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Development of Vibratory Microinjection System for Instantaneous Cell Membrane Piercing in Cytoplasmic Microinjection into Fertilized Eggs

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Abstract: To complete microinjection as quickly as possible, we have developed Vibratory Microinjection Systems (VMSs) that vibrate a micropipette in its longitudinal direction and can significantly reduce the time needed for pronuclear microinjection compared to ordinary (non-vibratory) microinjection. The longest breakdown of the time is the time required to pierce the cell membrane and the pronuclear membrane simultaneously. Because cytoplasmic microinjection, which pierces the cell membrane alone, is far more difficult and time-consuming than pronuclear microinjection, we next aimed to develop a VMS capable of penetrating the cell membrane instantly. In this new and latest version, two types of ultrasonic-wave vibrators were developed: the first for commercially available micropipettes (Femtotip) and the second for self-made micropipettes. The two vibrators differ only in their airtight structure, where the micropipettes connect to their respective vibrators: a female screw plus O-ring for the first vibrator (VMS6_1) and a silicone-rubber tube for the second (VMS6_2). The tube-type joint used in VMS6_2 only slightly damped or amplified vibrations from the vibrator to the micropipette tip, propagating them much more accurately than the screw-type joint in VMS6_1. In addition, VMS6_2 significantly shortened the time taken to pierce the cell membrane of a fertilized egg: an average of 1.52 s (N = 410) vs. 3.62 s (N = 65) in VMS6_1. The VMS6_2 group achieved a piercing time of zero in 86.1% of the allocated eggs, while only 10.8% of the VMS6_1 group did. In each vibrator, we also compared vibratory microinjection (VM; N = 475) and ordinary microinjection (OM; N = 457), which uses injection pressure in place of vibration. None of the eggs in the OM group achieved the zero-second piercing time. Compared to the OM, the VM group showed a significantly shorter piercing time, 1.80 vs. 10.69 s on average, and a significantly better survival rate, 90.3 vs. 81.8% on average. VMS6_2 not only improved on the already demonstrated superiority of VM to OM but also enabled instantaneous piercing of the cell membrane.

Keywords: Vibratory Microinjection System; instantaneous cell membrane piercing; vibration propagation mechanism; vibrator; ultrasonic vibration; cytoplasmic microinjection; self-made micropipette; Femtotip; longitudinal vibration

Citation: Miyawaki, F.; Hasegawa, J. Development of Vibratory Microinjection System for Instantaneous Cell Membrane Piercing in Cytoplasmic Microinjection into Fertilized Eggs. *Actuators* **2023**, *12*, 448. https://doi.org/10.3390/act12120448

Academic Editor: Eihab M. Abdel-Rahman

Received: 17 September 2023
Revised: 16 November 2023
Accepted: 22 November 2023
Published: 2 December 2023

1. Introduction

In order to produce transgenic animals, genetically engineered genes (foreign DNA) must be introduced into the nucleus of cells, where the DNA is integrated into the genome, although whether transgenic animals are actually born or not depends on whether at least one DNA construct is integrated into an aimed site of a target chromosome. Among several methods of introducing foreign DNA into cells, the microinjection method [1] can introduce not only DNA but also other types of macromolecules, such as RNA and protein, by injecting those macromolecules into cells. In addition, it allows us to select the site of introduction in a target cell, e.g., pronucleus or cytoplasm because the thin tip of

an injection micropipette containing such a solution is aimed at either target site inside the cell, advanced toward it, and punctured into it.

A brief description of microinjection follows. As shown in Figure 1a, only the tip of the injection micropipette is loaded with foreign DNA solution, and the rest of the micropipette, as well as the lumen of an injection holder, is filled with air pressurized at 30–50 hPa. This pressure is called 'compensation pressure' and is applied to prevent the capillary phenomenon that sucks culture media into the tip of the micropipette and thereby dilutes the macromolecule solution. Another air pressure of, for example, 150 hPa, called 'injection pressure', is sometimes applied to the air inside the injection micropipette to enable puncturing the target cell as well as the ejection of DNA solution into the cell. In addition, a pressure as high as 7000 hPa is sometimes applied to blow off a small coagula of DNA solution when it clogs the tip of the micropipette. The connection between a micropipette and an injection holder must, therefore, be airtight.

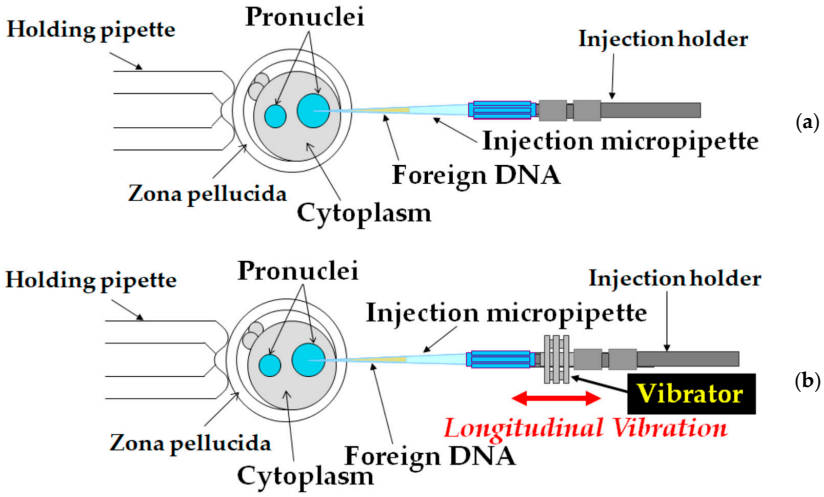

Figure 1. Pronuclear microinjection. (**a**) ordinary (non-vibratory) pronuclear microinjection; (**b**) vibratory pronuclear microinjection.

Figure 1a illustrates 'pronuclear microinjection' [2,3], which injects a small amount of foreign DNA solution into either of two pronuclei in a fertilized egg. This type of microinjection is much more popular than that which injects DNA solution into the cytoplasm (cytoplasmic microinjection) and is the most efficient in gene transfer per cell because the foreign DNA is directly introduced into a pronucleus.

In order to improve the microinjection method, we developed several versions of the Vibratory Microinjection System (VMS) [4–6], which aims to perform pronuclear microinjection by vibrating an injection micropipette longitudinally (Figure 1b). The components and working principles of the VMS are briefly described. The VMS chiefly comprises a vibrator, a driving unit (piezo driver), and a pressure device that pressurizes air inside two different air circuits and measures their respective air pressures. The vibrator that vibrates the micropipette in the longitudinal direction consists mainly of a plurality of piezoelectric elements and a housing. By applying AC voltage to the piezoelectric elements, an ultrasonic vibration is generated, which vibrates the entire vibrator. If the vibrator-level vibration is efficiently transmitted to the micropipette tip, the longitudinally vibrating tip of the micropipette should easily puncture the membrane structure of the cell.

Two other devices utilize piezoelectric actuators: the Piezo Impact Micro Manipulator® (PrimeTech, Tsutiura, Japan) and the PiezoDrill® (Vermont Optechs, Charlotte, USA). Both devices are used mainly for intracytoplasmic sperm injection, in which thick micropipettes, 8–10 µm in outer diameter of their tips, are used and inserted into the cytoplasm, whereas pronuclear microinjection needs injection micropipettes with tips as thin as approximately 1 µm in outer diameter. The Piezo Impact Micro Manipulator® moves a micropipette stepwise using an inertial force resulting from rapid deformation of piezoelectric elements [7].

To make this inertial force strong enough to move a micropipette, a mercury drop is usually put into the micropipette. The PiezoDrill works using a hydraulic mechanism and, therefore, needs to fill both a micropipette and its connecting tube with heavy hydraulic fluid (Fluorinert® or mercury). This fluid is driven by tapping the tube with a piezoelectric actuator vibrating at a very low frequency, such as 1–2 Hz [8]. The patent for the VMS [9,10] was approved both in Japan and the USA, and this proves that the VMS differs from the others in mechanism, function, and application. We developed several different types of vibrators whose structures and vibration characteristics were outlined [11].

The features of the VMS for pronuclear microinjection, compared with an ordinary (non-vibratory) pronuclear microinjection, were briefly summarized in the following three findings: (1) the VMS pierced the pronuclear membrane, together with the cell membrane, significantly more easily and quickly [6], (2) injected a DNA solution at a significantly higher speed [6], and (3) provided a significantly better development of embryos [5]. In these experiments, we used DNA constructs as small as 3.5 kb (kilobase) and 1.67 kb DNA at concentrations of 2.5 ng/μL and 2.0 ng/μL, respectively.

Thereafter, we attempted to introduce a much larger foreign DNA, up to 300 kb or more, constructed with bacterial artificial chromosomes (BAC) as a vector. Since this BAC DNA construct is capable of containing plural sets of genes, it is feasible to create transgenic animals with the expression of a cassette of transgenes. In the case of BAC, it is known that using polyamine buffer instead of the conventional TE buffer (10 mM Tris-HCl, pH 7.5, 0.25 mM EDTA) is more effective in preventing degradation of high molecular weight DNA and increases recombination efficiency [12]. However, since the molecular weight of BAC is much larger, it is known to often clog micropipettes, and the recommended concentration is 0.5 ng/μL [13].

