The 2009 IEEE/RSJ International Conference on
Intelligent Robots and Systems
October 11-15, 2009 St. Louis, USA

Self-Controlled Cell Selection and Loading System for & Microfluidic Systems

Huseyin Uvet, Akiyuki Hasegawa, Kenichi Ohara, Tomohito Takubo, Yasushi Mae, Tatsuo Arai

***Abstract*— In this paper, we presented a design for an automated cell supply system that can be used with complex microfluidic applications requiring single cell loading such as the current nuclear transplantation method. The aim of the system is to automatically transfer mammalian donor ($\sim$15μm) or egg ($\sim$100μm) cells one by one from a container to a PDMS micro-channel and then transport them to other modules. The system consists of two main parts; a single cell suction module, and a PDMS-based microfluidic chip controlled by an external pump. The desired number of vacuumed cells can be directed into the microfluidic chip and stored in a docking area. From the batch, they can be moved to next module by activating pneumatic pressure valves located on two sides of the chip.**

I. INTRODUCTION

Microfluidic technology and its applications are extensive and have made significant advantages over the past several decades[1]-[3]. Microfluidic-based technology offers a convenient platform for cellular analyses of biological systems, as the small scale of micro-channels and devices allows producing scalable system architecture [4]. Their inexpensive composition makes them a potential candidate for large scale production. Microfluidic technology covers not only the material phenomena but also the technology for manipulating and controlling the components as micro size particles in micro size artificial capillaries. Therefore, the integration of these technologies with micro robotic applications could be useful in the automation of cell manipulation for important areas such as single cell analysis, manipulation and treatment, including nuclear transplantation [5]-[8]. Integration of cell treatment steps is crucial to develop microfluidic devices for analysis of cell constituents, cell lysis and cell culture[9][10]. For example, experimental results show that microfluidic technology provides a significant advantage in the production of mammalian embryos. Besides these vital concepts, cell fusion and nuclear transplantation [11][12] are also important topics. Our project "Automated Nuclear Transplantation Using Micro Robotics" is our novel approach to find a solution to overcome difficulties in the nuclear transplantation process. In this project, several cell manipulation tasks such as positioning, cutting, sorting, filtering, and fusion are performed by different interconnected modules, by a so-called "Desktop Bioplant"[13]. This Desktop Bioplant, which includes micro channels and micro wells on a chip with appropriate sensors and actuators, is increasingly in demand for nuclear transplantation operations in biotechnology. Unfortunately, microfluidic methodologies have suffered from limited means to manipulate fluids and cells. In the case of the cell-fusion step of mammalian cloning, a microfluidic chip requires simultaneous control of the particles involved. In this process, two cells (a donor cell and an oocyte) are brought into very close contact and aligned via alternating current (AC), after which a direct current is applied for a brief period to complete the fusion [14][15]. Precise cell selection and transportation are essential when carrying out such an operation. In particular, single cell selection and transfer of target cells through a flow stream are of a great importance. Moreover, in-chip micro-injection [16] and single cell PCR [17] are other popular microfluidic methods which require manipulation of living cells one at a time.

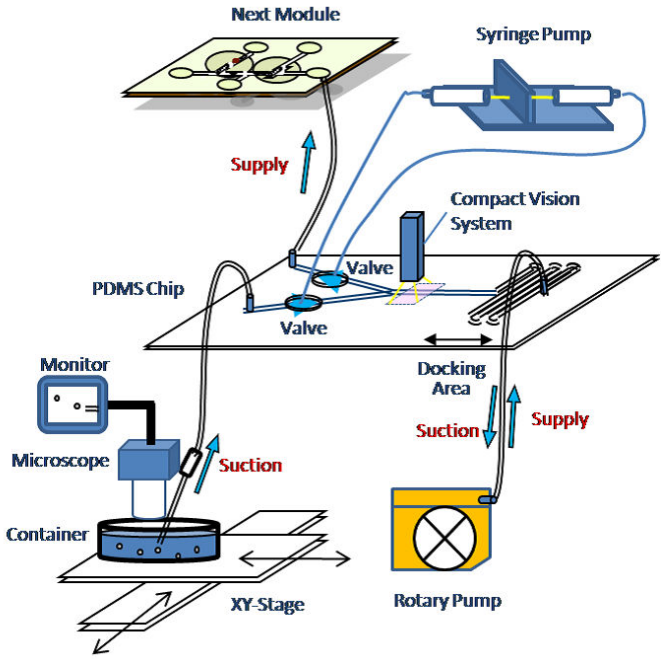

Fig. 1. Schematic view of the "Self-Controlled Cell Selection and Loading System"

