ_c$ as the camera coordinate frame, where $O_c$ is located at the center of the camera and $Z_c$ coincides with the optical axis of the microscope. Next, it defines $O_i - uv$ as the coordinate frame in the image plane, with three coordinates $u$, $v$ and the rotary angle $\theta_i$. The origin $O_i$ is located in the optical axis. The axes $u$ and $v$ are within the camera image's plane, perpendicular to the optical axis.




<Figure 10> Configuration of the new microinjection system

In a similar way to the microscope magnification model given in Zhao (2002), the relationship between the camera coordinates $[X_c, Y_c, Z_c, \theta_c]^T$ and the stage coordinates $[X, Y, Z, \theta]^T$ is given as :

$$\begin{bmatrix} X_c \\ Y_c \\ Z_c \\ \theta_c \end{bmatrix} = \begin{bmatrix} R & 0 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} X \\ Y \\ Z \\ \theta \end{bmatrix} + \begin{bmatrix} d \\ 0 \end{bmatrix} \tag{1}$$

$$R = \begin{bmatrix} \cos\alpha & \sin\alpha & 0 \\ -\sin\alpha & \cos\alpha & 0 \\ 0 & 0 & 1 \end{bmatrix} \in \mathbb{R}^{3\times 3}$$

where $R$ is the rotation matrix from the frame $O-XYZ$ to the frame $O_c - X_cY_cZ_c$, $\alpha$ is the angle between the two frames, and $d = [d_X, d_Y, d_Z]^T$ denotes the displacement between origins of the two frames. Since the two frames are fixed, both $R$ and $d$ are constant.

Then the relationship between coordinates in the image plane $[u, v, Z, \theta_i]^T$ and that in the camera frame $[X_c, Y_c, Z_c, \theta_c]^T$ is :

$$\begin{bmatrix} u \\ v \\ Z \\ \theta_i \end{bmatrix} = \begin{bmatrix} f_X\,0\,0\,0 \\ 0\,f_Y 0\,0 \\ 0\;\,0\,1\,0 \\ 0\;\,0\,0\,1 \end{bmatrix} \begin{bmatrix} X_c \\ Y_c \\ Z_c \\ \theta \end{bmatrix} - \begin{bmatrix} 0 \\ 0 \\ d_Z \\ 0 \end{bmatrix} \tag{2}$$

where $f_X = \lambda/\delta_u$ and $f_Y = \lambda/\delta_v$ are the display resolutions of the vision system in the two directions, $\lambda$ is the magnification factor of the microscope objective, $\delta_u$ and $\delta_v$ are the $u$-axis and $v$-axis intervals between CCD pixels. Note that in equation (2), the image coordinate vector is augmented from $3\times1$ to $4\times1$ by adding the coordinate $Z$ for easy processing later on.

Combining equations (1) and (2), the relationship between coordinates $[u, v, Z, \theta_i]^T$ and $[X, Y, Z, \theta]^T$ is given as :

$$\begin{aligned} \begin{bmatrix} u \\ v \\ Z \\ \theta_i \end{bmatrix} &= \begin{bmatrix} f_X\,0\;0\,0 \\ 0\,f_Y 0\,0 \\ 0\;\,0\;1\,0 \\ 0\;\,0\;0\,1 \end{bmatrix} \begin{bmatrix} R & 0 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} X \\ Y \\ Z \\ \theta \end{bmatrix} + \begin{bmatrix} f_X\,0\;0\,0 \\ 0\,f_Y 0\,0 \\ 0\;\,0\;1\,0 \\ 0\;\,0\;0\,1 \end{bmatrix} \begin{bmatrix} d \\ 0 \end{bmatrix} - \begin{bmatrix} 0 \\ 0 \\ d_Z \\ 0 \end{bmatrix} \\ &= \begin{bmatrix} f_X\cos\alpha & f_X\sin\alpha & 0 & 0 \\ -f_Y\sin\alpha & f_Y\cos\alpha & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} X \\ Y \\ Z \\ \theta \end{bmatrix} + \begin{bmatrix} f_X d_X \\ f_Y d_Y \\ 0 \\ 0 \end{bmatrix} \\ &= T \begin{bmatrix} X \\ Y \\ Z \\ \theta \end{bmatrix} + \begin{bmatrix} f_X d_X \\ f_Y \end{bmatrix} \end{aligned} \tag{3}$$

where $T \in \mathbb{R}^{4\times4}$ is the transformation matrix between the image frame and the stage frame. Since both frames are fixed, $T$ is time-invariant. $f_X$, $f_Y$, $d_X$, $d_Y$ are constant parameters.

Using Lagrange's equation of motion, the dynamics of the four DOF motion stages are given as:

$$\begin{bmatrix} m_X+m_Y+m_P & 0 & 0 & 0 \\ 0 & m_Y+m_P & 0 & 0 \\ 0 & 0 & m_Z & 0 \\ 0 & 0 & 0 & I_P \end{bmatrix} \begin{bmatrix} \ddot{X} \\ \ddot{Y} \\ \ddot{Z} \\ \ddot{\theta} \end{bmatrix} + N' \begin{bmatrix} \dot{X} \\ \dot{Y} \\ \dot{Z} \\ \dot{\theta} \end{bmatrix} + \begin{bmatrix} 0 \\ 0 \\ -m_Z g \\ 0 \end{bmatrix} = \tau - \begin{bmatrix} J^T f_e \\ 0 \end{bmatrix} \tag{4}$$

