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Evaluation of Ultrasonic-Range Vibratory Microinjection System at a Frequency of 35 kHz Using Fertilized Mouse Eggs

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Abstract: *To facilitate pronuclear microinjection, we have been developing the Vibratory Microinjection Systems (VMSs) that provides a micropipette with longitudinal vibration. The current VMS utilizes any frequency up to 100 kHz. We compared 35-kHz vibratory microinjection (VM) with ordinary microinjection (OM). Fourteen micropipettes were used to inject 420 BDF1 zygotes. Each micropipette finished its injection quota of 30 eggs, which were manipulated one by one alternately using the two types of microinjections, even when it repeatedly pulled out nuclear components. All microinjections were conducted at a compensation pressure of 30 hPa and digitally recorded for subsequent image analysis. VM resulted in slightly better embryonic development in 4-day culture than OM, but significantly shortened the time spent on microinjection and the time spent swelling the pronucleus by 25% and 30%, respectively. These pronuclear swelling times, together with the almost identical degrees of pronuclear swelling in both groups, suggested that VM injected a GFP solution at a 42% higher speed. VM significantly reduced the incidence of pulling out nuclear components, suggesting VM's capability of removing the nuclear components already adhering to the micropipettes. These results indicate VMS is a useful option which is capable of raising the efficiency of microinjection significantly by saving time, labor and cost of microinjection.*

Key words *: Microinjection, Longitudinal vibration, Ultrasonic-range vibration, Injection speed, Working efficiency*

Received: August 8, 2011
Accepted: September 20, 2011
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Introduction

The microinjection of DNA directly into the pronuclei of fertilized zygotes [1] is the most successfully used method of gene transfer in the mouse, and is also considered to be the most efficient method for gene transfer. However, because the production rate of transgenic mice is still low, various methods have been investigated and proposed for raising the production rate.

Miyawaki *et al.* have been aiming to improve the microinjection method itself by developing the Vibratory Microinjection System (VMS) [2–4] which is vibrating a micropipette longitudinally while it is being inserted into a pronucleus (Fig. 1). The VMS is different in terms of mechanism and movement from the Piezo Impact Micro Manipulator (PMM-150, PrimeTech, Japan) that moves a micropipette stepwise using a strong inertial force generated by rapid deformation of piezoelectric elements [5]. The Piezo Impact Micro Manipulator therefore needs some inertial mass, for example a mercury drop in the micropipette, to develop the inertial force strong enough to move the micropipette. By contrast, VMS utilizes pure vibration of piezoelectric elements to pierce cells and inject DNA solution into them, and does not need such inertial mass. VMS uses the vibration frequency of more than tens of thousands Hz whereas the Piezo Impact Micro Manipulator uses a vibration of about a few hundred Hz.

Miyawaki *et al.* demonstrated that the VMS using audible-range vibration (0–18 kHz) was significantly superior to the ordinary (non-vibratory) microinjection as indicated by the rate of embryonic development to the blastocyst stage as well as the incidence of pulling the nuclear components, such as RNA and/or DNA, out of fertilized eggs [3, 4].

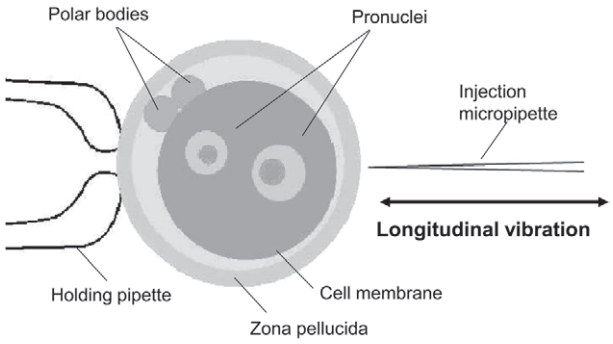

Fig. 1. Schema of vibratory microinjection.

Subsequently, in order to investigate the effectiveness of ultrasonic-range vibrations, Miyawaki *et al.* developed a new version of VMS. In this study, we evaluated this current version of VMS in comparison with the ordinary microinjection.