We found another serious problem even at such a low concentration of BAC DNA. Lethal cell damage caused by a "pulling-out" event, in which a micropipette pulls a part of nuclear components such as RNA and/or DNA out of the cell immediately after pronuclear microinjection occurred more frequently than when the small DNA constructs were used. The "pulling-out" event not only leads to cell death but also contaminates the tip of a micropipette with sticky nuclear components, thereby repeating the event in the following pronuclear microinjections. Once this event occurs, the micropipette has to be replaced. Furthermore, since the replacement is rather a laborious process, "pulling-out" events must be avoided wherever possible.

The only way to reliably avoid such a fatal and troublesome event is cytoplasmic microinjection. However, the recombination efficiency of microinjection into the cytoplasm is much lower than that of direct injection into the pronucleus because DNA injected into the cytoplasm must pass through the pronuclear membrane to move into the pronucleus. Therefore, we hypothesized that if a large amount of BAC DNA at a high concentration is already present around the site where the two pronuclei fuse and the pronuclear membrane disappears, these BAC DNAs would be more easily incorporated into the genome because the DNA does not need to pass through the pronuclear membrane. We, therefore, theorized that the above idea could be realized if we could develop a vibrator that was able to easily inject a concentrated solution of DNA constructs as large as 300 kb into the cytoplasm of fertilized eggs.

We then developed a fourth version of the vibrator that can easily microinject very large BAC DNA (>300 kb) into the cytoplasm at high concentration and confirmed that the above cytoplasmic microinjection could be achieved by microinjecting it into the narrow gap just before the fusion of the two pronuclei. This gene transfer was also confirmed to be effective and was named "inter-pronuclear cytoplasmic microinjection" [14]. Furthermore, we also examined what was optimal for BAC DNA at four different concentrations: 2.5, 5.0, 7.5, and 10.0 ng/μL and found that 7.5 ng/μL was optimal [14]. However, since the structure of the fourth version of the vibrator was quite complex, we attempted to simplify its structure and developed the sixth version of the vibrator [15].

The previous vibrators, including the sixth version [15], were developed for commercially available micropipettes, specifically Femtotip® (Eppendorf, Germany; Figure 2a). We used Femtotip because we trust their production control to keep the outer diameter of its tip within an error range of ±0.5 μm. The size of the tip of a micropipette can heavily influence the following factors: the readiness to insert a micropipette into a fertilized egg, the speed of injecting DNA solution, injurious effects based on microinjection, and others. For example, a larger bore tip can accelerate the speed of injecting DNA solution, thereby increasing the volume of the solution being injected. Therefore, since the VMSs must be evaluated with a fair comparison with normal (non-vibratory) microinjection, the most important requirement was to make the tip size difference as small as possible. Therefore, since it is impractical to use only the self-made micropipettes that are elaborated to approximately the same degree, we thought that Femtotips would eliminate or reduce experimental errors based on tip size variation.

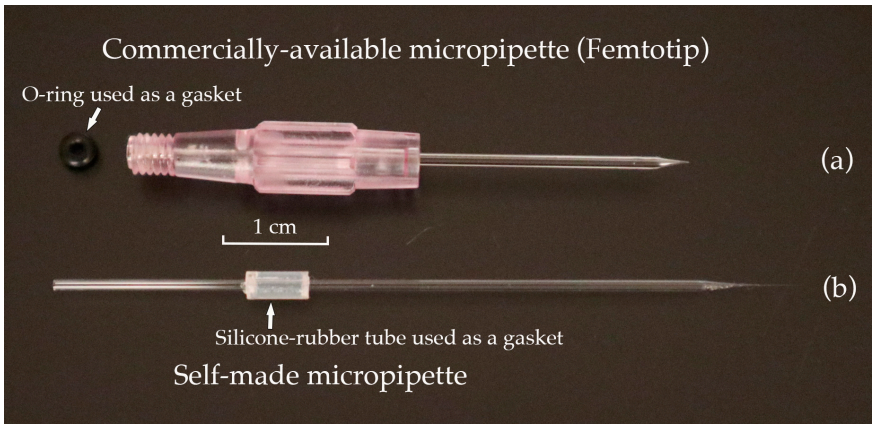

Figure 2. Two types of micropipettes. (**a**) a commercially-available micropipette (Femtotip). Its rear end has a male-screw structure, and a rubber O-ring is used as a gasket.; (**b**) a self-made micropipette. A silicone rubber tube is used as a gasket.

We switched from Femtotips to self-made micropipettes (Figure 2b), which are much more inexpensive and popular than Femtotips, because we have sufficient comparative data to demonstrate the superiority of vibratory microinjection over ordinary microinjection. The self-made micropipettes are prepared as follows. A glass capillary 1 mm in outer diameter is pulled with a puller while the middle part of the glass capillary is heated, and the gradually tapering middle part is sharply cut when it becomes thin enough to be appropriate for the tip of a micropipette.

When we planned to develop a vibrator suitable for a self-made micropipette, we decided to modify the sixth version of the vibrator that had been designed for Femtotip so that it could be connected to a self-made micropipette. In this study, we measured the speed of puncturing the cell membrane during cytoplasmic microinjection and the survival of fertilized eggs after microinjection. These two factors were compared not only between vibratory and ordinary microinjections but also between the two VMSs: the VMS with the sixth version of the vibrator connected to a Femtotip and that with the sixth version of the vibrator connected to a self-made micropipette.

2. Materials and Methods

2.1. Development of Two Sixth Version Vibrators

The vibrator for Femtotips was named VMS6_1 (Figure 3a), and the vibrator for self-made micropipettes was named VMS6_2 (Figure 3b). These two vibrators of version 6 were almost identical in the essential part associated with three multilayer piezoelectric actuators (PAC-133C, NGK SPARK PLUG Co., Ltd., Nagoya, Japan) and the housings of the vibrator. Like a bolt-clamped Langevin-type transducer, the three multilayer piezoelectric actuators were sandwiched between the front and the rear housings made of duralumin;

the size of the housing was 24.0 mm in diameter and 10.0 mm in thickness. The periphery of the housing was tightened more strongly than the center of the housing so as to obtain as much vibration displacement as possible at the center of the front housing. In addition, the dimensions of the piezoelectric actuators and the three components of the housing, i.e., front, rear, and side, were designed to be sufficiently smaller than the wavelength to suppress both resonance vibration and anti-resonance vibration. We continued to use this sandwich configuration from the second version of the vibrator to the sixth and latest version.

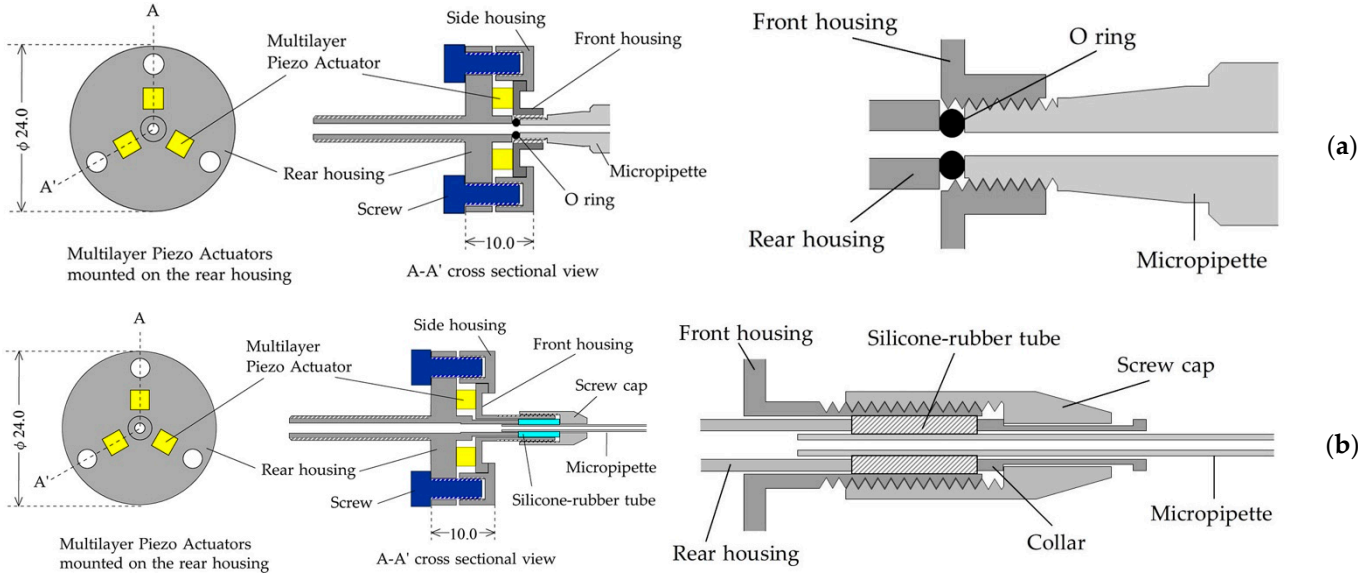

Figure 3. Schematic diagrams of the structure for the two vibrators used in this study. (**a**) the vibrator (VMS6_1) for a commercially available micropipette (Femtotip); (**b**) the vibrator (VMS6_2) for a self-made micropipette.

