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INJECTION AND CUTTING METHODS OF ANIMAL CELLS USING A MICROFLUIDIC CHIP

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Abstract— **We have developed a novel technique for injecting and cutting animal cells for an automatic cloning system. Animal cells that have been chemically softened are aspirated into a narrow teflon tube, which is then inserted into a microfluidic chip. The tube's tip is inserted into a microchannel and releases the aspirated cells in the microchannel. The microfluidic chip contains two orthogonal channels. One is a narrow microchannel that aspirates and fixes a cell; in the other channel, a fluid flows at high velocity for cutting the aspirated cell. The thickness of the aspiration channel can be adjusted to fix the aspirated cell. Half the volume of the cell is aspirated into the narrow microchannel and high-speed flow is then generated to cut the cell into two. In this report, the cutting method and the injection method are described. Then an imaging process is also investigated for an automatic cutting.**

I. INTRODUCTION

Recently, a research of cloning is actively investigated. It is expected that the cloning technology is useful for food problem, an organ transplant or making of the laboratory animal that has the same gene. Especially, regenerate medicine makes a major progress by development of "iPS Cell"[1][2] so stable supply of big apes which are used for clinical implantation is very important to solve the problem of the upkeep cost of large animals. Therefore, to improve productivity, an automatic cloning system is required to meet the strong demand.

Wakayama et. al. developed a cloning method of mice from enucleated oocytes injected with cumulus cell nuclei[3]. The cloning a cell involves: (1) cutting the cell and extracting the nucleus from the cell, (2) combining the cloned nucleus and the rest of the cell, (3) providing an electrical stimulus to fuse the two components together. The focus of this study is cutting the cell. In an earlier study of embryo manipulation, the nucleus of a cow's egg was removed and it was replaced with a modified nucleus of another cell; this procedure was performed with manual operation of human hand and mechanical manipulator[4]. However, it requires a lot of time and effort on the part of the operator and a system to work easily is requested. On the other hand, we researched micromanipulation with miclofluidic chip and some micromanipulation technique[5]-[8]. Therefore, we propose a new method for removing the nucleus an oocyte using these technique. Then, the success rate for injecting cells into a microfluidic chip is not high since there are air bubbles attached to the connector between the tube and the chip and cells become trapped in these bubbles. In this paper, we also report a tube-chip connector that is suitable for injecting and cutting cells.

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II. INJECTION AND CUTTING METHODS

As for manipulate the bovine oocyte, dielectrophoresis[5], high electric field[9], laser manipulation[8] and mechanical cutting[4] are thought as the cutting method but the dielectrophoretic force and laser manipulation force are week for the cutting. Using the high electric field, it is difficult to cut it without destroying the cell membrane. The mechanical cutting is normally used. On the other hand, liquid current is strong in the micro scale. Firstly we classify the cutting method, which is used blade and flow as shown in figure 1.

A sharp blade "BIO-CUT(FEATHER CO.,Ltd.)" is used for the cell cutting in the standard[1]. Bovine oocytes chemically softened are put on a plastic plate and cut by the blade. However, it is difficult to automate this process and to speed it up. The cutting power of the blade is too strong, so the cell is destroyed by a little operation mistake. Therefore, a highly accurate positional control of the blade is needed. Moreover, about the image data processing to automate, the cells are overlapped with the blade so the recognition of the cells and the blade are difficult. On the other hand, the calibration of the installation angle of the blade is needed.