Materials and Methods

Ultrasonic-range vibratory microinjection system

The ultrasonic-range VMS consists of a vibrator and a driving unit (Figs. 2a, b). The vibrator is composed of three identical stacked-type piezoelectric actuators and a housing, and has a central path through which foreign DNA solution flows (Figs. 2c, d). This vibrator was designed to be interposed between a micropipette and an ordinary injection holder. The major differences from the previous audible-range VMS are the type and number of piezoelectric actuators. In the previous version, one cylindrical stacked-type piezoelectric actuator was used and was capable of generating audible-range vibration.

We measured the amplitudes of the vibrator from three directions (three dimensions) with a displacement meter using reflection of red light: the longitudinal amplitude and two lateral amplitudes at the level of the vibrator. Generally speaking, the longitudinal amplitude has an approximately linear relation with the voltage applied to the vibrator. In this measurement, the voltage was set to 10 V peak to peak (p-p).

Pronuclear microinjection

1. Transgene

In this study, we used a gene expressing green fluorescent protein (GFP) as the transgene, and obtained GFP construct (1.67 kb) by cutting plasmid DNA pAcGFP1-N1 (4.7 kb; PT3716-5, TaKaRa, Japan) with restriction enzymes, Afl II (1003A, TaKaRa, Japan) and Ase I (R0526S, New England BioLabs, USA). This DNA construct was diluted with Tris EDTA buffer so that the concentration of the GFP gene solution became 2.0 ng/μl.

2. Injection pipette

To accurately evaluate the effects of VMS on the "evaluation indices" described below, the difference in opening diameter of the tip of micropipettes must be as small as possible. Instead of handmade micropipettes, we used commercially-available micropipettes (Femtotip®, eppendorf, Germany) because the diameter of their openings is within a small range of 0.3 to 0.7 μm under product control.

3. Injection protocol

Eight- to nine-week-old BDF-1 female mice (CLEA Japan, Inc., Japan) were superovulated by injecting 7.7 IU of serum gonadotrophin (ASKA Pharmaceutical, Japan) into the peritoneal cavity 72 h before microinjection and 7.7 IU of human chorionic gonadotrophin (ASKA Pharmaceutical, Japan) intraperitoneally 24 h before microinjection. Then, they were mated with BDF-1 male mice (CLEA Japan, Inc., Japan). On the next morning, fertilized one-cell eggs were collected from the female mice with a vaginal plug.

A total of 420 zygotes were used in this study. VMS was compared with the ordinary microinjection from several viewpoints. We were most interested in the difference in the speed of injecting DNA solution. Although the opening diameters of the micropipettes are within such a small range (0.3–0.7 μm), even these small individual variations in diameter may be too large to accurately compare the injection speed between the two types of microinjections. To eliminate this concern, injection was alternated between the vibratory and ordinary microinjections using one micropipette. The ordinary microinjection was conducted with the vibrator switched off while the vibrator was kept interposed between the micropipette and the injection holder as shown in Fig. 2b. A set of 30 fertilized eggs were microinjected one by one using one micropipette alternately with and without vibration (15 eggs for each group).

Unlike the previous audible-range VMS, the current VMS was evaluated by adjusting the voltage applied to the vibrator according to individual micropipettes because we aimed to keep constant the vibration states of individual micropipettes, in particular amplitudes of the longitudinal vibration, and because we have found that the vibration states at the same applied voltage can vary from micropipette to micropipette. However, we were not able to see the longitudinal vibration, but we found that lateral vibration of the tip of micropipettes became visible when the applied voltage exceeded their respective

levels. We assumed that we might keep the amplitudes of the longitudinal vibration of micropipettes almost identical when those of the visible lateral vibration of the micropipettes were adjusted to the same level by changing the applied voltage. However, since the lateral vibration is unintentional and unfavorable, we set the applied voltage to a level which started generating a slight lateral vibration of the tip of each micropipette.

The temperatures of the room and M2 medium were kept at 26°C. We used FemtoJet® (eppendorf, Germany) as an injector to control the compensation and injection pressures. The injector was connected to the rear end of an ordinary injection holder using a stiff, thin tube. In each micropipette used in this study, the compensation pressure, which is the pressure preventing the culture medium from flowing into the micropipette according to the phenomenon of capillary action, was fixed at 30 hPa. Both vibratory and ordinary microinjections were carried out without any further pressure added to the compensation pressure, mean that no injection pressure was used.

