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Development of Automatic Micromanipulation System for Biological Cell Sorter

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Abstract: *A square case containing ova and culture medium was vibrated with a voice coil motor to concentrate the ova at the center of the case. Only those ova which conform to the memorized shape are then selected with a picture processing device and are positioned one by one at the point of a holding pipette. A system with the functions mentioned was experimentally constructed. This system can automatically position any ovum in a certain position, thereby shortening the time of the process to search out an ovum and position it at the point of the holding pipette.*

Key words: *Micromanipulator, Image processing, Vibration, Piezo, Micromanipulation.*

In life science research on mammals, minute work at the cellular level is done with a micromanipulator. Nowaday micromanipulators for cell handling are being used for the following procedures: partition of nuclear transplantation of cells or fertilized ova to generate individuals with the same characteristics, fertilization of ova by injection of sperm into the ooplasm or ovum perivitelline space [1, 2], in vitro fertilization by excising a part of the zona pellucidum [3, 4]. These procedures are used as a means to improve the breed of domestic animals and to elucidate the fertilization mechanism. Microfertilization with a micromanipulator used is also being carried out in the treatment of sterility. The present micromanipulator for the cell operation generally used in microfertilization, etc. transmits the movement of the operator's hand to a minute apparatus by remote control by using oil pressure. Especially for operation in a minute area, operator skill is necessary, and the degree of skill is the greatest factor in the success of minute work. The aim of the automation of this work is to improve work efficiency, but a fully-automatic micromanipulator does not yet exist, though there are examples of semi-automatic micromanipulators. The following are problems in the automation of microfertilization with micromanipulators and DNA injection into the nucleus, etc. ①: Because the cell is elastic, the micropipette cannot be inserted smoothly, and the cell is transformed and destroyed. Normally, the skilled operator judges the form of the micropipette point and the transformation of the cell caused by the elasticity and changes in the injection method. ②: There are differences in the shape etc., of individual cells. So the operator has to judge the qualities of cells. ③: It is necessary to introduce a complex mechanism into the automation of the oil pressure drive. ④: It is necessary to search out several cells in one drop of medium sequentially and quickly. The automation of minute work by means of a micromanipulator was difficult for the abovementioned reasons. But we have developed a micromanipulator with a micromovement mechanism (Piezoelectric Impact Drive Mechanism) utilizing rapid deformations of piezoelectric elements to drive the micropipette, and did several kinds of experiments involving minute work under the microscope with the micromanipulator [1–3]. As a result, because this micromanipulator uses impulse force caused by a rapid disformation of the piezoelectric element to drive the micropipette, it is confirmed that the micropipette can be inserted very smoothly into the cell surface membrane and the zona pellucidum of the ovum. In this paper, we report a trial system for gathering ova from any position in the medium to the center of the microscope view by shaking the Petri dish container and square case etc., selects the ova whose shape conforms to the memorized one by using the picture processing device and positions the ova at the micropipette point by electric stages.

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Materials and Methods

Materials

The mice used in these experiments were 8 weeks old. They had PMSG injections, and had hCG injections in two days. In the 24 hours after the hCG injections, their oviducts were taken out and the ova were collected.

Methods (Experimental equipment)

Experimental device: The main parts of this device are the excitation machine as the prepositioning part, the picture processing device as the cell selecting part, and the micropipette micro movement with it's piezoelectric impact drive mechanism.

Prepositioning part: The device which shakes the Petri dish and square case filled with medium by using a voice coil motor was made for trial purposes. The voice coil motor is controlled by an analog PID controller which compares the voltage from the transmitter with the displacement voltage detected by the eddy current gap sensor (EX-500 of KEYENCE). The small square case is fixed in a large square case installed on the shaft of the voice coil motor (Fig. 1). A voltage wave is input by the transmitter to control the voice coil motor, and the square case is shaken. This vibration is used to gather the ova.

Cell selection part: Fig. 2 shows the construction of the experimental device. In order to position the sample table, the image information from the inverted microscope is taken with the CCD camera and the XY stage is driven by means of the image recognition automatic positioning device. The object lens is driven by the pulse motor of the coupler installed on the microscope focus handle. Fig. 3 is a general view of the device.