Wire cutting and pillar cutting are suitable for automatic cutting because it works by the batch processing without vision sensing. However, if a hard cell is blocked in the space,

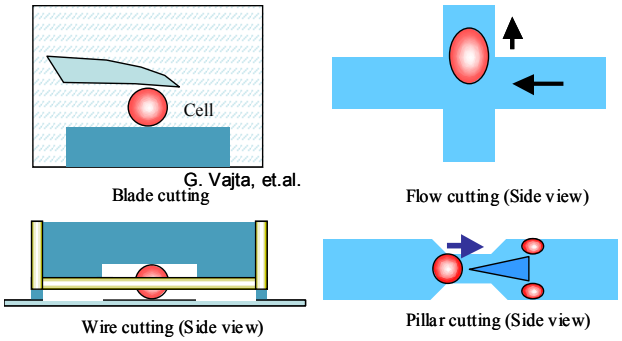

Fig.1 Classification of cutting method

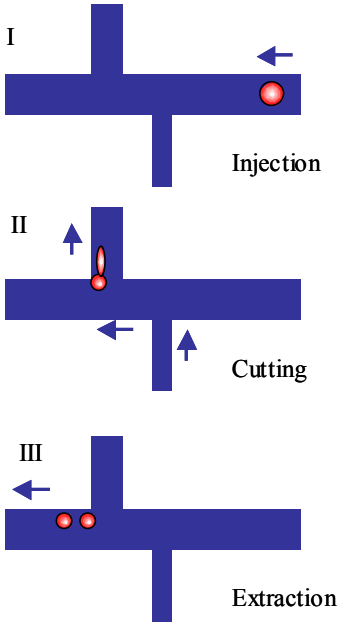

Fig. 2 Flow cutting process

the other softened cells are damaged. After all, to solve this problem, the vision is needed.

Flow cutting is performed using narrow pipette. A half size oocyte is aspirated in a pipette, and snap the pipette to add a momentary vibration. We propose a new cell cutting method using microfluidic chip. Microfluidic chip and the solution are transparent so observation is easy.

A. Cell cutting method

Figure 2 shows a process on flow cutting. Fluidic force is suitable for the cell cutting. The cells that have been chemically softened are injected in a narrow microchannel which is fabricated in a microfluidic chip. The microfluidic chip contains two orthogonal channels. One is a narrow microchannel that aspirates and fixes a cell; in the other channel, a fluid flows at high velocity for cutting the aspirated cell. The thickness of the aspiration channel can be adjusted to fix the aspirated cell. Half the volume of the cell is aspirated into the narrow microchannel and high-speed flow is then generated to cut the cell into two.

B. Cell injection method

To use microfluidic chip, an injectionmethod is needed because a bovine oocyte, which the diameter is about 100μm, is transported through a microtube with a large amount of liquid compared with the cell. Then we propose a new injection method using narrow teflon tube. Cells that have been chemically softened are aspirated into a narrow teflon tube, which is then inserted into a microfluidic chip through a silicon tube connected the chip. The tube's tip is inserted into a microchannel and releases the aspirated cells in the microchannel.

III. SYSTEM

Figure 3 shows a schematic diagram of the microfluidic chip and the teflon tube pipette. The microfluidic chip is made from polydimethylsiloxane (PDMS) using a molding method[10]. A glass plate, which the thickness is 150μm, is bonded. The PDMS has a property to bond a glass. It first punctured in the punch of 1mm to make Injection port, and the hole and the tube, which the inside diameter is 1mm, were fixed through the axle, and the space was buried to fixed the tube with PDMS. The cells injected from the injection port are