where $m_X$, $m_Y$ and $m_Z$ are masses of the $X$, $Y$ and $Z$ positioning tables, $m_P$ is the mass of the working plate, $I_P$ is the inertia of the rotational axis and the working plate, $N' \in \mathbb{R}^{4\times4}$ denotes a diagonal matrix of the positioning table that reflects damping and viscous friction effects, $[\tau_X, \tau_Y, \tau_Z, \tau_r]^T$ denotes the torque inputs to the driving motors, $J$ is the Jacobian matrix and $f_e = [f_{eX}, f_{eY}, f_{eZ}]^T$ is the external force applied to the actuators during the cell injection. During the cell injection process, the rotation axis $\theta$ does not change such that only $[f_{eX}, f_{eY}, f_{eZ}]^T$ are considered. Note that $f_e = 0$ if the injector does not contact the cells.

Define $q = [u, v, Z, \theta_i]^T$ as the generalized coordinate. Substituting equation (3) into (4) yields:

$$\begin{aligned} M\ddot{q} + N\dot{q} + G &= \tau - \begin{bmatrix} J^T f_e \\ 0 \end{bmatrix} \\ M &= \begin{bmatrix} m_X+m_Y+m_P & 0 & 0 & 0 \\ 0 & m_Y+m_P & 0 & 0 \\ 0 & 0 & m_Z & 0 \\ 0 & 0 & 0 & I_P \end{bmatrix} T^{-1} \\ G &= \begin{bmatrix} 0 \\ 0 \\ -m_Z g \\ 0 \end{bmatrix} \end{aligned} \tag{5}$$

where $M$ denotes the inertia matrix of the system, $N = N' T^{-1}$ represents the system damping and viscous friction effects, and $G$ is the gravitational force vector.

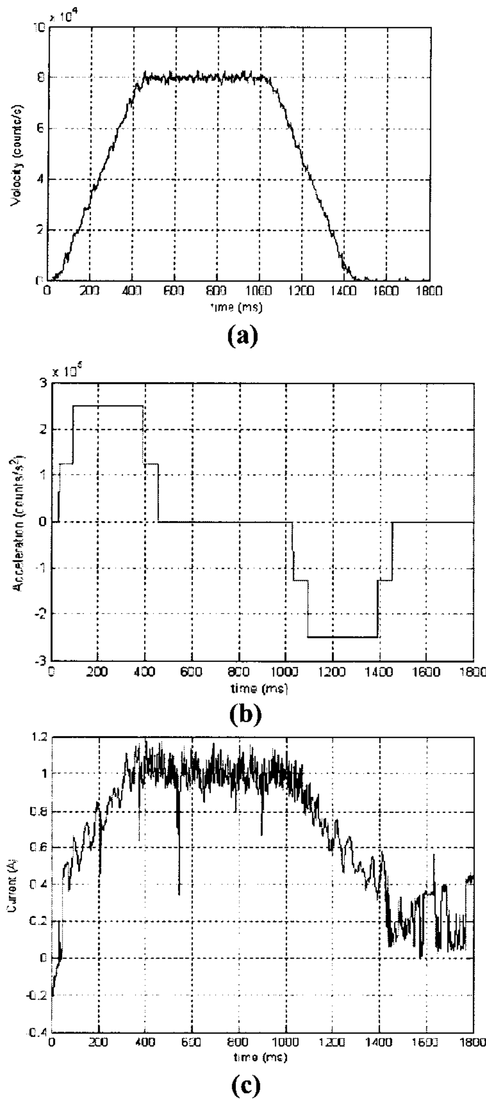
## 6. Experimental Identification of System Model Parameters

The modeling accuracy directly relates to the performance of the control system. The dynamic model of the cell injection system is shown in equation (5). The modeling parameters $M_{XYZ}$, $N_{XYZ}$, and $G_{XYZ}$ can be obtained experimentally through system identification.

The model parameters of three translational axes were obtained by conducting several computations with velocity, acceleration and the current of the axial motion. Velocity, acceleration and current, as shown in <Figure 11> (a), <Figure 11> (b) and <Figure 11> (c), were obtained by executing the linear motion for each axis and recording through the DCT0040 motion control system user interface. The motion parameters used in the test are displacement (10000 counts~90000 counts), velocity (10000 counts/s~90000 counts/s) and acceleration ($2.0\times10^5$counts/$s^2$). The accuracy of the each step by motors in x-y table is 0.3175μm.

As shown in <Figure 11>, the pippet will be injected for 450μm with positive acceleration, for 600μm with constant

speed and for 450µm negative speed. With these differences of acceleration, we can expect to improve the survival ratio of zebrafish embryos using procedure which is similar to the method of biologist.



<Figure 11> The output profiles of system parameters identifying experiments ; (a) Velocity profile ; (b) Acceleration profile ; and (c) Operation current

## 7. Conclusion

A robotic cell injection system for automatic batch injection of suspended cells is proposed. The proposed system utilizes cells arranged and held in circular arrays for insertion. A suspended cell holding device is designed and fixed on an actuated rotary plate, permitting suspended cells such as zebrafish embryos to be transported, one by one, into the injection site field of view. This newly developed cell injection system is composed of three modules and each part is optimized for bio-manipulation tasks. Then the kinematic and dynamic modeling of the cell injection system is developed. Experiments were performed to identify the modeling parameters of this micromanipulation system.

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