Image recognition positioning device: (X and Y axes)

PS3000 of Flovel was used as the image recognition positioning device for the X and Y axes (front, back, left and right). The target pattern is registered beforehand (Fig. 4). PS3000 searches for the registered pattern in the picture, and when the pattern is found, the XY stage is driven to move the pattern to the screen standard position. The image for searches is the gray scale, and pattern recognition uses the normal correlation method.

Image recognition positioning device: (Z axis)

AF2000 of Flovel was used as the image recognition positioning device on the Z-axis (top and bottom).

This device can drive the microscope focus handle and proceed FFT (fast Fourier Transform) of the image

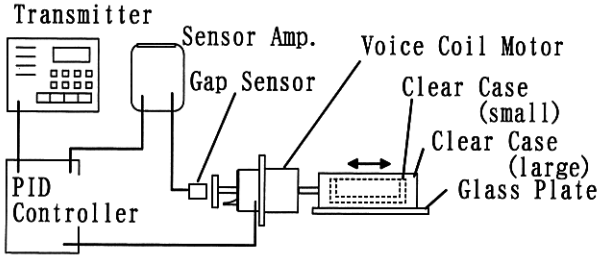


Fig. 1. Outline of the system

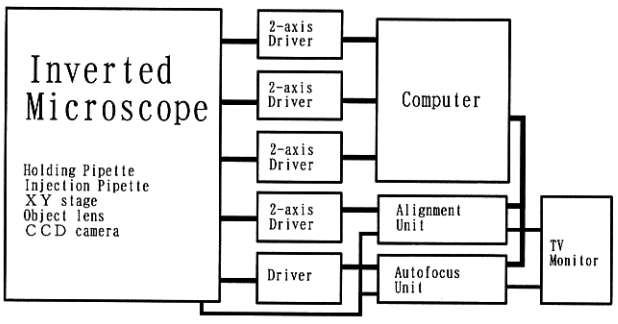


Fig. 2. Outline of the system

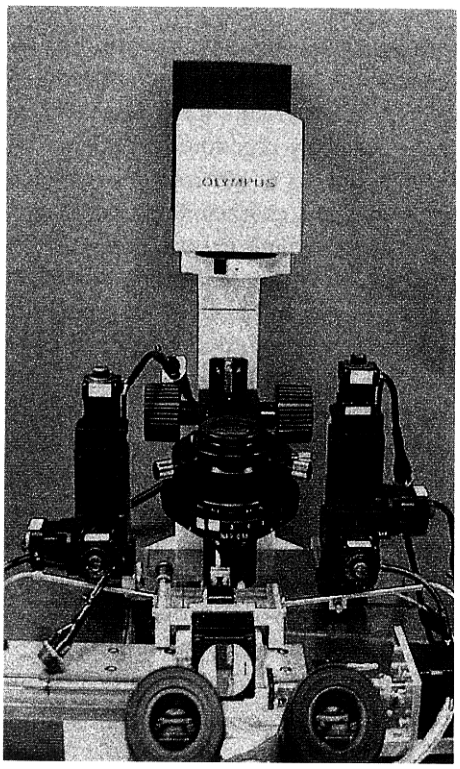


Fig. 3. General view of the device

at the same time, and fix Z coordinates to that including the greatest numbers of high-frequency wave elements. The focus can be obtained by this operation.

Micropipette micromovement part with piezoelectric impact drive mechanism: The drive unit for the minute apparatus (micropipette) which can be controlled electrically is necessary for automation of the cell operation. Here, "Minute movement mechanism used rapid deformations of the piezoelectric elements" [3] (piezoelectric impact mechanism) developed by the authors was used for the micro-movement of the micropipette. Fig. 5 shows the driving principle.

(1) The main body is connected to the weight via one piezoelectric element. Static friction power stops the main body. The piezoelectric element is shrinking.
(2) The main body and the weight are moved in opposite directions by the action of impact power by a rapid expansion of the piezoelectric element when the voltage is rapidly applied to the piezoelectric element.
(3) The piezoelectric element is slowly shortened at the speed at which the main body does not move, and only the weight returns.
(4) The inertial force of the weight acts on the main body when the piezoelectric element shortens, gradually increasing the speed, and then stops suddenly, and the main body moves.
(5) The main body stops by the static friction power.

The main body moves in the stepping movement, repeating from (1) to (5).

