Development of Automated Nuclear Transplantation System

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Abstract: *In nuclear transplantation, especially in enucleation, rotating the oocyte to a certain position has been an important work. Therefore, we developed an oocyte rotation system by using electrostatic force, which is known to be excellent in handling minute objects. This system has become the basic technology for the automated nuclear transplantation system. Mouse oocyles were rotated at a speed of about 60 deg/s by electrostatic phenomena (i.e. electrorotation and dielectrophoresis), and the direction of rotation could be reversed. In order to confirm the electrostatic effect on the fertilization and developmental ability of oocytes, we performed IVF and nuclear transplantation with rotated oocytes. The result was that the fertilization and developmental ability of the rotated oocytes was the same as that of the control, proving that rotation by electrostatic force does not influence the fertilization and development ability of oocytes.*

Key words: *Mammalian Oocyte, Nuclear transplantation, Micromanipulator, Electrostatic force, Electrorotation, Dielectrophoresis*

In recent years, life science research on mammals has greatly developed [1–4]. In this research, nuclear transplantation is done in order to improve the breed of domestic animals and to elucidate the fertilization and development mechanism. Nuclear transplantation is generally done by the following procedure with a micromanipulator.

1. Bring the oocyte into the field of view of the microscope.
2. Hold the oocyte with a holding pipette.
3. Carefully rotate the oocyte with an injection pipette to the position where the nucleus can be reached easily with the injection pipette.
4. Insert the injection pipette into the perivitelline space.
5. Aspirate the nucleus (Enucleation).
6. Inject the nuclear donor into the perivitelline space (Fusion method) or the cytoplasm of the enucleated oocyte.

At present micromanipulators use hydraulic pressure, oil pressure, or air pressure to manipulate the oocyte. Great operator skill is required for such micro operations. The dexterity of the operator determines the feasibility of these operations. The automation of these operations, therefore, improves efficiency.

We have now developed an injection system with a micro movement mechanism (Piezoelectric Impact Drive Mechanism) [5]: a micromanipulator with a motor driven stage, an oocyte positioning system by means of image processing and a shaker for the petri dish container [6].

In this paper we report the experimental system for rotating oocytes by means of electrostatic force. By integrating this system and the previously developed system, an automated nuclear transplantation system is developed.

Materials and Methods

Principle of the oocyte rotation system

It is very difficult to manipulate micro objects mechanically because the influence of friction, thermal expansion and vibration is very great on the micro scale. In addition, it is difficult to fabricate minute mechanical

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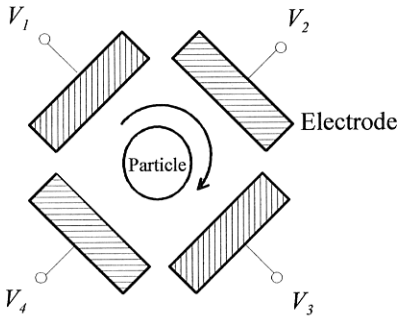

Fig. 1. Electrode alignment that generates a rotating electric field: $V_1 = V_0 \sin \omega t$, $V_2 = V_0 \sin (\omega t + \pi/2)$, $V_3 = V_0 \sin (\omega t + \pi)$, $V_4 = V_0 \sin (\omega t + 3\pi/2)$.

$$T = 4\ \pi a^3\ \varepsilon_m\ \mathrm{Im}\left[\frac{\kappa_p - \kappa_m}{\kappa_p + 2\ \kappa_m}\right] E^2 \qquad (1)$$

$$\kappa_p = \varepsilon_p - j\sigma_p/\omega$$
$$\kappa_m = \varepsilon_m - j\sigma_m/\omega$$

a: particle radius
ε_p: dielectric constant of particle
ε_m: dielectric constant of surrounding solution
σ_p: conductivity of particle
σ_m: conductivity of surrounding solution
E: intensity of electric field
ω: angular frequency of applied voltage

$$F = 2\ \pi a^3\ \varepsilon_m\ \mathrm{Re}\left[\frac{\kappa_p - \kappa_m}{\kappa_p + 2\ \kappa_m}\right] \nabla E^2 \qquad (2)$$

systems.

The system we developed employs electrostatic force. Electrostatic force is known to be excellent in handling minute objects. A microelectrode that generates the electrostatic force can easily be fabricated by photolithography. In addition, direct damage to cells is small because manipulation which uses electrostatic force can be done without contacting cells. Since a cell contains much water and polar molecules such as protein, DNA and RNA, polarization is great, so that it is easy to manipulate a cell by the electrostatic force. Our system utilizes two electrostatic phenomena known as electrorotation and dielectrophoresis.