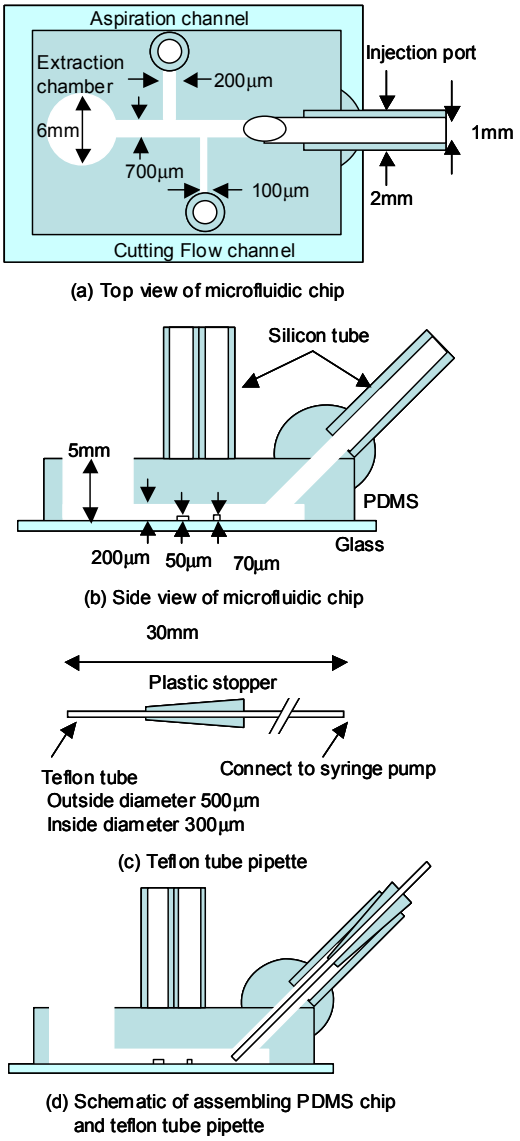

Fig.3 Schematic diagrm of microfluidic chip

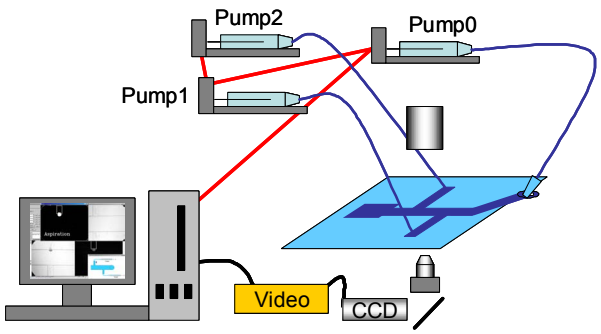

Fig.4 Over view of the system

aspirated into the aspiration channel, and high speed flow is generated from the cutting channel to cut cells (Fig.3(a)). The thickness and the width of the channels were adjusted by experimenting with bovine oocyte and we decided the thickness of the aspiration channel is 50μm. After the cutting, it is necessary to collect them for fusion and examination the characteristic of the cutting cell. Cut cells are transported to the extraction chamber and pick up by glass pipette. We used a teflon tube for pipetting the cells since it is reasonably flexible and is thus suitable for inserting into the silicon tube connected to the microfluidic chip(Fig.3(c)). Normally, micro animal cells are manipulated by glass pipette, which is made from Pasteur pipette. The pipette is heated with a gas burner and extended manually so the size of the made pipette is various. An expert can make a glass pipette adjusted the diameter suitable for a work but it is difficult. Then, infusion and withdrawal of a pipette are controlled by breath but it should be skilled too. The volume of the pipette is a few ml and there is a large amount of air in the tube where the mouth ties to the pipette so the response is late. To improve these problems, we use micro teflon tube which the inside diameter is 300μm for the pipette. The volume of the tube is about 2.1μl so a response is high. To fix and to prevent a backflow of solution from injection port, a plastic chip is installed to the teflon tube pipette. Figure 3(d) shows a schematic diagram of the modified microfluidic chip and the teflon tube pipette. A plastic stopper is used to stop the silicone tube of the microfluidic chip. The aspirated cells in the Teflon pipette are then released into the microchannel. Figure 4 shows the overview of the system. We use inverted microscoe(OLYMPUS Co.,LTD.) for the observation. The micro fluidic chip is set on the XY stage. Three syringe pumps(SDK-2, KD-SCIENTIFIC Co.,LTD) are connected to the teflon tube pipette, the cutting flow channel and the aspiration channel. These pumps are controlled from a PC. A CCD camera and video system are connected and the vision from the CCD is captured to the PC.