Impact drive stage PMM-MB-B by PRIME TECH LTD. with the impact drive mechanism was used as a minute apparatus drive unit at this time. The appearance of the micropipette insertion in the lapin ovum which uses piezoelectric impact driving mechanism is shown in Fig. 6. With the piezoelectric impact drive, the pipette without transforming can be inserted the ovum.

Electric stage: The electric stage is driven by the image recognition positioning.

In the design ball screws are combined with the pulse motors and the minimum amount of movement is 2 μm. It is sufficiently accurate because the diameter of the positioned ovum is 70–150 μm.

Cell operation procedure: The positioning operation was done according to the following procedures.

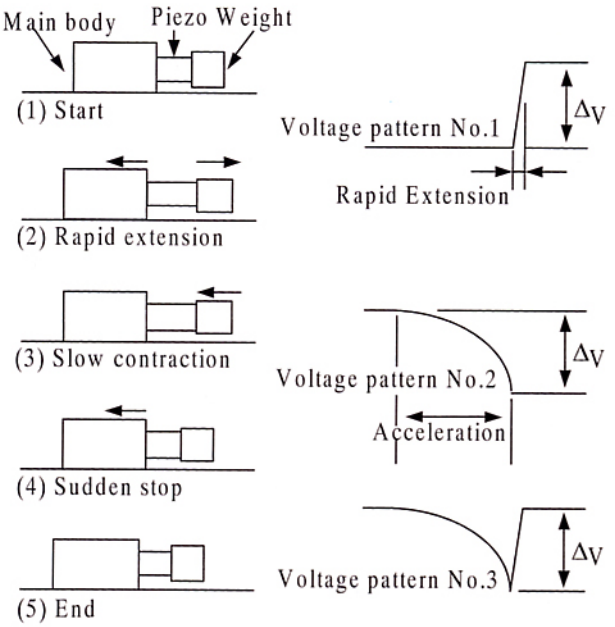


Fig. 5. Principle of movement of the piezoelectric impact drive mechanism

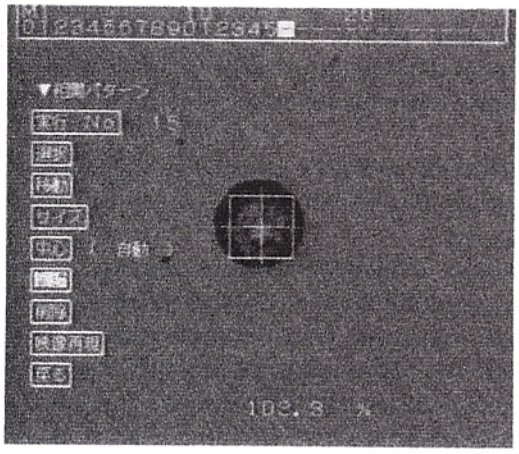


Fig. 4. Registration of the target

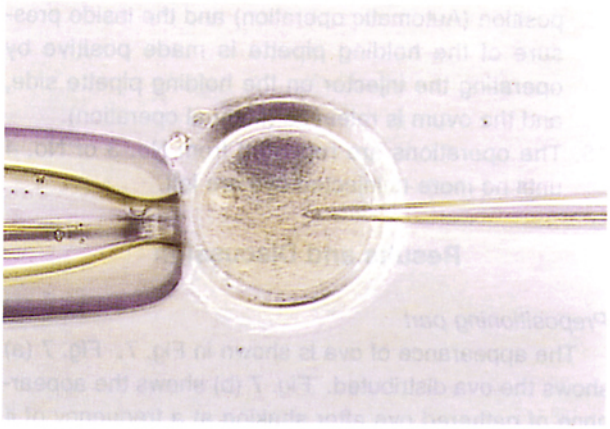


Fig. 6. Micropipette insertion

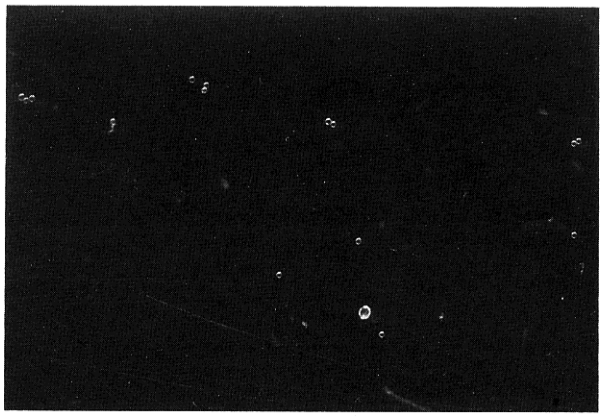
1. The injection pipette and the holding pipette are installed in the micromanipulator and set at the standard position.
2. Medium and ova filled the square case (Manual operation).
3. The square case is shaken to concentrate the ova to the center of the case (Automatic operation).
4. One ovum is selected with the picture processing device (Automatic operation).
5. The selected ovum is transported to the working area by the electric XY stage (Automatic operation).
6. The injection pipette is moved to the position of the sperm. The inside pressure of the injection pipette is changed to negative by operating the injector on the injection pipette side and the sperm is inhaled (Manual operation).
7. The holding pipette is moved to the position of the selected ovum in the working area.
8. The inside pressure of the holding pipette is changed to negative by operating the injector on the holding pipette side and the ovum is inhaled and held (Manual operation).
9. The injection pipette is moved to the extension line at the point of the holding pipette (Automatic operation).