The two vibrators differ only in their airtight structure, where the micropipettes connect to their respective vibrators. The joint structure between VMS6_1 (Figure 3a) and the rear end of Femtotip (Figure 2a) is common to all the vibrators of versions 2 to 5. We confirmed that the presence of an ordinary rubber O-ring worked as a gasket to seal the screwed joint structure because the screw–joint structure without an O-ring was not airtight.

In the case of the joint structure between VMS6_2 and a self-made micropipette, we used ordinary products that are widely used when a self-made micropipette is connected to an ordinary injection holder (Figure 1a): a silicone rubber tube (Figure 2b; HI01PK01, NARISHIGE, Tokyo, Japan) and a screw cap with a collar (Figure 3b) for an injection holder (HI-9, NARISHIGE, Japan). In this case, the silicone rubber tube firmly holding the shaft of a self-made micropipette (Figure 2b) functions as a gasket.

2.2. Vibration Characteristics

2.2.1. Vibration Characteristics on the Level of a Vibrator

Longitudinal vibrations at the front edge of the front housing of each vibrator were measured at a vibration frequency from 97.6 Hz to 50 kHz at an interval of 97.6 Hz with an optical fiber displacement sensor; each vibrator was driven by an alternating voltage of 10 Vpp with an offset voltage of 10 V.

2.2.2. Vibration Characteristics on the Tip of a Micropipette

To evaluate how the vibrations at the front edge of the two vibrators propagated to the tip of their respective micropipettes, we tried to measure the movements of the tip of each micropipette. However, the oscillatory motion of the bare tip, which had an outer diameter of about 0.5~0.7 μm, could not be detected at frequencies in the ultrasonic region. Therefore,

we measured the motion of a small, lightweight object attached to the bare tip as a reference value, although it is different from the vibration characteristics of the actual micropipette.

We constructed 2 mm cubes from UV curable resin with a 3D printer and glued stainless steel foil on those surfaces to make 2 mm cubic mirrors with a weight of approximately 10 mg. After one of the cubic mirrors was glued to the tip of each micropipette, we measured three-axial vibrations at the cubic mirror with three optical fiber displacement sensors. These measurements were made at a frequency from 30 to 50 kHz, the frequency band used for microinjection, at intervals of 12.2 Hz; each vibrator was driven by an alternating voltage of 10 Vpp with an offset voltage of 10 V.

2.3. Cytoplasmic Microinjection

2.3.1. Preparation for Animal Experiments

The BAC DNA used in this study was confirmed to be as large as approximately 340 kb (kilobase: a unit equal to 1000 base pairs of DNA or RNA) or more with pulsed-field gel electrophoresis (Figure 4).

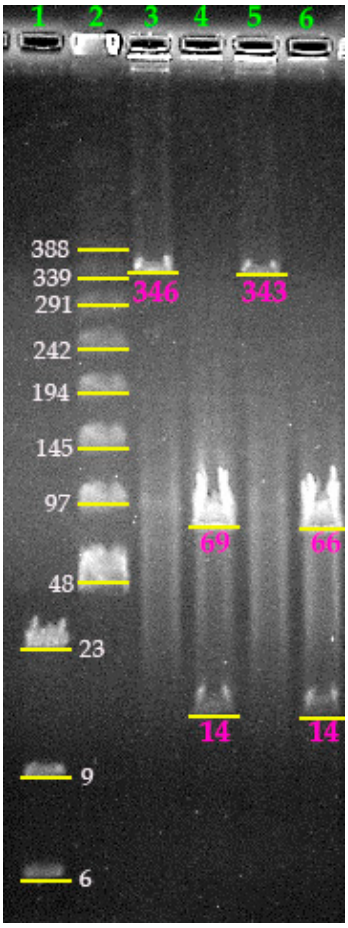

Figure 4. Pulsed-field gel electrophoresis. Lane numbers 3 and 5 show the positions of the original BAC DNA used in this study, indicating that the size of the BAC DNA is approximately 346 or 343 kb. Lane numbers 4 and 6 represent DNA fragments in which the original BAC DNA has been cleaved by a restriction enzyme such as Not I. Lane numbers 1 and 2 indicate the location of the small and large markers, respectively.

Lane numbers 1 and 2 indicate the results of standard markers, the sizes of which are known. The yellow lines indicate their respective positions, and the number shown next to each yellow line is the size (kb) of the marker.

Lane numbers 3 and 5 show the positions of the original BAC DNA used in this study, indicating that the sizes of the BAC DNA are approximately 346 and 343 kb, respectively. Since these two sizes were almost identical, the results from the electrophoresis were considered to be reproducible. Lane numbers 4 and 6 represent DNA fragments in which the original BAC DNA has been cleaved by a restriction enzyme such as Not I. The results were also reproducible.

We used the BAC DNA at a concentration of 7.5 ng/μL [14], which was 15 times thicker than the recommended concentration of 0.5 ng/μL [13].

A total of 1001 fertilized eggs collected from 40 8-week-old female BDF1 mice were enrolled in this study. Experiments were conducted on 23 experiment days. A total of 152 eggs out of the 1001 eggs were microinjected with 4 Femtotips on 4 experiment days (1 Femtotip on each day), and the remaining 849 eggs were treated with 19 self-made micropipettes on 19 experiment days (1 self-made micropipette on each day). On each experiment day, the collected eggs were divided into 2 sets at the maximum. The eggs in each set were put in a drop of M2 culture medium in a Petri dish (Figure 5a,b), and the drop was covered with mineral oil which prevented evaporation of water from the drop of culture medium. The temperature of the Petri dish was kept at 37 °C with a plate-type heater (Thermo Plate, TOKAI HIT, Fujinomiya, Japan; Figure 5a,b).

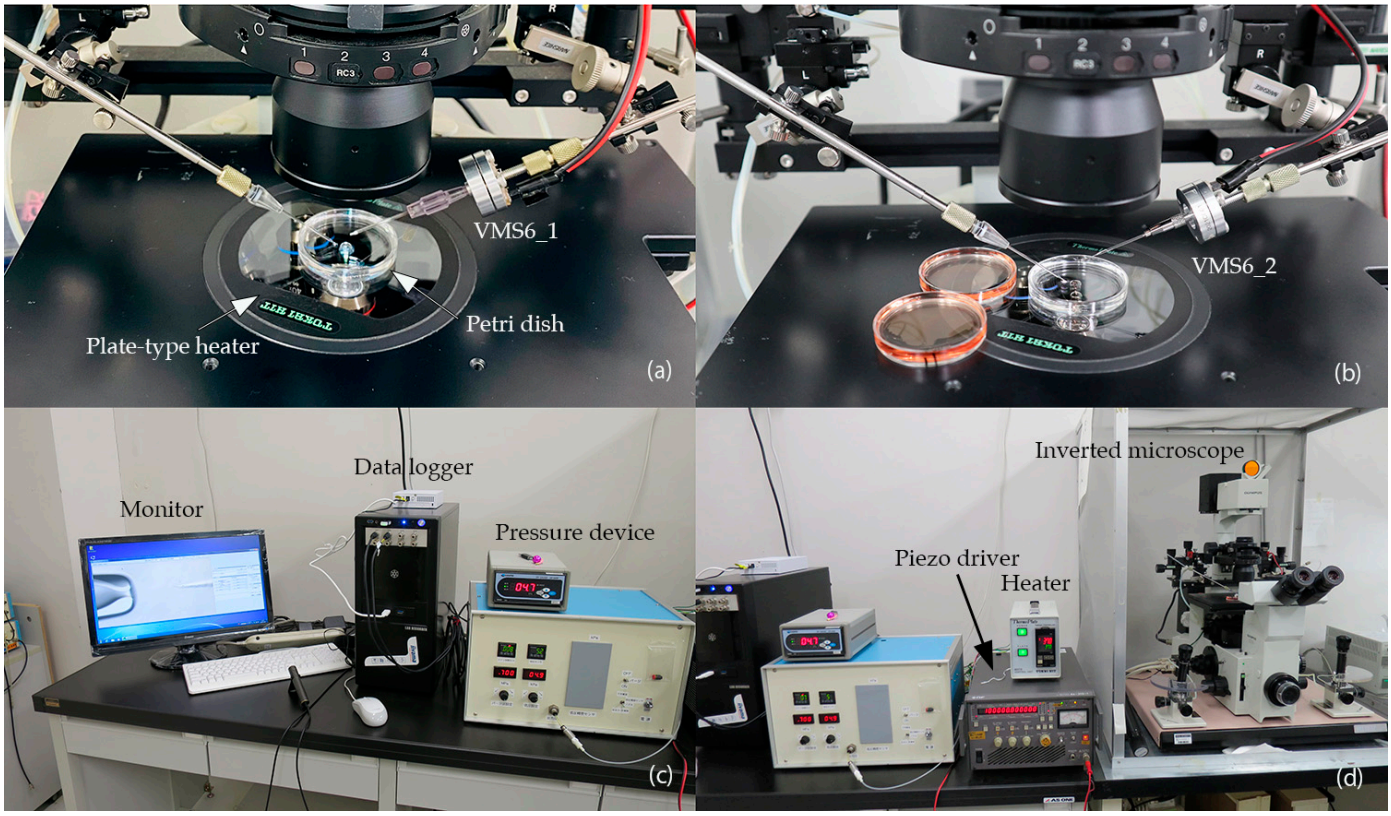

Figure 5. Laboratory equipment. (**a**) VMS6_1 for a Femtotip, a plate-type heater, and a Petri dish; (**b**) VMS6_2 for a self-made micropipette; (**c**) an order-made pressure device and a data logger; (**d**) a piezo driver, a heater, and an inverted microscope.