The process of microinjection was recorded with a high precision CCD video camera and stored as digital movies on a personal computer. After all the zygotes had been injected, we examined if they were alive or not. Then, they were cultured in M16 medium in an incubator (37°C, 95% O_2 and 5% CO_2) for 5 days. Embryonic development was assessed periodically.

Evaluation indices

We evaluated the vibratory and the ordinary microinjections using the 5 indices described below. The first 4 indices were obtained from analysis of the digital movies using an application program ImageHyper II® (DigiMo, Japan).

1. Measurement of “depression rate”

We measured how deeply a fertilized egg was depressed with a micropipette at the insertion site just before it penetrated the zona pellucida and the cell membrane. We used this measure, termed “depression rate”, as an index of how easily the micropipette pierced the membranes. The “depression rate” was defined as

(D–X)/ D x 100.

where D was the original width of the egg on the extension line of the axis of the micropipette, and X was the egg width on the extension line of the micropipette axis at the moment when the egg was maximally deformed (Fig. 3).

2. Measurement of “injection time”

If a micropipette is inside a fertilized egg for a longer period of time than usual, we believe that it will cause more damage to the egg. Accordingly, we measured how long a micropipette was inside each egg and termed this period of time the “injection time”. We defined the injection time as the period of time between the following two moments: the moment just before the micropipette penetrated the zona pellucida of an egg, and the moment when the micropipette was withdrawn from the egg. Our way of measuring the injection time was to search for the two frames capturing the above two moments in the digital movies and to count the intervening number of frames. Since the movies were recorded at 30 frames per second, the time interval between any two consecutive frames was 33.33 ms. We thus calculated the injection time.

3. Measurement of “DNA injection time” and ratio of pronuclear swelling

In order to estimate the speed of injection of the GFP gene solution into the pronucleus, we measured the time spent in swelling the pronucleus, which we termed the “DNA injection time”, and the degree of pronuclear swelling (Fig. 4). More precisely, the DNA injection time was defined as the period of time from the moment just before the pronucleus started swelling to the moment when it finished swelling, and it was measured using the digital movie in the same way as the injection time. The diameters of pronuclei before and after microinjection were accurately measured using the application program ImageHyper II® (DigiMo, Japan).

4. Incidence of “pulling-out” events

When a micropipette is withdrawn from a fertilized egg after pronuclear microinjection, the micropipette sometimes pulls a portion of the nuclear components, e.g. RNA and/or DNA. The reason for this is considered to be that the nuclear components, especially RNA, are sticky and easily adhere to the tip of the micropipette. This type of event, which we termed a “pulling-out” event in this paper, is not only lethal to eggs, but also needs to replace the micropipette with a new one because it is well known that once a micropipette has caused a pulling-out event, it repeats the event. We measured how often pulling-out events occurred in the two groups.

5. Embryonic development in culture after microinjection

Since fertilized mouse eggs reach the blastocyst stage 3.5 days postcoitum, the development of the fertilized eggs was periodically observed until five days after injection. We checked the expression of the GFP gene in the embryos, but have not described the results relating to GFP expression in this paper because we were not confident at that time that all of the green light observations made in this experiment were of fluorescence emitted by GFPs.