Electrorotation is the rotation of a particle by the interaction between the surrounding rotating electric field and the polarization of the particle. Applying a 90 degree phase-shifted voltage to the four electrodes shown in Fig. 1 it is possible to generate the rotating electric field. The torque of electrorotation is expressed in equation (1) [7].

Dielectrophoresis is the translational movement of a particle in a non-uniform electric field. This is the result of the interaction between the gradient of the surrounding non-uniform electric field and the polarization of the particle. The force of dielectrophoresis is expressed in equation (2) [7]. Notations in equation (2) are the same as those in equation (1). If F is positive, the particle is attracted to the strong region of a surrounding electric field (positive dielectrophoresis). On the contrary, if F is negative, the particle is attracted to the weak region of a surrounding electric field (negative dielectrophoresis).

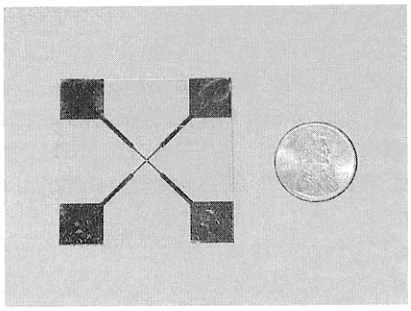

Fig. 2. Glass substrate deposited aluminum microelectrodes: The size of the substrate is 4 cm.

The set of four electrodes in Fig. 1 generates a non-uniform electric field causing dielectrophoresis. In the case of negative dielectrophoresis, the particle is centered. On the other hand, the particle is attracted to one of the four electrodes in the case of positive dielectrophoresis. When the surrounding conductivity is higher than that of the particle, the dielectrophoresis is negative at all frequencies.

Experimental set-up

The microelectrodes were fabricated by depositing aluminum on a crystal glass substrate as shown in Fig. 2. The gap between opposite microelectrodes is 300 μm. By means of a function generator (AG1200, Yokogawa Electric Co., Tokyo, Japan), a sine wave is generated and applied to these electrodes with a 90 degree phase-shift. The amplitude of the applied voltages is $4V_{0\text{-}p}$ and the frequency is 1 MHz.

The chamber containing the microelectrodes is attached to the stage of an inverted microscope (TS100, Nikon Co., Tokyo, Japan). Oocyte movement is observed with a CCD camera (CS5270, Tokyo Electric Industry Co., Ltd., Tokyo, Japan) and recorded by a video camera (NV-CX1, Matsushita Electric Industrial Co., Ltd., Osaka, Japan). A motor driven micromanipulator (Suruga Seiki Co., Ltd, Shizuoka, Japan) was used to transfer oocytes into a drop of medium.

Oocyte rotation experiment

Oocytes were collected from female B6D2F1 and ddY strain mice. These mice were superovulated by the administration of 5IU PMSG (Teikoku Zoki Mfg. Co.) and 5IU hCG (Teikoku Zoki Mfg. Co.). 15 hours after the administration of hCG, unfertilized oocyte were collected from oviducts.

The medium used in the oocyte rotation experiment was HEPES-buffered (Hb) HTF (Lot No. 996280810, Irvine Scientific) or Hb-CZB [8] medium. Oocytes were moved to the center of four microelectrodes one by one with a micromanipulator, and rotated by electrostatic force. The rotation of oocytes was observed.

Investigation of electrostatic effects on fertilization and development ability

In vitro fertilization: Rotated oocytes ware transferred into HTF (Lot No. 996280301, Irvine Scientific) or CZB [8] medium, and inseminated with sperm obtained from caudae epididymides of B6D2F1 males. We performed the IVF according to the procedure of Summers *et al.* [9] and the concentration of sperm for IVF was 1×10^6/ml. Male and female pronuclei were observed 6 hours after the insemination. Fertilized oocytes were cultured in HTF or CZB medium.

Nuclear transplantation: Nuclear donor cells were fibroblasts obtained from the abdominal skin of C3H strain males (8 weeks old). These cells were cultured in DMEM (Lot No. 0662307, Nissiu Pharmaceutical Co.) containing 0.5% (v/v) FBS (Lot No. 7005K, Atlanta Biologicals) to arrest the G1/G0 phase [1, 2, 4, 10]. Fibroblasts were collected from the abdominal skin of 8-week-old C3H male mice. 3-8 times passaged fibroblasts were cultured in DMEM containig 0.5%(v/v) FBS to arrest the cell cycle in the G0/G1 phase.