The eggs in the M2 drop were alternately treated with vibratory or ordinary (non-vibratory) microinjection using one injection micropipette. In order to enable the alternate microinjections with only one injection micropipette, the maximum number of eggs was limited to 30 in each set. The vibrator was set between a micropipette and an injection holder (Figures 1b and 5a,b). When the vibratory microinjection was performed, the vibrator was activated with a piezo driver (As-310-1, NF Corporation, Yokohama, Japan; Figure 5d), but no injection pressure was applied. On the other hand, in the case of ordinary

microinjection, an appropriate value of injection pressure, which varies depending on the bore size of the micropipette, was set for each micropipette and applied with an order-made pressure device (Figure 5c) while the vibrator was switched off. A compensation pressure of approximately 50 hPa was applied constantly with the pressure device in both microinjections to prevent the culture medium from entering the tip of the micropipette due to the capillary phenomenon.

The process of all the microinjections was shot and recorded at 30 frames/second with a data logger (VCC-H2500C, Digimo, Tokyo, Japan; Figure 5c) using a high-vision CCD camera. Along with the video recording, both the injection and compensation pressures were measured and recorded at a sampling rate of 50 kHz using software (Synchromotion Viewer version 10.019, Digimo, Tokyo, Japan). Since the maximum sampling rate for one system of pressure measurement in this data logger is 100 kHz, the maximum sampling rate for each pressure is 50 kHz when two systems of pressure are measured simultaneously. These two pressures were generated in two different air circuits in the order-made pressure device (Figure 5c) and, at the same time, measured with a high-precision sensor installed in each circuit.

2.3.2. Evaluation Indices of Cytoplasmic Microinjections

- "Piercing time"

We measured "piercing time", which was defined in this study as the time taken to pierce the cell membrane of a fertilized egg, from a digital video shot at 30 frames/second with the data logger. The protocol for measuring the piercing time was as follows. The tip of a micropipette was advanced toward the egg until the cell membrane was indented deeply to over half of the original size of the egg. Then, in the case of vibratory microinjection, a foot switch of the piezo driver (Figure 5d) was turned on, and this moment was defined as the start of piercing time. In the case of ordinary microinjection, the start of piercing time was defined as the moment when the injection pressure was applied. Since the data logger was set to sample video and pressure at sampling rates of 30 Hz and 50 kHz, respectively, one frame of video has approximately 1666 values of pressure. Although the pressure resolution was much higher than that of the video, the moment when the injection pressure was actually applied was determined at the same resolution as the video, namely 33.3 msec. The end of piercing was defined as the moment when we confirmed a visible change in the structure or color tone of cytoplasm.

- Survival after microinjection

After all the fertilized eggs allotted in each set had been injected, we examined if they were alive or not.

- Statistical analysis

Statistical significance was assumed at p less than 0.05. Student's t-test and Pearson's chi-square test were applied for statistical analysis of piercing time and survival after microinjection, respectively.

- Ethical approval

The animal experiments were conducted at Tokyo Denki University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

3. Results

3.1. Development of Two Sixth Version Vibrators

We developed two types of vibrators (Figure 6) according to the designs shown in Figure 3.

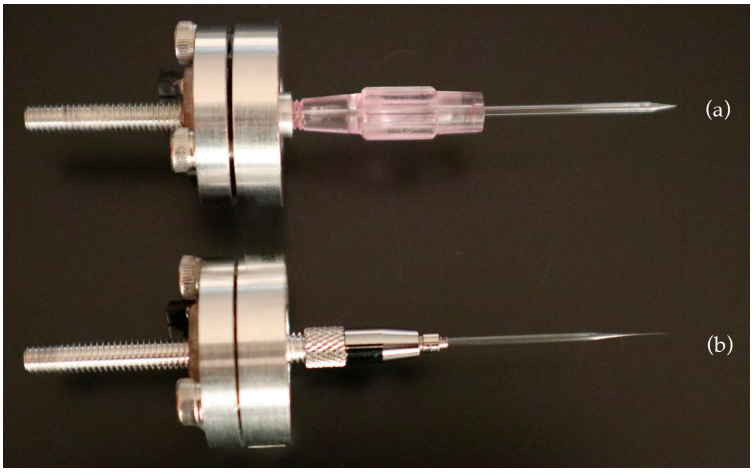

Figure 6. Two types of vibrators, version 6, in this study. (**a**) the vibrator named VMS6_1 for a commercially-available micropipette (Femtotip); (**b**) the vibrator named VMS6_2 for a self-made micropipette.

3.1.1. Vibration Characteristics on the Level of a Vibrator

The vibration characteristics of the two vibrators are shown by plotting the longitudinal displacements on the vertical axis and the vibration frequencies on the horizontal axis at an interval of 97.6 Hz (Figure 7).

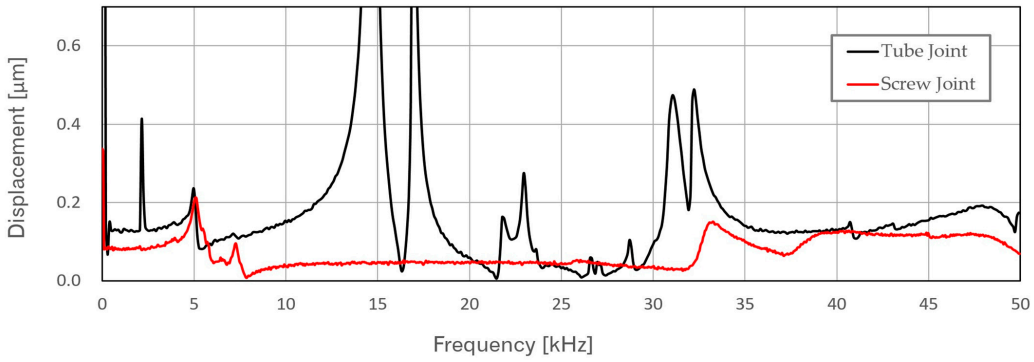

Figure 7. Vibration characteristics of two types of vibrators. Tube Joint: the vibrator (VMS6_2) for a self-made micropipette; Screw Joint: the vibrator (VMS6_1) for a Femtotip.

Both vibrators exhibited relatively flat characteristics over the frequency range from 35 kHz to 50 kHz, with no extremely high peaks indicative of resonance vibration. These flat characteristics were what we had aimed for. Because the first version of the vibrator was driven at a resonance frequency, it was frequently too strong for microinjection [4]. In addition, the use of resonance vibration was unrealistic because the driving frequency and applied voltage needed to be fine-tuned according to differences in types of micropipettes, individual differences in micropipettes of the same type, the volume and concentration of DNA solution in a micropipette, and other variations. Therefore, we aimed to devise and develop a vibrator that would be effective for microinjection over a wide frequency range.

In the case of the tube joint (VMS6_2), two large peaks (9.44 μm at 14.65 kHz and 1.21 μm at 16.89 kHz) were observed outside the microinjection frequency range, and two smaller peaks (0.47 μm at 31.05 kHz and 0.49 μm at 32.23 kHz) were found in the frequency range of 30 to 50 kHz used for microinjection.

Such a peak was not seen in the case of the screw joint (VMS6_1). Since the only major structural difference between the two was the front housings, these peaks were considered to be due to the thin pipe part of the micropipette attachment (Figure 3b). However, since the length of this part is about 1/30th of one wavelength, it cannot be explained by ordinary resonance. Numerical analysis is now underway to investigate its cause.

3.1.2. Vibration Characteristics on the Tip of a Micropipette

Figure 8a,b shows the measurement scenes of a Femtotip and a self-made micropipette, respectively. The orientation of their 3D coordinates is shown in Figure 8c, where the *Z* axis is the longitudinal vibration direction of the micropipette, while the X and Y axes are the lateral vibration directions.

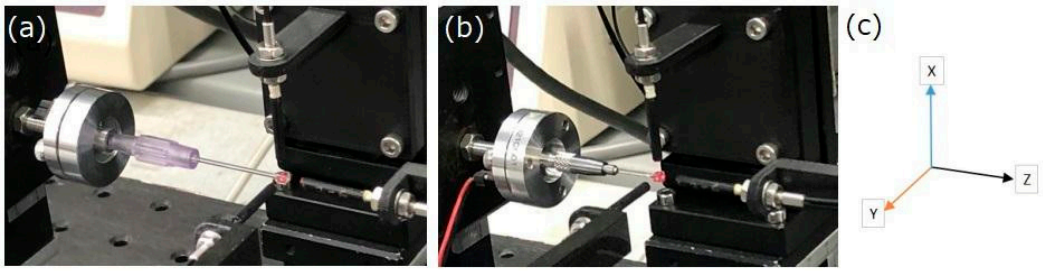

Figure 8. Measurement of vibrations at the tip of two types of micropipettes from three-dimensional directions with the help of a 2 mm lightweight cube. (**a**) Femtotip with a cube; (**b**) Self-made micropipette with a cube; (**c**) Orientation of the 3D coordinates.