IV. EXPERIMENT

A. *Experiment of injection*

Firstly, an experiment of injection is performed. It is necessary to improve the probability of injection to improve the success rate of cutting. In the section 2, a need of the injection is described but repossession is important too. Figure 5 shows a process of the injection and result of the experiment. The experiment system is as shown in figure 4. A microfluidic chip, syringe pumps, teflon tube pipette and all the tubes which are connected to the microfluidic chip and teflon tube pipette are filled by solution because to remove air bubbles to improve the response of the flows from syringe pumps. The surface-active agent is mixed with the solution because the beads are fix surface of tube, wall of the PDMS chip or glass. In the experiment on cutting, protein is mixed with the solution for the adhesion prevention. 20 microbeads (polystyrene bead, the diameter is about 100μm) are aspirated from a petri dish, which has a gentle slope to agglutinate a micro objects with vibration, and lined up in the teflon tube pipette with 3mm distance(Fig.5 (a)). The beads are coherent but the flow around the top ob the teflon tube pipette is a gradient flow so two or more beads can be prevented being inhaled. The teflon tube aspirated the beads is injected through a silicone tube connected to the microfluidic chip as shown in figure 5(b). The length of the teflon tube pipette and the silicone tube are adjusted to reach the top of the teflon tube in the microchannel. If the teflon tube is not reach the microchannel, the flow from the teflon tube is stagnated and the beads are stayed in the injection port. All beads flow sequentially in the microchannel and are extracted in the extraction chamber(Fig.5 (c)). At the exit of the microchannel, the space is broadens and the extracted beads are stayed around the exit. As shown in figure 5 (c), we were able to confirm beads 20 pieces all. Then, the beads are aspirated again with a glass pipette(Fig.5 (d)), and extracted in a petri dish. We finally succeeded in the collection of beads 20 pieces all. A similar experiment was conducted three times and all injections and extractions are succeeded. Then, using this process, we performed an experiment of cutting of 56 bovine oocyte and all injections and extractions are succeeded too. Thus, a high success rate was proven and it was shown that this technique was effective.

B. *Experiment of cutting*

In the section 4.1, we confirmed a high probability of the injection and we do not consider the possibility of the injection failure. Figure 6 shows a result of experiment of cutting. In this