Rotated oocytes were exposed to 5 μg/ml cytochalasin B (Lot No. 106H4073, SIGMA) for 15 min. Oocyte enucleation was performed under a micromanipulator. An enuclation pipette (internal diameter 20 μm) was inserted into the perivitelline space through the zona pellucida, and the nucleus was aspirated into the beveled pipette. And fibroblasts were injected to the perivitelline space of enucleated oocytes.

Nuclear transplantation was performed by electro fusion between the fibroblast and the enucleated oocyte. Electrofusion was performed with a Shimadzu cell fusion apparatus (SSH-1; Shimadzu Co.). The cell fusion chamber (FTC-03; Shimadzu Co.) was built of two electrodes 2 mm apart. Cell fusion buffer was a 0.3 M mannitol solution containing 0.05 M $CaCl_2$ and 0.1 M $MgSO_4$. Fibroblast and enucleated oocyte pairs were placed between the electrodes of the cell fusion chamber overlaid with the cell fusion buffer. Pairs were aligned in the direction of the electric field by means of 30 $V_{0\text{-}p}$, 1MHz alternating voltage for about 30 sec until alignment, and a DC pulse of 0.8 kV/cm for 40 μsec induced membrane fusion.

Reconstituted embryos were cultured in HTF or CZB droplets covered with mineral oil in 5% CO_2: 95% humidified air at 37°C. Then the developmental states of these embryos were observed.

Results and Discussion

Oocyte rotation

The oocyte was rotated at a speed of 60 deg/s. During the rotation, the oocyte was kept at the center of four electrodes due to negative dielectrophoresis as shown in Fig. 3.

It was possible to reverse the direction of rotation of the oocyte by interchanging V_1 and V_3. Since the viscosity is greater than the inertia of the oocyte, oocyte rotation stopped exactly when the applied voltage became zero. We can therefore precisely control the angular position of the oocyte.

Effects on fertilization and developmental ability

The result of IVF is that the fertilization percentage of experimented oocytes is 86.0% (43/50), that of the control is 86.7% (26/30), and the percentage of experimented oocytes that had developed to the morula and blastocyst stage was 52.0% (26/50) and that of the control was 50.0% (15/30).

The result of nuclear transplantation is that the fusion percentage of the experimented oocyte is 72.5% (29/40), that of the control is 70.0% (21/30), and the percentage of the experimental oocytes that developed is 2.5% (1/40) and that of the control is 3.3% (1/30).

These results confirm that rotation by electrostatic force does not affect the fertilization and developmental ability of oocytes.

Conclusion

In this paper we have proposed a system that can rotate oocytes at the rate of 60 deg/s. It is confirmed that the rotation by electrostatic force does not adversely affect the fertilization and developmental ability of oocytes. This system is therefore well suited for cell manipulation.

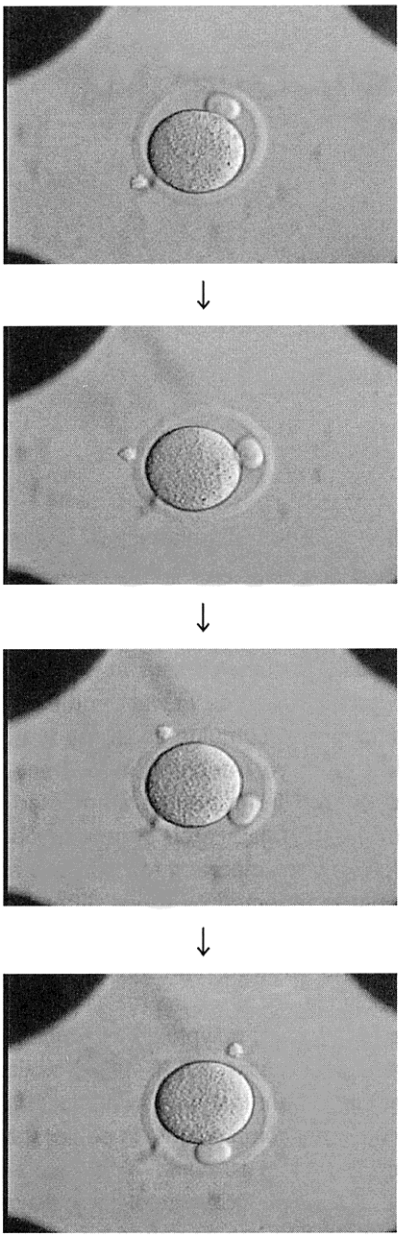

Fig. 3. Oocyte rotation in HTF at a speed of about 60 deg/s.

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