Figure 9 shows the 3D vibration characteristics of a 2 mm lightweight cube attached to the tip of a Femtotip driven by VMS6_1 in the microinjection frequency region from 30 to 50 kHz at an interval of 12.2 Hz. The vibration in the *X*-axis direction (lateral vibration), i.e., unintentional vibration, was larger than the intentional vibration in the *Z*-axis direction (longitudinal vibration), and relatively large displacements in both directions were observed between 41.5 to 46 kHz; the peaks were 3.86 µm at 43.31 kHz for the lateral vibration and 2.70 µm at 43.14 kHz for the longitudinal one.

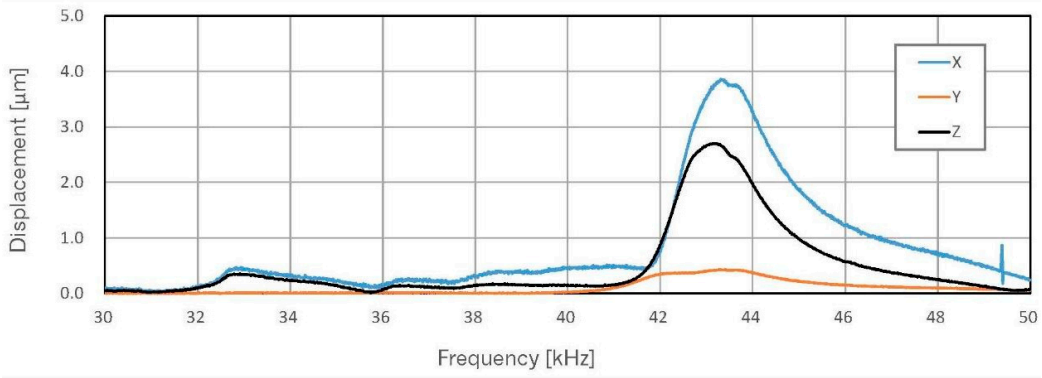

Figure 9. Three-dimensional vibration characteristics of a 2 mm lightweight cube attached to the tip of a Femtotip.

In the case of the 3D vibration characteristics of a 2 mm lightweight cube attached to the tip of a self-made micropipette driven by VMS6_2 (Figure 10), the intentional longitudinal vibration in the *Z*-axis direction was larger than the unintentional lateral vibrations in the *X*-axis and *Y*-axis directions, and the characteristics were relatively flat except for 32 to 34 kHz; the peaks were 0.66 µm at 32.15 kHz for the longitudinal vibration and 0.50 µm at 32.19 kHz for the lateral one (*X*-axis).

To conveniently evaluate the vibration propagation from the vibrator to the tip of a micropipette, the *Z*-axis displacement at the tip level (Dz_T), i.e., the cube, was divided by the *Z*-axis displacement at the vibrator level (Dz_V) at each frequency from 30.1 to 49.9 kHz at 97.6 Hz intervals. The value was termed 'propagation rate' (PR).

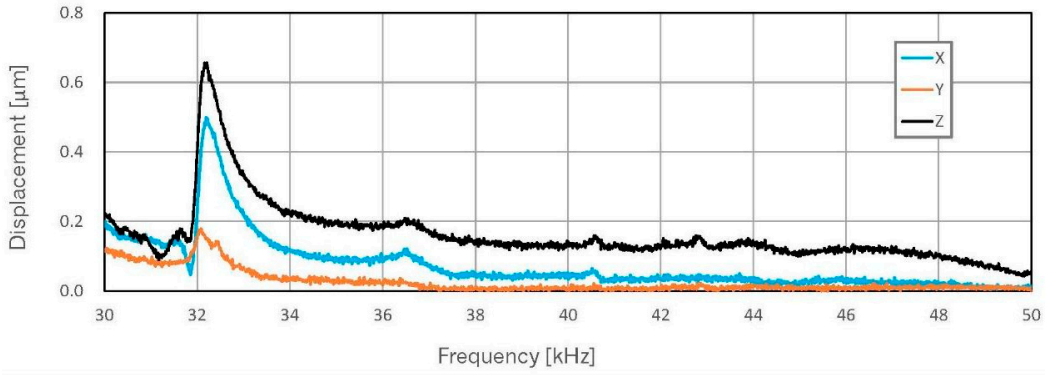

Figure 10. Three-dimensional vibration characteristics of a 2 mm lightweight cube attached to the tip of a self-made micropipette.

For the combination of the Femtotip and the VMS6_1 vibrator, the PR was 4.309 ± 5.704 (average ± standard deviation, N = 204) in the frequency range of 30.1 – 49.9 kHz (Table 1), suggesting that the propagation was amplified by a factor of 4.3. A finer analysis shows that in the frequency region from 30.1 to 41.3 kHz (Figure 9), where the *Z*-axis displacement at the tip level was relatively smaller than in the rest of the region, the average PR was 1.571 (±0.609, N = 116); in the remaining frequency region from 41.4 to 49.9 kHz, where Dz_T was larger (Figure 9), the average PR was 7.919 (±7.229, N = 88).

Table 1. Comparison in vibration propagation between two joint structures.

Joint Structure	Vibration Propagation Structure	Frequency Range (kHz)	*Z*-Axis Displacement at the Tip: Dz_T (µm)	*Z*-Axis Displacement at the Vibrator: Dz_V (µm)	Propagation Rate (Dz_T/Dz_V)	*p* (*t*-Test)
Screw + O-ring (Femtotip)	Screw	30.1~49.9	0.478 ± 0.680 ^{Sa}	0.100 ± 0.030 ^{Sb}	4.309 ± 5.704 ^{Sc}	
		30.1~41.3	0.143 ± 0.081	0.091 ± 0.037	1.571 ± 0.609	
		41.4~49.9	0.919 ± 0.850	0.112 ± 0.011	7.919 ± 7.229	$^{Sa\text{-}Ta}$ < 0.0001
Silicone rubber tube (Self-made)	Tube	30.1~49.9	0.162 ± 0.092 ^{Ta}	0.172 ± 0.077 ^{Tb}	0.985 ± 0.369 ^{Tc}	$^{Sb\text{-}Tb}$ < 0.0001
		30.1~34.5	0.263 ± 0.139	0.264 ± 0.115	1.115 ± 0.476	$^{Sc\text{-}Tc}$ < 0.0001
		34.6~49.9	0.131 ± 0.034	0.144 ± 0.022	0.945 ± 0.323	

Average ± standard deviation. Sa-Ta denotes the *p* value of the *t*-test between Dz_T in Screw Joint and Dz_T in Tube Joint. Sb-Tb denotes that between Dz_V in Screw Joint and Dz_V in Tube Joint. Sc-Tc denotes that between the propagation rate in Screw Joint and the propagation rate in Tube Joint.

For the combination of the self-made micropipette and the VMS6_2 vibrator, two peaks observed in the D_Z_V (Figure 7) were nearly identical to the peak observed in the D_Z_T (Figure 10). The average PR was 0.985 (±0.369, N = 204; Table 1) in the frequency range of 30.1 – 49.9 kHz, suggesting that the vibrator-level vibrations propagated well to the tip level without being amplified, although slightly attenuated. In the frequency region of 30.1 and 34.5 kHz, where Dz_V formed two peaks (Figure 7), the average PR was 1.115 (±0.476, N = 47), suggesting fairly good propagation with slight amplification. In the remaining frequency region, the average PR was 0.945 (±0.323, N = 157), resulting in good propagation with slight attenuation.

3.2. Cytoplasmic Microinjection

3.2.1. Detailed Records of Cytoplasmic Microinjections

A total of 152 fertilized eggs were microinjected on 4 experimental days with one Femtotip each day. Twenty-six eggs were excluded from the following analysis because those eggs had been pierced and injected before either vibration or injection pressure was applied. Thereby, 65 eggs were treated with vibratory microinjection using VMS6_1 (VM1

group), and 61 eggs were treated with ordinary microinjection using an average injection pressure of 175 hPa ranging from 137 to 226 hPa (average ± standard deviation: 175 ± 39) (OM1 group).

A total of 849 fertilized eggs were microinjected on 19 experimental days with one self-made micropipette each day. Forty-three eggs were excluded for the above-mentioned reason. The vibratory microinjection using VMS6_2 was conducted on 410 eggs (VM2 group), and the ordinary microinjection using an average injection pressure of 152 hPa, which ranged from 110 to 232 hPa (152 ± 30), was performed on 396 eggs (OM2 group).