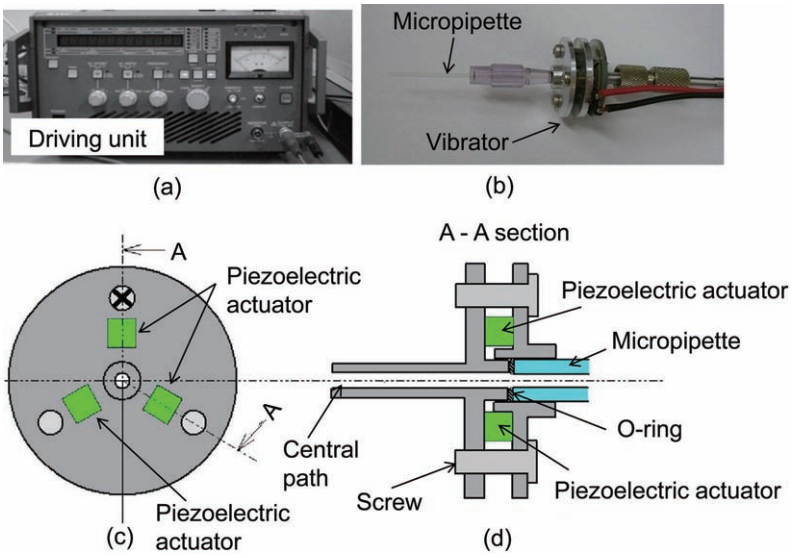

Fig. 2. Vibratory microinjection system.
(a) the driving unit, (b) the vibrator with a micropipette, (c) front view of the vibrator, (d) side view of the vibrator.

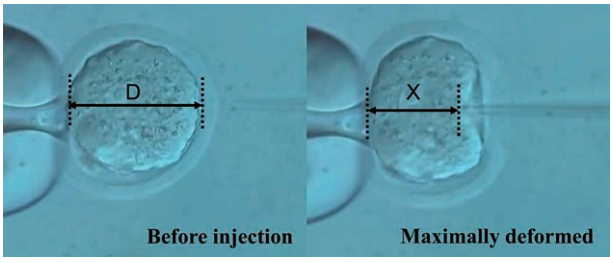

Fig. 3. Measurement of "depression rate".

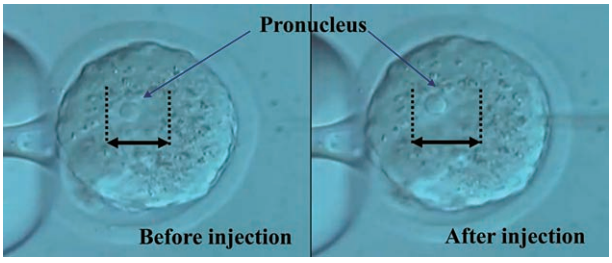

Fig. 4. Measurement of "pronuclear swelling".

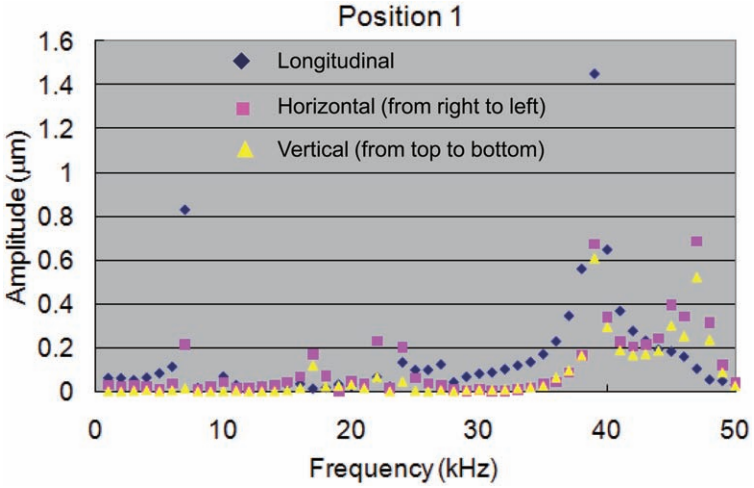

Fig. 5. Relation between amplitude and frequency at an applied voltage of 10 V peak-to-peak.
Position 1 is the position shown in Fig. 2c, namely, the actuator marked "X" was positioned at the top.

Table 1. Evaluation indices used in this experiment

	VM	OM	*P*
Depression Rate (%)	29.6 ± 5.1 (N = 210)	33.0 ± 4.8 (N = 210)	< 0.0001 Student's *t* test
Injection Time (sec)	3.39 ± 1.87 (N = 210)	4.55 ± 2.78 (N = 210)	< 0.0001 Student's *t* test
DNA injection time (sec)	1.87 ± 0.94 (N = 187)	2.65 ± 1.17 (N = 189)	< 0.0001 Student's *t* test
Ratio of pronuclear swelling	1.148 ± 0.077 (N = 187)	1.149 ± 0.070 (N = 189)	NS Student's *t* test
Pulling-out events	11 eggs (5.24%) (N = 210)	24 eggs (11.43%) (N = 210)	0.02 χ^2 test

Average ± standard deviation. VM and OM denote vibratory and ordinary microinjections, respectively. NS: not significant.