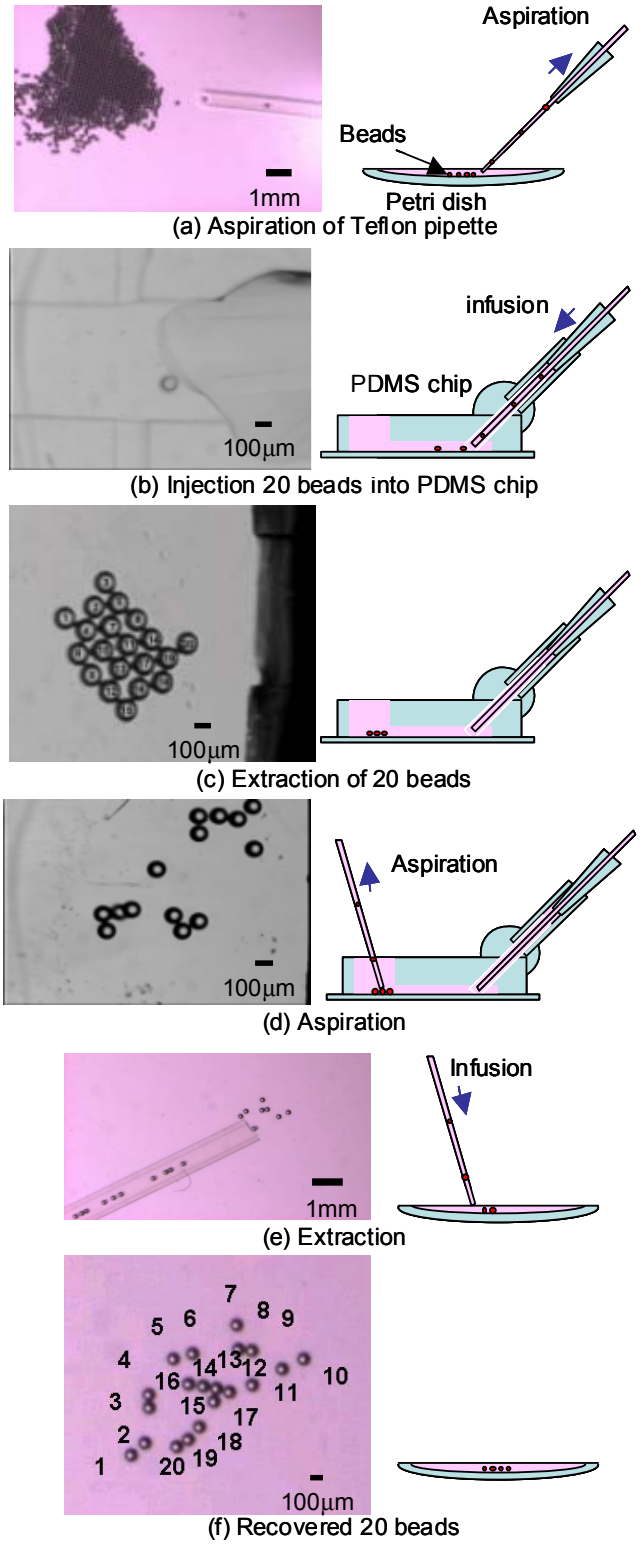

Fig.5 A result of an experiment of injection

experiment of injection. The cells are picked up by the teflon tube pipette from a watch glass and the cells are released into the microchannel (Fig.6(a)). The diameter of the bovine oocyte is about 100μm. The cells were transported around the study we used bovine oocyte that had been chemically softened. Injection and extraction of the cells are done as shown as the aspiration channel by the flow from the cutting flow channel. Then half the volume of the cell is aspirated into the aspiration channel. The cell is transformed, and is fixed at the cross point of the aspiration channel and main channel. Then high speed flow, which the velocity is 25μl/m, is run from the cutting flow channel. It was then cut by a fluid flowing in the cutting channel (Figs.6(c)-(f)). The cell was thinly extended and cut to ensure that the cell membrane was not destroyed. After cutting, the cells were transported to the extraction chamber and picked up using the glass pipette (Fig.6(g)). Figure 6(h) shows the cut cells restored to spheres, indicating that the cell membrane had not been destroyed. In this study, 55 bovine oocytes were cut in this manner with a 98% success rate.

C. *Result and discuss*

In the cutting experiment, not only flow cutting but also blade cutting is performed to compare the each cutting success rate. Table 1 shows the result of the success rate of the cutting. As for the cutting success rate, the flow cutting is higher than the blade cutting. The difference of the success rate is caused that the power of the blade cutting is too high so cutting fails due to a little operation mistake. With the flow cutting, the aspirated cell is transformed and held by the aspiration channel. Then the cell is stretched gradually by the flow so it is easier to cut it without a broken of the cell membrane. From this result, the cutting method using microchannel flow can be used without trouble as the technique of cutting. On the other hand, size of the cut oocyte is problem. Figure 7 shows ratios of size of cut cells. The cut cells are restored to spheres and the size of the cell is measured by a vision by the CCD camera. In this time, volume of the aspirated cell is guesstimated by the operator of the pump so the size of the cut cells had a scattering result. The calculation of the aspirated cell by the image from CCD camera and automatic pump control system is needed.

V. IMAGE PROCESSING

To realize an automatic cell cutting, a image processing of the cutting point and pump control are needed. Then measurement of diameter of a target oocyte and volume of an aspirated cell is important to control the size of the cut cells. In this time, only the image processing part is described. The programming language used Visual C++ and Open CV is used as an image processing reference. Firstly, it is necessary to select the technique for recognizing the cell. One is template matching and another one is binarization of background difference image.