10. The injection pipette is moved with the piezo micromanipulator, and the tip is thrust into the ooplasm.
11. The inside pressure of the injection pipette is made to positive, and the sperm is exhaled into the ovum (Manual operation).
12. The tip of the injection pipette is removed from the ovum (Automatic operation).
13. The operator observes whether there is a sperm inside the ovum (Manual operation).
14. The holding pipette is moved to the ova release position (Automatic operation) and the inside pressure of the holding pipette is made positive by operating the injector on the holding pipette side, and the ovum is released (Manual operation).
15. The operations are repeated from No. 3 or No. 4 until no more uninjected ova are left.

Results and Discussion

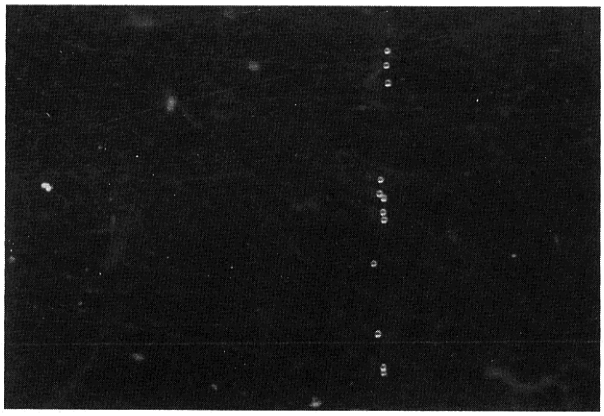
Prepositioning part

The appearance of ova is shown in Fig. 7. Fig. 7 (a) shows the ova distributed. Fig. 7 (b) shows the appearance of gathered ova after shaking at a frequency of 4 Hz and an amplitude of 2 mm.

The frequency was fixed at 4 Hz, and the behavior



(a) Before shaking the square case



(b) After shaking the square case

Fig. 7. Appearance of gathered ova

of the ova (mouse ova) related to the different vibration amplitudes was observed. Fig. 8 shows the result. The axis of ordinates shows the time taken for the ova to line up in a row from the dispersed state since the vibration was started. The usual indication of the completion of lining up is that ova which moved toward the center line have formed a line less than 1 mm in width.

1.8–2.0 mm amplitude gave the best result. The ova gathered at the center line in 5–7 sec and formed a straight line.

The flow of liquid in the square case was examined. Under the conditions (4 Hz frequency, 2 mm amplitude) in which pseudoova (made of polystyrene, ϕ 100 μm) gather to the center line in 10 sec, this examination is carried out with beads (Zirconia beads, ϕ 100 μm) floating on the demineralized water and vermilion ink sinking in the demineralized water.