To the best of our knowledge, there is no successfully implemented automated cell selection and loading system for microfluidic devices. The "Self-Controlled Cell Selection and Loading System" reported here performs the following steps; (1) supplies cells into a polydimethylsiloxane (PDMS) module one by one, (2) stores an aligned cell group in a dock, (3) transfers them to other modules. The entire system

This work is supported by the Bio-oriented Technology Research Advancement Institution under the Research and Development Program for New Bio-industry Initiatives.

Huseyin Uvet is with Department of Systems Innovation, Osaka University, Japan, ebn@arai-lab.sys.es.osaka-u.ac.jp

includes (a) micro manipulator with micro-glass pipette, (b) Three-layered PDMS microchip with pneumatic pressure valves controlled by external pumps and (c) monitoring systems. On top that, all parts are managed by a computer to provide full automation from beginning to end. Here, the pneumatic pressure valves are formed at the intersection of "Y" character micro-channels. When the external pump infuses air in the valves, the membrane between layers deflects, closing the fluidic flow. The pressure valves are digitally controlled in order to be programmable. According to the different parts and functions of the systems, this paper is divided into three main sections, (1) materials and methods, (2) experimental results (3) discussion and conclusion. In the first section, materials used to create the microfluidic chip and methods for preparing cells as well as a new design of compact vision structure are briefly described. Secondly, an automated cell detection/tracking and controlling algorithm is presented along with its applications in the micro-fluidic chips, micro-manipulators and pumps. In the final section, experimental results indicating efficiency and usability of the total mechanism are described.

II. MATERIALS AND METHODS

The proposed system is able to singly pick cells from a container and transport them to a microfluidic chip. The structure allows manipulating cells in microfluidic channels and docking them in desired locations at controllable numbers. However, due to the complex physical properties of egg and donor cells, manipulating cells through a microfluidic chip poses certain challenges. This system is comprised of the parts shown in Figure 1. External pumps, micro manipulators and camera systems are connected to a computer and run automatically based on a "Cell Detection/Tracking and Control" algorithm. All experiments were performed at room temperature and one microfluidic device was used several times, cleaning the device with water after every experiment.

A. Cell Types and Preparation

In this paper, we primarily focus on the cloning of somatic cells. Experiments were performed using fibroblasts as donor cells and egg cells as recipient cells. Egg cells were isolated from either cow or pig ovaries. Ova were cultured more than 24 hours after harvesting from the ovary. In order to detach cumulus cells from the harvested ova, cells were treated with hyaluronidase (Nacalai Tesque Inc.). The pellucid zone of ova was removed by pronase treatment and near-circular egg cells isolated. The isolated egg cell diameter was 100-150μm. Fibroblasts 10-30μm in diameter were isolated from female cow ears. The fibroblasts were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum (FBS) in 35 mm dishes in a 5% CO2 incubator. When cells became confluent, the culture medium was changed to 0.5% FBS-supplemented media. Fibroblasts were harvested from the dishes by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA).

B. Penicillin-Streptomycin and Bottom Surface Treatment

Fibroblasts readily adhere to the surface of plastic culture dishes. We detached fibroblasts by trypsin-EDTA treatment, then waited over 30 minutes and attempted to aspirate the fibroblasts. However, over time, detached cells re-adhere to the culture dish and cannot be aspirated. To avoid this problem, we attempted two different methods to treat the culture dish. One method is to paint the dish with a small amount of liquid PDMS (without the use of catalyst). This method is also known as siliconizing [18], ie. coating a surface with silicon. The other method is to pour PDMS (using a catalyst) and cover the dish with a thin solid PDMS layer. In the case of non-treated containers, less than half of the fibroblasts could be aspirated. On the other hand, the cells in the PDMS-coated dish were all easily aspirated. While the solid PDMS layer also aided fibroblast aspiration, there were irregularities in the PDMS surface, and additional time is also required.