6. Statistical Analysis

The data were statistically analyzed using Student's *t*-test or χ^2 test. Statistical significance is assumed at $P < 0.05$. Data are expressed as mean ±standard deviation.

Results

The relationship between vibration frequency and amplitude

The vibration property of the vibrator was found to be slightly different according to which piezoelectric actuator was positioned at the top (Fig. 2c), although the three identical piezoelectric actuators were arranged equally around the central path. One example of the relations between frequency and amplitude is shown in Fig. 5, in which the longitudinal amplitude, together with the two lateral vibrations, is plotted against frequencies ranging from 0 to 50 kHz at intervals of 1 kHz.

Since the lateral vibrations were unintentional, we chose the frequency showing a large difference in amplitude between longitudinal and lateral vibrations. According to the data shown in Fig. 5, the best frequency is 39 kHz. However, we chose 35 kHz in this study for the following reasons. The amplitude of vibration at the tip of a micropipette connected to the vibrator would be different from that at the level of the vibrator as shown in Fig. 5. In addition, the tip amplitude would vary from micropipette to micropipette. We therefore searched around 39 kHz for the frequencies which gave rise to lateral vibrations at relatively lower voltages because the lateral vibrations, unlike the longitudinal vibrations, were visible under a microscope. Thus, we selected 35 kHz among other potential candidates as the vibration frequency in this study.

We set the applied voltage for each micropipette to a level which made unintentional lateral vibration of the micropipette tip slightly visible. The voltages ranged from 5 to 10 V p-p and this relatively wide range of the voltages applied to the vibrator indicated that the vibration property varied considerably from micropipette to micropipette even when the frequency was the same.

Measurement of "depression rate"

The depression rates were 29.6 ± 5.1% (average ±standard deviation; N=210) in the vibratory-microinjection (VM) group and 33.0 ± 4.8% (N=210) in the ordinary-microinjection (OM) group ($P < 0.0001$, Student's *t*-test, Table 1).

Measurement of "injection time"

Injection times were 3.39 ± 1.87 sec (N=210) in VM group and 4.55 ± 2.78 sec (N=210) in OM group ($P < 0.0001$, Student's *t*-test, Table 1). The VMS shortened the injection time by 1.16 sec (by 25%).

Measurement of "DNA injection time" and ratio of pronuclear swelling

Since these measurements took a lot of time and effort, we randomly selected eggs for these measurements. The DNA injection times were 1.87 ± 0.94 sec (N=187) in VM group and 2.65 ± 1.17 sec (N=189) in OM group ($P < 0.0001$, Student's *t*-test, Table 1). The VMS shortened the DNA injection time by 0.78 sec (by 30%). On the other hand, the ratios of swelling the pronuclei were almost identical: 1.148 ± 0.077 (N=187) in VM group and 1.149 ± 0.070 (N=189) in OM group (Table 1). We speculated that the volumes injected into the pronuclei were also identical in the two groups because no statistically significant difference was observed in the original

Table 2. Embryonic development in culture 4 days after microinjection

	VM		OM		*P*
	Number	Rate (%)	Number	Rate (%)	
Blastocyst	98	46.7	92	43.8	0.556
Dead	79	37.6	82	39.1	0.763
Others	33	15.7	36	17.1	
Total	210	100	210	100	

Statistical analysis was done by χ^2 test.

diameters of the pronuclei between the groups. The original diameters are not shown here because the values were measured relatively (using the number of dots on a PC screen) but not absolutely (with a real scale). Assuming that the average volume of the injected GFP solution was identical and its value was *x*, the injection speeds for both groups could be calculated by dividing *x* by the respective DNA injection times. As a result, the relative speed of the vibratory microinjection with respect to the ordinary microinjection was calculated as 1.42.

Incidence of "pulling-out" events

A total of 420 eggs were manipulated with 14 micropipettes. The substances inside a pronucleus were pulled out in 11 of 210 eggs (5.24%) in VM group whereas the pulling-out events were observed in 24 of 210 eggs (11.43%) in OM group ($P = 0.02$, χ^2 test, Table 1).