The method of finding a combination of vibration frequency and an applied voltage suitable for vibratory microinjection is as follows. The movement of a micropipette is checked within the vibration frequencies of 30 kHz to 50 kHz at an interval of 1 kHz at an applied voltage of 10 Vpp every time we start using a micropipette. Firstly, we check the lateral movements of a micropipette. Since the movement is injurious to eggs, we exclude the frequencies that cause lateral movements. Thereafter, we aim to choose one frequency from the remaining frequencies and, in addition, search for a suitable applied voltage basically within 10 Vpp, but sometimes up to 15 Vpp. However, because longitudinal vibrations (movements) are invisible, we cannot confidently determine which combination of vibration frequency and applied voltage is best suited for vibratory microinjection. Furthermore, we must choose a particular combination as soon as possible because the time spent on actual microinjections is limited. As a result, we chose a particular combination using a kind of intuition born of long experience and tested the effectiveness of the vibration condition on a few unfertilized eggs, which are always present in collected eggs. Since this test does not always work, we are sometimes forced to stop the preliminary step without any acceptable vibration condition and proceed to actual microinjections. Even if we find a good combination, we sometimes have to change it during actual microinjections, possibly because of a change in acoustic impedance of the micropipette when its tip becomes dirty and/or becomes narrowed with cytoplasmic substances as microinjections repeat.

We also searched for an injection pressure suitable for ordinary microinjection on every experiment day. This is adjusted by observing the speed of injecting DNA into the cytoplasm of a few unfertilized eggs. However, we sometimes had to change the initially determined injection pressure because of a narrowing of the tip of the micropipette.

We have listed all the vibration conditions and injection pressures and others in Appendix A. On some experiment days, as shown in Appendix A, the vibration conditions and injection pressures changed from the initial settings. Especially on 1 October 2021, we could not find any good vibration conditions. Slight changes of less than 10 hPa in injection pressure between sets 1 and 2 were frequently observed, but those changes were because of the limitations of setting and adjusting low injection pressure in the order-made pressure device (Figure 5). The air circuit for injection pressure is designed to generate pressure as high as 7000 hPa but not to allow for the setting of pressures smaller than 10 hPa.

3.2.2. Evaluation Indices of Cytoplasmic Microinjections

- "Piercing time"

Sequential images of a typical case for each of the 4 groups are demonstrated in Figures 11–14.

Figure 11 shows a case of ordinary microinjection with a Femtotip (OM1 group) at an injection pressure of 156 hPa. The reason we chose this case was that its piercing time was 10.97 s, which was the closest to the average piercing time of 9.58 s among 61 cases in the OM1 group. Figure 11a captures an image just before the application of the injection pressure. Some pressures were already recorded in the panel of injection pressure, but those were residual pressures inside the air circuit. Figure 11b shows the moment when the injection pressure is applied. The red arrow indicates a sudden increase in pressure from the residual pressure level. Figure 11b was shot just one frame (33.3 msec) after Figure 11a. The yellow arrow in Figure 11c indicates an accumulation of the DNA solution ejected from the tip of the micropipette. The DNA solution was still present outside the egg and formed

an oval indentation of the cell membrane. This type of accumulation is called a "bubble" in the field of microinjection. The bubble formation is a sign that the cell membrane has not been penetrated. Figure 11d captures the moment of piercing. The green arrow indicates a slight change in the pattern of the cytoplasm. The bubble of DNA did not visibly shrink at this moment but disappeared immediately after this frame. After a sufficient amount of DNA was injected into the cytoplasm, the micropipette was withdrawn, as shown in Figure 11e (495 msec after the achievement of piercing).

The video used to create Figure 11 has been uploaded as Supplementary Video S1.

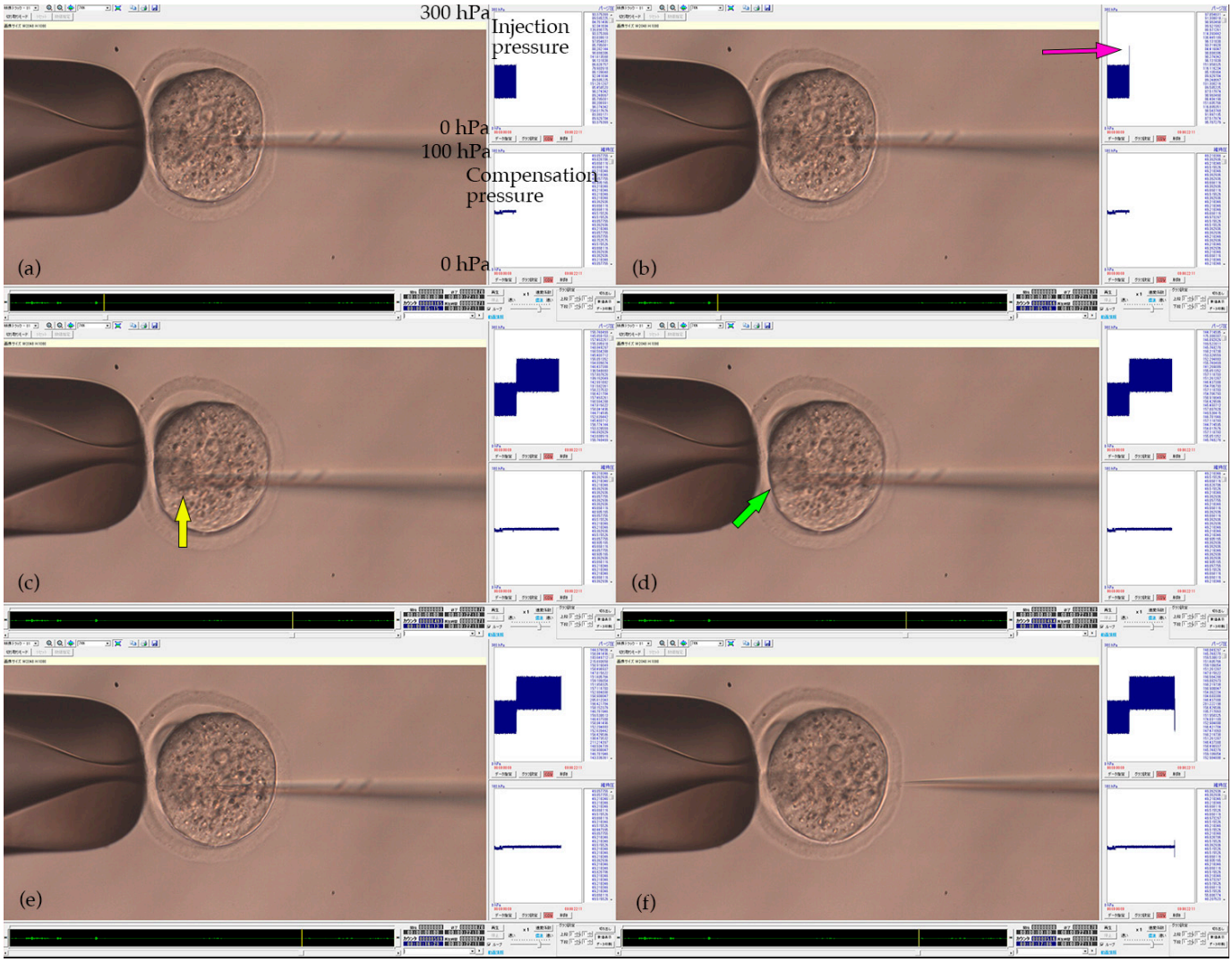

Figure 11. Ordinary microinjection with a Femtotip (OM1 group) at an injection pressure of 156 hPa. (**a**) Image just before application of the injection pressure, although some residual pressures inside the air circuit have been recorded; (**b**) Image at the moment when the pressure is applied. The red arrow indicates a sudden increase in pressure: one frame (33.3 msec) after (**a**); (**c**) One frame before piercing the cell membrane. A bubble of DNA solution is indicated by the yellow arrow; (**d**) Image at the moment of piercing. The green arrow indicates a slight change in the pattern of the cytoplasm. The bubble of DNA does not visibly shrink. The piercing time is 10.97 s; (**e**) Image 495 msec after (**d**). After a sufficient amount of DNA has been injected into the cytoplasm, the micropipette is being withdrawn; (**f**) Complete withdrawal of the micropipette.

The case of vibratory microinjection with a Femtotip driven by VMS6_1 (VM1 group) is shown in Figure 12. The piercing time in this case was 3.77 s and was the closest to the average piercing time of 3.62 s among 65 cases in the VM1 group. An oval-shaped bubble of DNA solution was observed more clearly than in Figure 11c (Figure 12c), and this image was shot one frame before piercing. After one frame (33.3 msec), the size of the bubble shrank, and slight deformation of the cytoplasm was visible (Figure 12d). Figure 12e, 825 msec after the moment of piercing, captures the moment when the micropipette starts to be withdrawn after a sufficient amount of DNA has been injected into the cytoplasm.