C. Cell Suction System

In the acquisition process of a single cell, first of all, the pump (ISMATech Inc.) was connected to the dock side of the PDMS chip via a Teflon tube (0.5mm$\times$1.0mm). The main route starting from the dock side port (outlet port) to glass micro-glass tube was on the same line. Therefore, flow speed and flow direction in the micro-channel, as well as the speed on the tip of the micro-glass tube, were handled by this pump. During experiments, we recognized that small diameter tubes decrease the possibility of creating of cell stacks on the intersection point of the inlet port and the Teflon tube. Hence, the micro-glass tube and the cell delivery channel were connecting using another Teflon tube (0.3mm$\times$0.2mm). We used a micro-glass tube 0.30 mm outer diameter, 0.18 mm inner diameter as a suction mouth for the egg cells. A micro-glass tube can easily vacuum single cells without inflicting any damage. In addition, it has a noncomplex fabrication procedure that makes it easy to apply into common procedures. For the fibroblast, foregoing glass tube was heated to decrease its diameter (inner dia. $\sim$50μm). The tip of the micro-glass tube was processed by micro forge (MF-830, Narishige Inc.) and polishing machine (EG-44, Narishige Inc.).

D. The Manufacturing of the Microfluidic Chip

The microfluidic chip employed in this research has been designed to perform two main functions; aligning aspirated cells in docking area, and transferring them to next module. The molds of the fluidic chip (main-mold), valves (2nd mold) and air-chamber (3rd mold) were patterned on a silicon wafer with different heights, using SU8-based photolithography, which has been described elsewhere [19]. On the main mold, channels for delivering cells were 200 μm wide and 160 μm deep, dimensions sufficient to contain an egg cell. It has "Y" shape character and is completed with a snake-like dock which is shown in Figure 2. After preparation of the mold, the PDMS device and the valves were fabricated using classic multilayer soft lithography technique. The entire chip

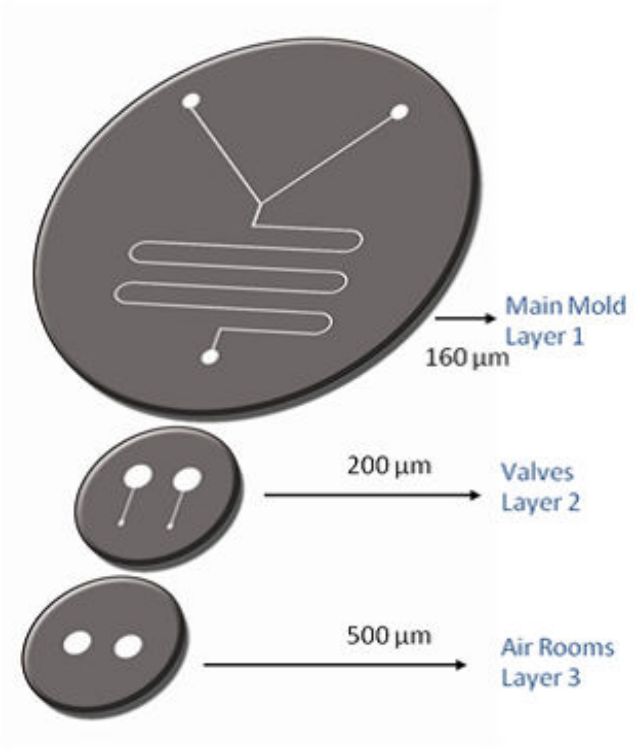

Fig. 2. Three positive relief molds were fabricated with different heights to form the main fluidic channels and valves. The molds were created with a single photolithography step.