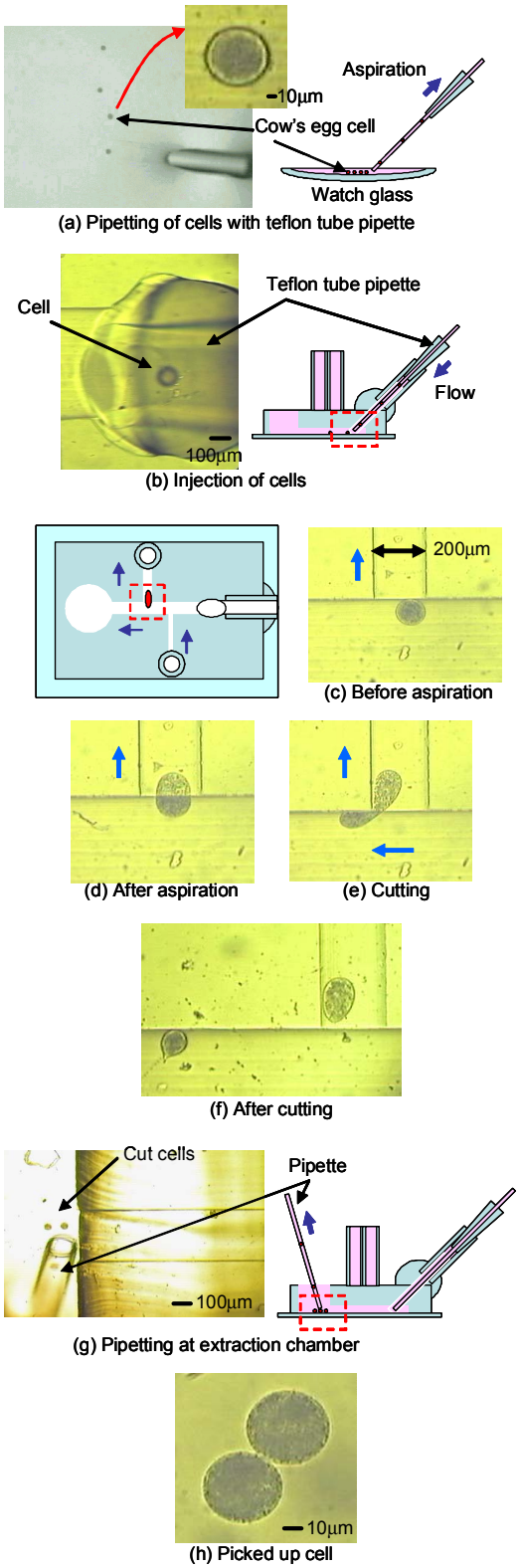

Fig.6 A result of the experiment

Table 1 Success rate of cutting with microchannel flow and blade

	Flow cutting	Blade cutting
Cutting success rate	55/56 (98%)	54/59 (92%)