Fig. 9 shows the results. From the cross section

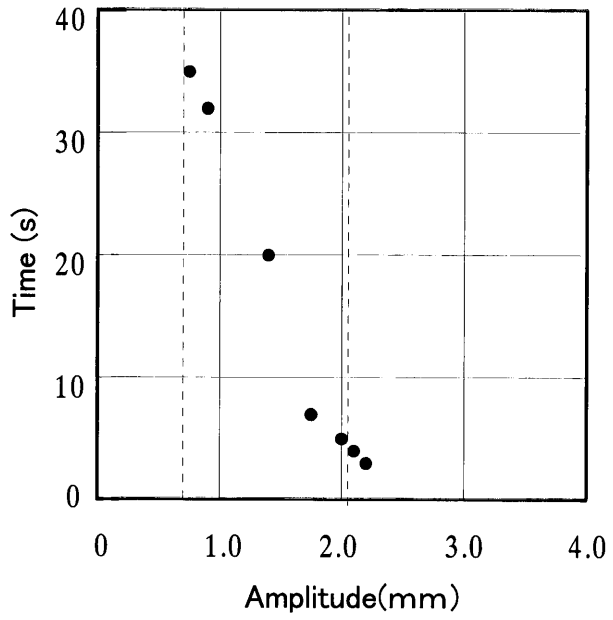


Fig. 8. Relation of time until ova line up in the row to amplitude (4 Hz)

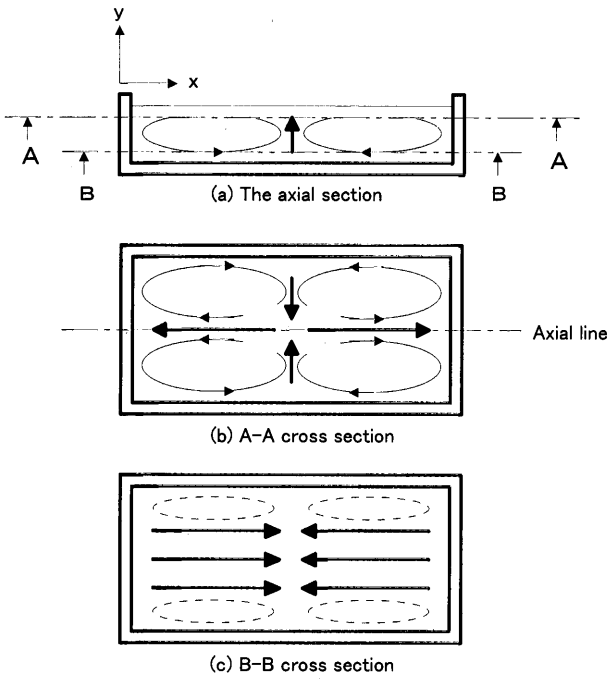


Fig. 9. Lipuid flow

(Fig.9 (a)), two convections with the boundary in the center of the case were observed. Rising flow occurs in the part where pseudoova line up. This rising flow becomes the surface flow, divides in the directions of the vibrations, and flows in straight lines. Simultaneously, a straight line flow toward the center in a horizontal direction was seen on the surface. In the lower layer which directly influences pseudoova, a straight line flow occurs facing the center from the side of the square case. This flow occurs alternately, synchronized with the vibration of the case. That is, when the case moves left, the liquid in the left half flows to the right (a), and when the case moves right, the liquid in the right half flows to the left (b), as shown in Fig. 9. We think that pseudoova are gathered to the center by this flow. The width of the flow is 15–20 mm. The flow near the case side appears disordered.

Cell selection part

The experimental setup constructed of the image recognition devices and the electric XYZ stage was found able to search out an ovum and to position it to the micropipette automatically.

Conclusion

The experiment in which a mouse ovum inside a square case is moved into position for the voice coil motor to shake the square case filled with medium.

Ova were lined up in a row at the center in 5–7 sec when the case was shaken at an amplitude of 2 mm and frequency of 4 Hz. Next, the position of the ovum is detected by the picture processing device, and the holding pipette installed in the XYZ stage for driving pipettes is moved to the ovum, inhales the ovum and holds it (At this stage, inhaling is a manual operation). It takes less than 5 sec for the holding pipette to be positioned after the picture processing is started, and the procedure saves more than 10 sec compared with those without prepositioning to the center of the square case. In the above experiments, it was confirmed that the time taken to search cells and to position the holding pipettes was shortened by positioning automatically any ovum in a certain position in the liquid by shaking the case. In an injection experiment with real mice ova, 30 ova were injected and 27 of them were fertilized. Consequently it was confirmed that this procedure was very useful in injecting a lot of ova.

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