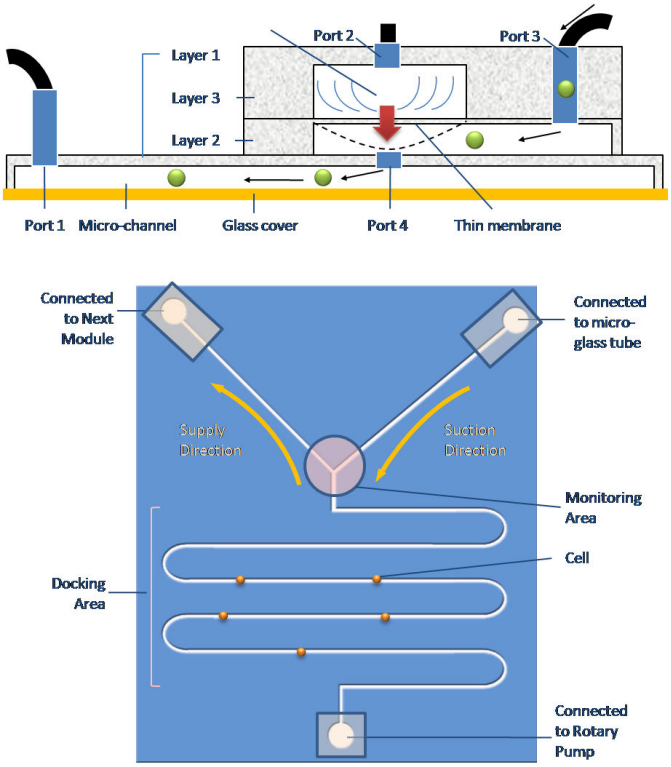

Fig. 3. Port 1 is connected to a rotary pump that controls flow speed and direction in the fluidic channel. A thin PDMS membrane is actuated with Port 2 connected to syringe pump. When the air-chamber is filled with air, it closes the gate (Port 4) for the fluidic channel and stops cell flow from the loading inlet (Port 3). The "Y" character channel controls supply suction and supply directions by switching valves.

is illustrated in Figure 3. Firstly, a thin layer of PDMS ($\sim$300μm), the first layer, was spin coated on the main mold, then cured for less than 20 min. at 100oC in an oven. The same method was repeated for the second layer, which also contains the valve. As incorrect placement of the second layer ($\sim$250μm), containing a thin membrane, may obstruct flow permanently, the first and second layers were carefully aligned together. The valve layer was completed by adding a PDMS slab containing an air-chamber (depth: 500μm). Then, the combined PDMS layers were treated by air plasma using expanded plasma cleaner for 1 minute together with a slide-glass rinsed in ethanol. The slide-glass was placed on the prosthesis and a pressure was applied until they firmly bound to each other. Finally, the total microfluidic chip was sealed with PDMS and cured a second time.

E. The Valve Control Principle

Within the PDMS chip, a valve region is pushed by applying air pressure to the air-chamber and obstructing the micro-channel. The air pressure control valves for "open" and "close" actions are activating by a micro-syringe pump (Kd-Scientific KDS270). The KDS pump links two valves so that one becomes "closed" if the other becomes "open". In addition, the control of the current direction in the micro-channel is handled by the rotary motion of a high precision tubing pump (ISMATech).

F. Vision Systems

Two camera systems were assigned to monitor two important sections of the entire mechanism. The first camera (Pointgrey Firefly camera) with microscope (Olympus Inc. ZX) was placed on the cell container in order to detect egg or donor cells position in a container. The second camera (Compact optical setup with Pointgrey Dragonfly camera) was boarded on the microfluidic chip with the purpose of actuating valves and changing flow direction. The compact vision system provides good image quality, allowing data on the oocyte cell and donor cell to be extracted from the acquired images [20]. In this second version, we changed the optical setup and designed a task specific system which has a 1.5 mm monitoring area and light source on the same side as the CMOS camera connection. It can be placed and aligned on a chip with the aid of xyz micro stages. As shown in Figure 4, the new system is small and can be easily combined with the microfluidic chips easily. This makes it possible to observe the cell container and microfluidic chip simultaneously in a short distance. Otherwise, we would have to use two commercial microscopes, which require a large workspace and the use of a long tube to connect the glass tube to the inlet port of the PDMS chip.