Embryonic development in culture after microinjection

In VM group, the survival rate and the mortality rate immediately after the microinjection were 77.1% (162/210) and 22.9% (48/210), respectively, and 73.8% (155/210) and 26.2% (55/210) in OM group. The survival rate was slightly higher in VM group than that in OM group ($P = 0.43$, χ^2 test).

Twenty-four hours after microinjection, the rates of developing to the 2-cell stage were 61.9% (130/210) for VM group and 58.1% (122/210) for OM group ($P = 0.426$, χ^2 test). The number of dead eggs increased in both groups, but no statistically significant difference was observed between the groups ($P = 0.754$, χ^2 test).

After 4 days of culture, 98 of 210 eggs (46.7%) in VM group and 92 of 210 eggs (43.8%) in OM group had developed to the blastocyst stage ($P = 0.556$, χ^2 test, Table 2). Seventy-nine eggs (37.6%) in VM group and 82 eggs (39.1%) in OM group were dead ($P = 0.763$, χ^2 test). VM group showed slightly better embryonic development (Table 2).

Discussion

VM group showed a significantly smaller depression rate than OM group (Table 1, Fig. 3), thereby indicating that the VMS is capable of piercing the zona pellucida and the membranes such as the cell membrane and pronuclear membrane more easily.

The DNA injection time in VM group was significantly shorter than that in OM group while the ratio of pronuclear swelling was almost identical in both groups (Table 1, Fig. 4), suggesting that the VMS has a higher speed of injecting DNA solution into pronuclei. It is well known that microinjection of large DNA constructs using BAC (bacterial artificial chromosome) vector is difficult because of their high viscosity and easy fragmentation although such large DNA constructs are considered to be promising for the production of transgenic animals. We believe that VMS may increase the success rate of injecting large DNA constructs using BAC vector.

The injection time in VM group was significantly shorter than that in OM group (Table 1). This probably results from the above-mentioned two features of VMS: easier piercing and higher injection speed. The shorter injection time furthermore indicates that the VMS can raise the working efficiency of microinjection, because the shorter injection time suggests that a larger number of zygotes can be microinjected per unit time.

VM group showed a significantly lower incidence of pulling-out events (Table 1). In this study, the same micropipette alternated between vibratory and ordinary microinjections and was used until its quota (30 eggs) was finished, even when the pulling-out event repeated before the quota was finished. It is well known that once a micropipette pulls nuclear components out, it generally repeats this pulling-out event in subsequent operations. If this rule had been the case in this study, the incidences of "pulling-out" events should have been almost identical in both groups. VM group, however, had a significantly lower incidence (Table 1). Since the sticky nuclear com-

ponents adhering to the tip of micropipette are thought to cause another pulling-out event, we consider that the application of the longitudinal ultrasonic-range vibration to the micropipette removed the nuclear components already sticking to the micropipette. The low incidence of pulling-out events indicates that the VMS can save labor through fewer changes of micropipettes as well as money when expensive, commercially-available micropipettes are used.

There was no statistically significant difference in embryonic development between the groups (Table 2). The previous version of VMS, that is, the audible-range vibratory microinjection system, showed significantly better results of embryonic development than the ordinary microinjection did [3, 4]. The expression rate of Venus gene, which was used as a reporter gene in the previous experiment, was higher in the VM group although it was not statistically significant. In addition, the previous VMS demonstrated a much smaller depression rate than the current version of VMS. These differences between the previous and current versions may arise from instability of the current vibrator, which was aimed at achieving finer tuning of amplitude than the previous version and was designed to need larger applied voltages to produce a given amplitude. Possibly due to its instability, the current version was found to produce unintentional lateral vibration at lower applied voltages than the previous version, hence preventing us from raising the applied voltage to the level that might have achieved results similar to those of the previous version.

The current version of VMS, the ultrasonic-range VMS, may not be so good as the previous version, and remains to be improved. However, the current version of VMS was capable of significantly raising the working efficiency of microinjection, and we conclude that it is an efficient option for gene transfer.

Acknowledgements

This study was supported in part by a grant of Strategic Research Foundation Grant-aided Project for Private Universities from the Ministry of Education, Culture, Sport, Science and Technology, Japan (MEXT), 2008–2012 (S0801023), and also by Research Institute for Science and Technology of Tokyo Denki University Grant Number Za06-01 / Japan.

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