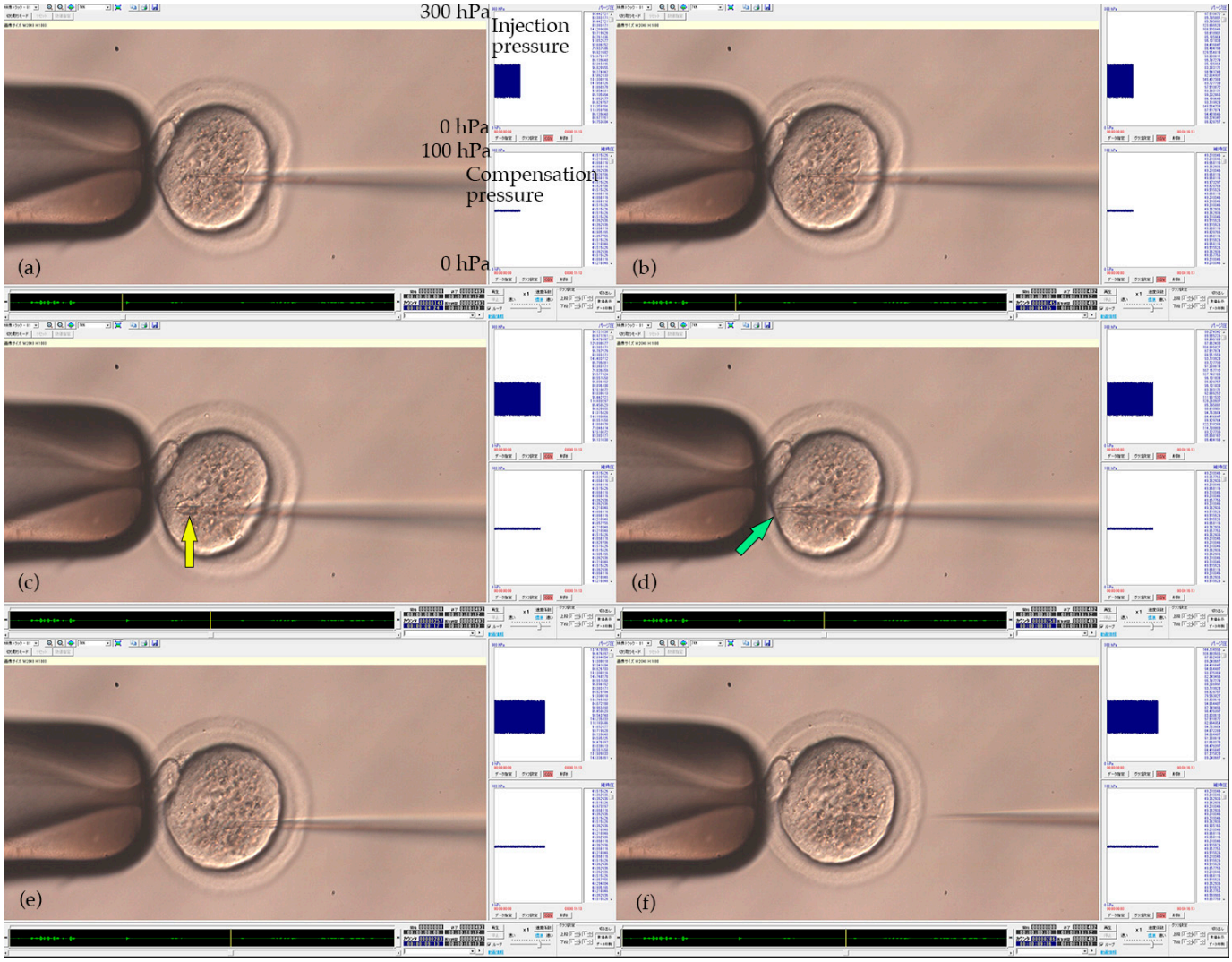

Figure 12. Vibratory microinjection with a Femtotip driven by VMS6_1 (VM1 group). (**a**) Image just before switch-on; (**b**) Image at the moment of switch-on: one frame (33.3 msec) after (**a**); (**c**) One frame before piercing the cell membrane. Note that a large bubble of DNA solution is indicated by the yellow arrow, and no change in compensation pressure is observed; (**d**) Image at the moment of piercing. The green arrow indicates slight deformation of the cytoplasm together with shrinkage of the bubble of DNA. The piercing time is 3.77 s; (**e**) Image 825 msec after the piecing (**d**). The moment when the micropipette starts to be withdrawn after a sufficient amount of DNA has been injected into the cytoplasm; (**f**) Complete withdrawal of the micropipette.

Since no injection pressure was used in the vibratory microinjection, the bubble formation also suggests that the ultrasonic vibration might have generated some energy that was able to eject some amount of the thick DNA solution from the micropipette. No substantial change in pressure was observed in the panel of compensation pressure (Figure 12), but this does not serve as evidence that the vibration did not generate any pressure because the vibration frequency, in this case, was 39 kHz and sampled at a rate of 50 kHz, which is the maximum sampling rate to measure both the injection and compensation pressures. In preliminary studies, however, we confirmed that ultrasonic vibration produced no change in pressure inside a micropipette by measuring the pressure at sampling rates that followed the sampling theorem (Nyquist theorem), for example, a sampling rate of 100 kHz, which is the maximum sampling rate for one system of pressure measurement in the data logger used in this study, and a vibration frequency of 50 kHz or less.

The video used to create Figure 12 has been uploaded as Supplementary Video S2.

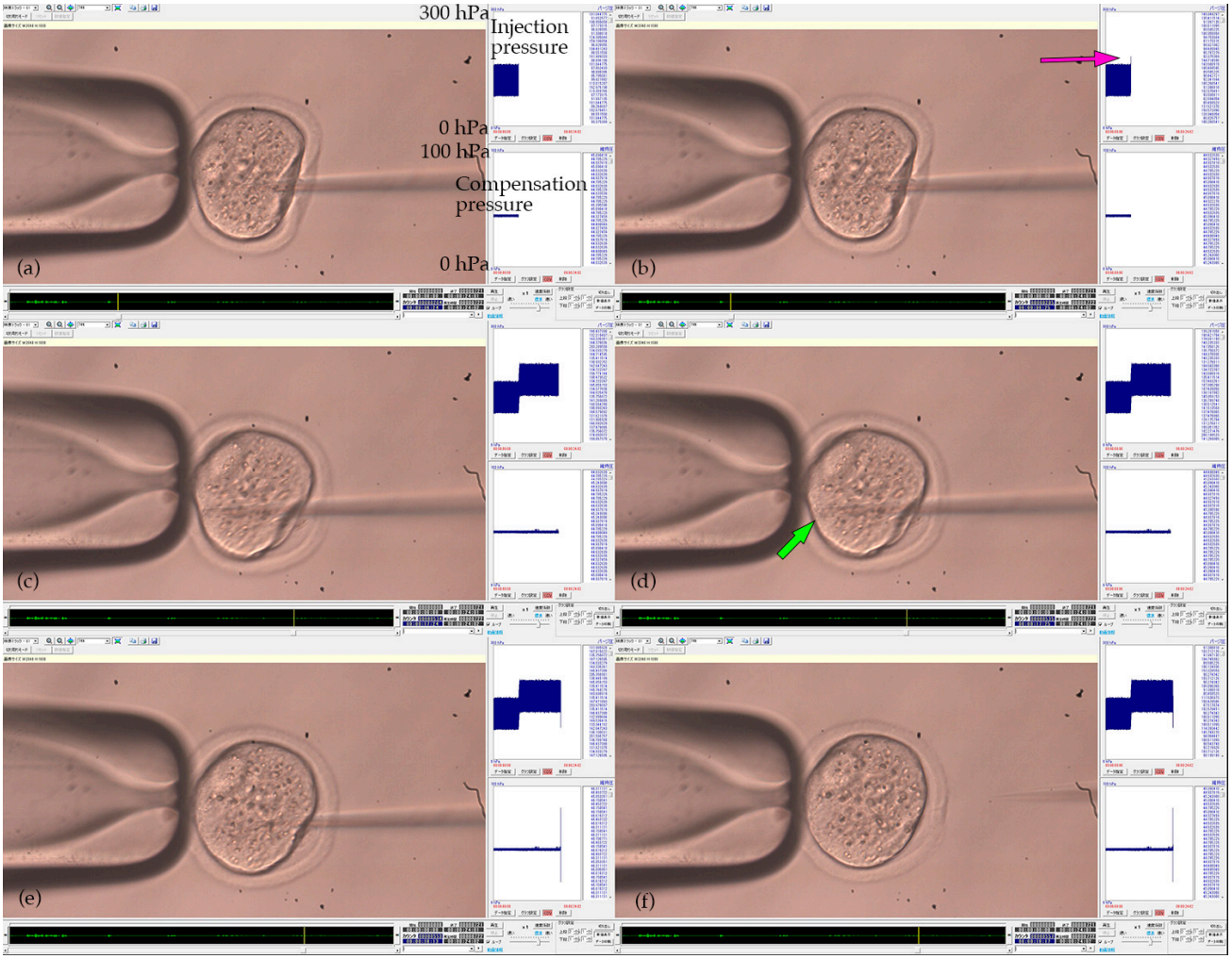

Figure 13. Ordinary microinjection with a self-made micropipette (OM2 group) at an injection pressure of 146 hPa. (**a**) Image just before application of the injection pressure, although some residual pressures inside the air circuit have been recorded; (**b**) Image at the moment when the pressure was applied. The red arrow indicates a sudden increase in pressure: one frame (33.3 msec) after (**a**); (**c**) One frame before piercing the cell membrane; (**d**) Image at the moment of piercing. The green arrow indicates a slight change in the pattern of the cytoplasm. The piercing time is 11.00 s; (**e**) The moment when the micropipette starts to be withdrawn, 594 msec after the piercing (**d**). The injection pressure is off; (**f**) Complete withdrawal of the micropipette.