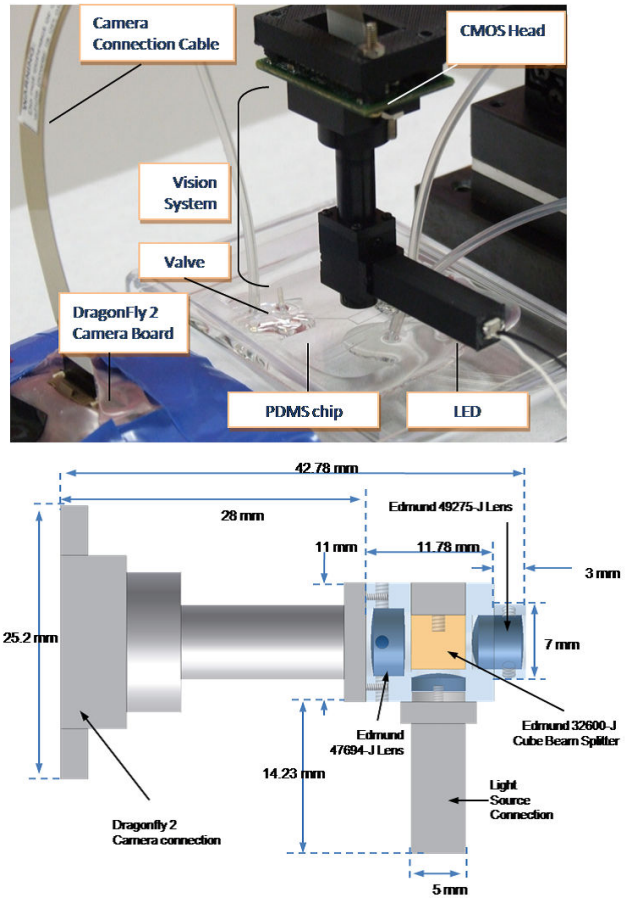

Fig. 4. Compact Vision system design

G. Cell Detection/Tracking and Control

The detection/tracking and control algorithm was mainly programmed for single cell applications. Its execution steps can be described as follows:

a) Start the first camera and detect the position of single cells in the cell container.
b) Align the glass tube to the cell position and begin suction (ISMATech pump).
c) Toggle to the second camera and count the number of cells that pass the cross section in the PDMS chip.
d) If the desired number of cells passes the cross point, switch valves and let the cells flow to the next module.
e) Switch valves and cameras if a second group of cells is required.

A background subtraction algorithm was employed throughout the detection phase in order to eliminate redundant artifacts and to surpass optics-based aberrations. The background subtraction method was essentially applied to moving regions, and the object positions were automatically found after input images were compared with a background image. In this way, it is also possible to detect and track multiple objects. After the edge is identified, the algorithm makes a circular approximation to the edge of the object and draws a circle around it, which is taken as the diameter of the object. The vision system was calibrated by calculating the

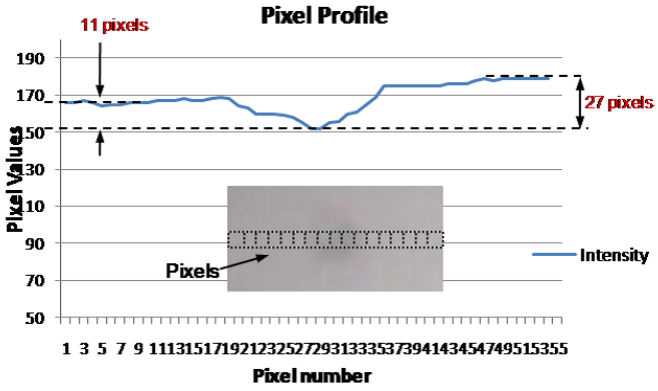

Fig. 5. Pixel intensity changes according to pixel number to extract donor cell from background. Minimum intensity change is used as default threshold value.

ratio of the field of view and sensor size with the aid of an Olympus Ronchi Ruling glass (100 lp/mm). We essentially applied same idea to the two camera systems, but cells are only floating in the container viewed by the microscopic camera. On the other hand, for the compact vision system, we consider the boundary of any moving region as an edge of the object, because cells always come into the observation area one by one. However, the program was plagued by some environmental problems such as bubbles and other unwanted objects which appear similar to the donor cell. As egg cells are $\sim$10 times bigger than donor cells, requiring a deep channel depth (150μm), the donor cells can easily go out of focus range and the image become blurry. Furthermore, the donor cell is very transparent under visible light. Even though its shape is near circular, because of the light conditions, in most cases, it is complicated to extract its features from the background (Figure 5). After entering the micro-channel, according to light dispersion over the donor cell, high density parts become darker, while bright areas frequently converge to background color. Whereas the system could often capture the edges of the donor cell through consecutive frames, in some cases, for the reasons explained above, the system could not locate the edges of the donor cell.