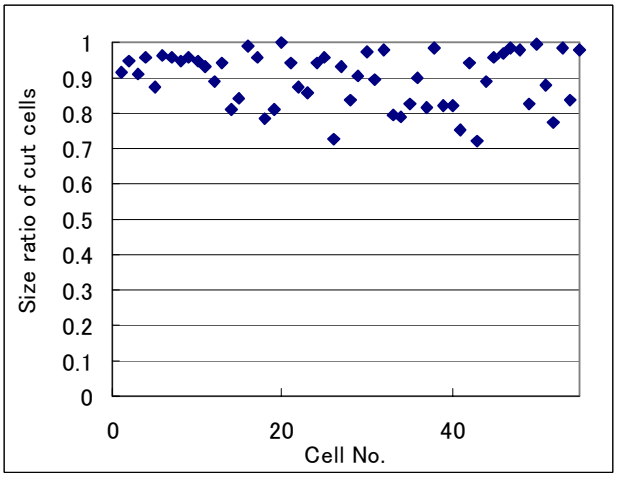

Fig.7 Graph of size ratio of cut cells

The vision of the microscope is sickly. Then a cell has similar shape but, each cell is slightly different shape. Therefore, the template matching is not suitable for this observation and binarization is used. The edited image of the cutting experiment is used as a sample video. The background is displayed in the beginning. Next, the appearance of the cutting experiment is displayed. The stage is not moved and the microfluidic chip is fixed to the stage. It covers to the microscope so that the lighting condition should not change. Figure 8 shows a result of the image processing. The cross point of the main channel and aspiration channel is centered in the observational area. Then, the channel is filled by the solutions and a background is captured(Fig.8(a)). A target cell is come in the observation area and the image is captured(Fig.8(b)). The differential image(Fig.8(c)) is calculated by subtraction the background image from the captured image. A cell is recognized by the binarized image(Fig.8(d)). Then, the diameter of the cell and a volume of the aspirated cell are calculated. A micro scale is used to measure a length of the one pixel and it is converted into 3.75 microns. From these data, automatic cut should be performed and we performed it(Fig.9) but the response of the syringe pump is not good. The delay is about 0.2 seconds because the pumps are controlled by serial communications only and there is a delay until the movement of the pump is reflected in the fluid. To solve the delay and response problem is our nearly future work.

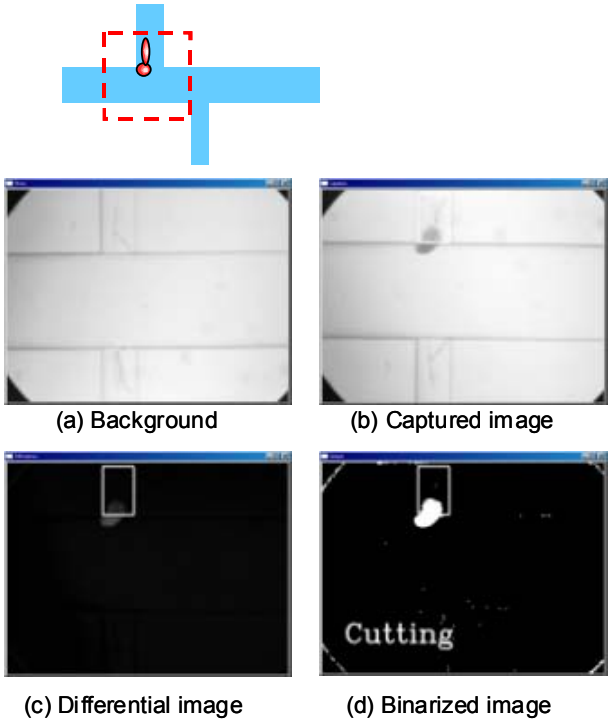

Fig.8 Binarized image of cutting

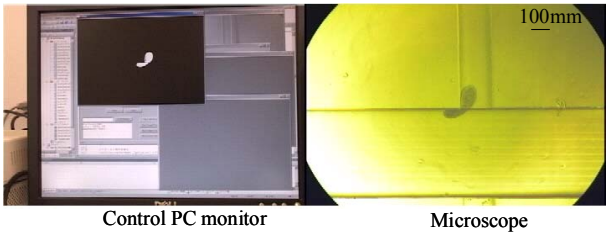

Fig.9 An experiment of auto cutting

VI. CONCLUSION

We have developed a novel technique for injecting and cutting animal cells for an automatic cloning system. The technique of cutting was classified, and it proposed the fluid cutting as a proper procedure. It proposed the injection method, and a microfluidic chip and cutting system were constructed. It succeeded at a high probability in the injection experiment, and the effectiveness of this technique was shown. It succeeded at a high probability, and the effectiveness of the cutting technique was shown in the cutting experiment. In the image data processing, it succeeded in the recognition of the cell but to solve the delay and response problem is our nearly future work.

ACKNOWLEDGMENT

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