III. RESULTS

A. Egg Cell and Fibroblast Suction

We used egg and fibroblast cells as shown in Figure 6. After a number of treatments explained in the previous section, the egg and the donor cells dispersed randomly manner in different containers. We program an initial desired number of cells for suction and it searches for the cells in the container. As soon as the program detects a cell or a cell group, it takes the position of the cells on the screen view, draws circles around each detected cell, and displays their positional information (Figure 6). The suction mouth of the micro glass-tube aligns with the nearest cell on the bottom surface, which is numbered as “0”, and then the ISMATech pump starts flow. Once the suction of the cell numbered “0” is completed, the suction mouth returns to the

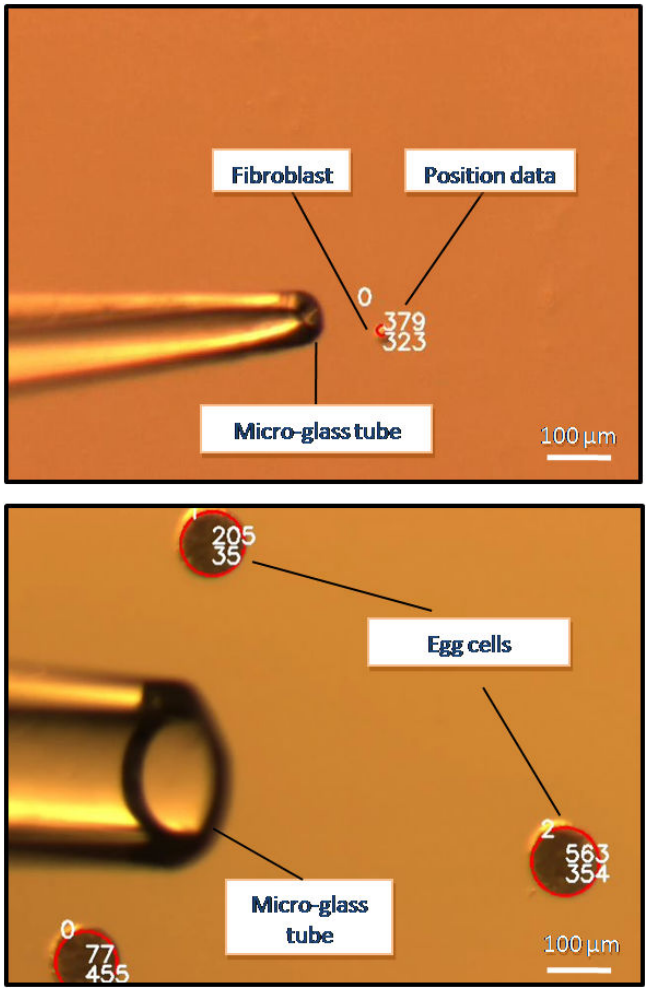

Fig. 6. Fibroblast and egg-cell suction from a container. The tip size of the glass tube for the fibroblast is approximately 50μm, and for the egg-cell approximately 180μm. The detection algorithm locates the cells and aligns the micro-glass tube with them.

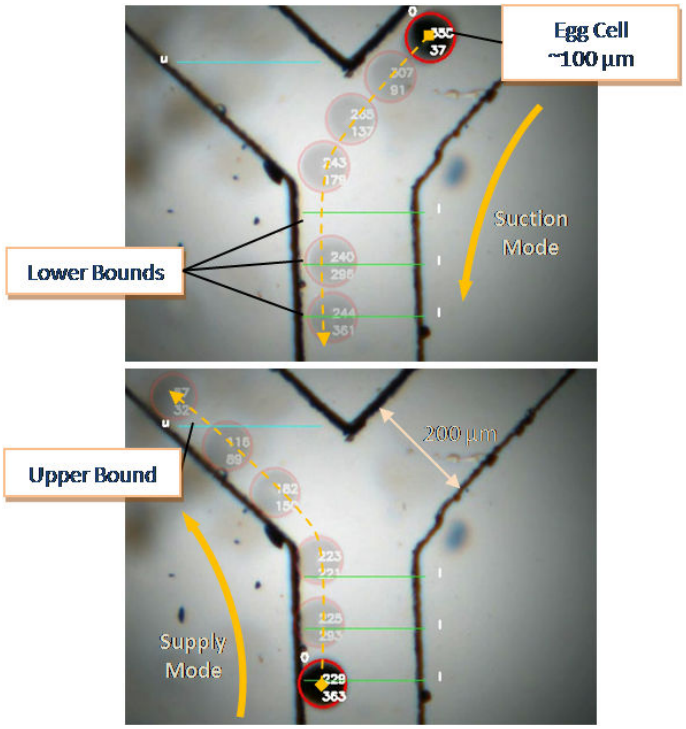

Fig. 7. Experimental result for tracking and controlling a single egg cell in the microfluidic chip. The images were taken by the compact vision system. Dashed lines with transparent egg cell figures for both images show trajectories in case of suction and supply modes.

original starting point, takes off from the surface to "safe height" and carries on suction of the medium without cells. After 15 seconds (an experimentally calculated delay used in order to put a specific distance between each cell) the suction mouth returns to the bottom surface and moves to next cell, numbered as "1". This procedure repeats until the last cell is vacuumed from the screen view. If the program reaches the total number of desired cells, it stops searching, moves to the "safe height" and continues with the suction, even if there are still some cells on the screen.

B. Direction Control

Once the aspiration of the desired number of cells is completed, the camera system on the cell container is toggled to the compact vision system placed on the cross section of the "Y" character micro-channels (Figure 7). The task of the compact system is, by switching the valves, to ensure that the collected cells are gathered at the dock and transported to the next module. When a new cell shows up from the upper-right hand corner, the program detects cells and starts tacking. To avoid environmental failure in the PDMS chip and to maintain efficiency, four control parameters are added to the "Cell Detection/Tracking and Control" program. These parameters are shown on the screen as sequential lines. First three lines (green color) are for confirmation of that a detected cell safely passes to dock side (Figure 7). The second blue line, the upper-left hand side, counts the number of cell transferred. In the beginning, three green and one blue parameters toggle as "false". As soon as the center of the detected circular area of the cell passes a single line, it toggles to "true". After it passes three green lines in order, the program counts this as one cell and waits for second cell. When the desired total number of cells is reached, cells in the dock are transported to the next module. Each time a single cell passes the blue line this is recorded for the final confirmation. To start cell transfer to the next module, the system runs as follows:

a) ISMATech rotary pump connected to the end of the "Y" shape channel stops flows.
b) KDS syringe pump applies pressure to the right pneumatic valve (infusion) while releasing the left valves (withdraw). In experiments, time elapsed during valve switching was measured to be approximately three seconds.
c) The main flow direction is reversed.
d) ISMATech pump starts running again and transfers cells in the batch to the micro-channel on the left.

IV. DISCUSSION AND CONCLUSION

The system presented here allows individual cells to be aspirated from a container and transferred to other modules. The components, comprising of pumps and micro manipulators, were connected to a computer that automatically controlled all functions carried out in the microfluidic device. The pneumatic pressure valves were successfully applied onto the fluidic chip. We also demonstrated a potential application of this system, the automation of nuclear transplantation, valves were successfully applied onto the fluidic chip. We also demonstrated a potential application of this system, the automation of nuclear transplantation, by singly transporting egg and donor cells. Cell coupling for nuclear transplantation can be automated by microfluidic devices. The detection method developed for this study is a simple and robust technique well-suited for microfluidic systems. Experimental results showed that the synchronized camera modules together with this algorithm were able to handle pumps and micro-manipulators simultaneously. The performance of the algorithm was tested with different tasks. Data analyzed from the images was used to control the motion of the particles in the channels. This device is by no means a final system for nuclear transplantation. Its efficiency may be increased by including "automated cell fusion" functions within networks of micro-channels. Future research will focus on improving the algorithm and control, with the goal of executing simultaneous donor and egg cell manipulation in different fluidic chips before fusing them in a microfluidic chip.

V. ACKNOWLEDGMENTS

The authors would like to thank Prof. Fuhimoto Arai and Prof. Yoko Yamanishi (Tohoku University) for their technical assistance and useful discussions and David Bennett for editing of the manuscript.

REFERENCES

[1] T. T. Vilkner, D. Janasek and A. Manz, "Micro total analysis systems. Recent developments", Anal. Chem., Vol. 76, pp. 3373–3386, 2004.

[2] A. Unger, H. P. Chou, T. Thorsen, A. Scherer and S. R. Quake, "Monolithic microfabricated valves and pumps by multilayer soft lithography", Science, Vol. 288, pp. 113–116, 2000.

[3] E. W. H. Jager, O. Inganas and I. Lundstrom, "Microrobots for micrometer-size objects in aqueous media: potential tools for single-cell manipulation", Science, Vol. 288, pp. 2335–2338, 2000.

[4] G.M. Walker, H.C. Zeringue, D.J. Beebe, "Microenvironment design considerations for cellular scale studies", Lab Chip, Vol. 4, pp. 91–97, 2004.

[5] Ramesham, R., Ghaffarian, R., "Challenges in interconnection and packaging of microelectromechanical systems (MEMS) Electronic", Components and Technology Conference 50th, pp. 666–675, 2000.

[6] YC Lee, BA Parviz, JA Chiou, S Chen, "Packaging for microelectromechanical and nanoelectromechanical systems", IEEE Trans. on Advanced Packaging, Vol. 26, No. 3, pp 217–226, 2003.

[7] Y. Cui, Q. Wei, H. Park C. M. Lieber, "Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species", Science, Vol. 293, pp. 1289–1292, 2001.

[8] M. A. Schwarz and P. C. Hauser, "Recent developments in detection methods for microfabricated analytical devices", Lab Chip, Vol. 1, pp. 1–6, 2001.

[9] Scho#holzer, F., D. Hahn, B. Zarda, and J. Zeyer, "Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of Lubricus terrestris", L. J. Microbiol. Methods, Vol. 48, pp. 53–68, 2002.

[10] Elfwing, A., Y. LeMarc, J. Baranyi, and A. Ballagi., "Observing growth and division of large numbers of individual bacteria by image analysis.", Appl. Environ. Microbiol., Vol. 70, pp. 675–678, 2004.

[11] Changqing Yia, Cheuk-Wing Lia, Shenglin Jia and Mengsu Yang, "Microfluidics technology for manipulation and analysis of biological cells.", Analytica Chimica Acta, 2006. 560(1/2): p. 1-23. 3.

[12] Alison M Skelley, Oktay Kirak, Heikyung Suh, Rudolf Jaenisch, Joel Voldman, "Microfluidic control of cell pairing and fusion", Nature Methods Vol. 6, pp. 147–152, 2009.

[13] Tatsuo Arai, et. al, "Automated Embryonic Cell Manipulation Using Micro Robotics Technology", International Conference on Intelligent Robots and Systems, Workshop(IEEE IROS 2007), 2007.

[14] M. P. MacDonald, G. C. Spalding and K. Dholakia, "Microfluidic sorting in an optical lattice", Nature, Vol. 426, pp. 421–424, 2003.

[15] Glenn M. Walker, Henry C. Zeringue and David J. Beebe, "Microenvironment design considerations for cellular scale studies", Lab Chip, Vol. 4, pp. 91–97, 2004.

[16] Andrea Adamo and Klavs F., "Microfluidic based single cell microinjection", Lab Chip, Vol. 8, pp. 1258–1261, 2008.

[17] Nicholas M. Torielloa, Erik S. Douglasa, Numrin Thaitrongb, Sonny C. Hsiaob, Matthew B. Francis, Carolyn R. Bertozzib and Richard A. Mathiesa, "Integrated microfluidic bioprocessor for single-cell gene expression analysis", PNAS, No. 105, Vol. 51, pp. 20173–20178, 2008.

[18] Gunnar P. H. Dietz, Mathias Bahr, "Synthesis of Cell-Penetrating Peptides and Their Application in Neurobiology", Methods in Molecular Biology, ISBN: 978-1-58829-666-5, 2008.

[19] Zhang J., Tan K. L., Gong H. Q., "Characterization of the polymerization of SU-8 photoresist and its applications in micro-electro-mechanical systems (MEMS)", Polymer testing, Vol. 20, No6, pp. 693–701, 2001.

[20] Huseyin Uvet, Tatsuo Arai, Yasushi Mae, Tomohito Takubo, Masato Yamada, "Miniaturized Vision System for Microfluidic Devices", Advanced Robotics, Vol. 22 No. 11, pp. 1207–1223, 2008.