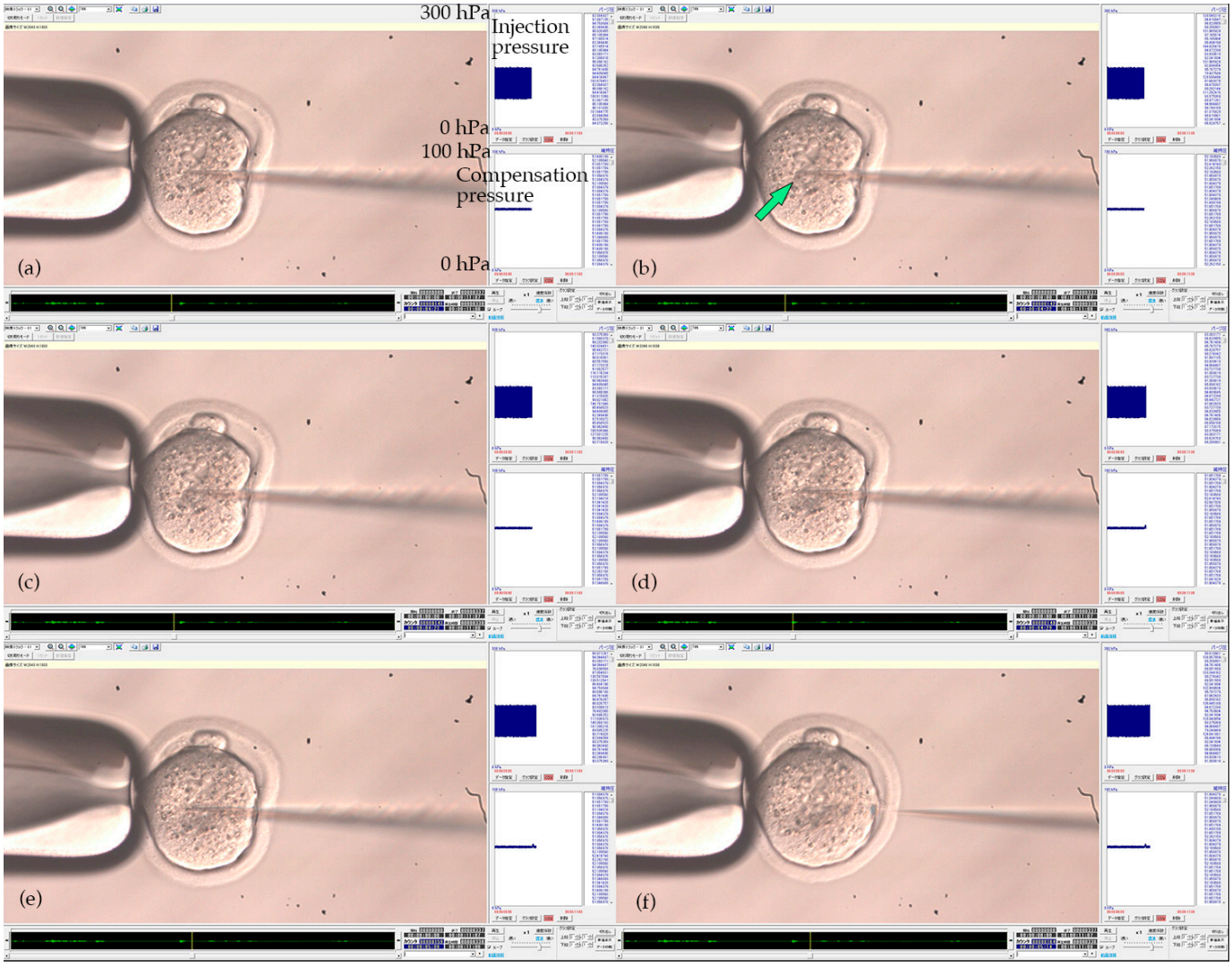

Figure 14. Vibratory microinjection with a self-made micropipette driven by VMS6_2 (VM2 group). (**a**) Image just before switch-on; (**b**) Image at the moment of switch-on: one frame (33 msec) after (**a**). The green arrow indicates a subtle change in the brightness of the cytoplasm around the tip of the micropipette, indicating that a small amount of DNA has been injected. The piercing time is 0 s; (**c**) One frame after (**b**). The area of cytoplasm that received the DNA solution ejected from the tip of the micropipette looks like a crater; (**d**) The crater-like appearance enlarges 231 msec after the piercing (**b**); (**e**) The moment when the micropipette starts to be withdrawn, 561 msec after the piercing; (**f**) Complete withdrawal of the micropipette.

Among the 396 cases of ordinary microinjection with a self-made micropipette (OM2 group) at an average injection pressure of 152 hPa, Figure 13 demonstrates the case with a piercing time closest to the average time of 10.87 s in the OM2 group. The piercing time and the injection pressure, in this case, were 11.00 s and 146 hPa, respectively. Figure 13b captures the moment when the injection pressure was applied. Note that the red arrow indicates the beginning of the injection pressure application. Figure 13c,d captures 33.3 msec before and at the moment of piercing, respectively. The green arrow in Figure 13d indicates a slight change in the pattern of the cytoplasm. Figure 13e shows the image when the micropipette began to be withdrawn 594 msec after the moment of piercing.

The video used to create Figure 13 has been uploaded as Supplementary Video S3.

Among the 410 cases of vibratory microinjection with a self-made micropipette driven by VMS6_2 (VM2 group), we could not find any case with a piercing time close to the average time of 1.52 s in the VM2 group. Since the piercing time was zero in 353 out of 410 eggs (86.1%) in the VM2 group, a case with a piercing time of 0 s is demonstrated in Figure 14. The green arrow in Figure 14b indicates a subtle change in the brightness of the cytoplasm around the tip of the micropipette at the moment of the vibrator's switch-on, indicating that a small amount of DNA was instantaneously injected into the cytoplasm. In Figure 14c–e, the area of the cytoplasm that received the DNA solution ejected from the tip of the micropipette looked like an enlarging crater. The image in Figure 14d was obtained 231 msec after the piercing (Figure 14b). Figure 14e captures the moment when the micropipette started to be withdrawn, 561 msec after the piercing (Figure 14b).

The video used to create Figure 14 has been uploaded as Supplementary Video S4.

The results of piercing time are summarized in Table 2 and Table 3. 'Joint structure' in the tables refers to the mechanically connecting parts between a micropipette and a vibrator. 'Vibration propagation structure' denotes the component that contributes the most to the propagation of ultrasonic waves generated by a vibrator to a micropipette. 'Injection mechanism' indicates the physical element that is capable of piercing the cell membrane, except for the sharpness of the tip of the micropipette, and is capable of ejecting DNA solution into the cytoplasm.

Table 2. Comparison of time taken to pierce the cell membrane between vibratory and ordinary microinjections in each joint type.

Joint Structure	Vibration Propagation Structure	Injection Mechanism	Group	Total No. of Eggs	Piercing Time (s)	*p* (*t*-Test)
Screw + O-ring (Femtotip)	Screw	Vibration	VM1	65	3.62 ± 3.72	0.0002
	---	Air pressure	OM1	61	9.58 ± 11.85	
Silicone rubber tube (Self-made)	Tube	Vibration	VM2	410	1.52 ± 5.98	<0.0001
	---	Air pressure	OM2	396	10.87 ± 11.09	
Femtotip + Self-made	Screw or Tube	Vibration	VM1 + VM2	475	1.80 ± 5.77	<0.0001
	---	Air pressure	OM1 + OM2	457	10.69 ± 11.19	

Table 3. Comparison of time taken to pierce the cell membrane between two joint types in each microinjection.

Injection Mechanism	Joint Structure	Vibration Propagation Structure	Group	Total No. of Eggs	Piercing Time (s)	*p* (*t*-Test)
Vibration	Screw + O-ring (Femtotip)	Screw	VM1	65	3.62 ± 3.72	0.0062
	Silicone rubber tube (Self-made)	Tube	VM2	410	1.52 ± 5.98	
Air pressure	Screw + O-ring (Femtotip)	---	OM1	61	9.58 ± 11.85	NS (0.4058)
	Silicone rubber tube (Self-made)	---	OM2	396	10.87 ± 11.09	

NS: non significant

In the case of Femtotips, the average piercing times in VM1 and OM1 groups were 3.62 s (±3.72, N = 65) and 9.58 s (±11.85, N = 61), respectively ($p = 0.0002$, Student's *t*-test). The vibratory microinjection was almost 3 times quicker to pierce the cell membrane than the ordinary microinjection that was assisted with the average injection pressure of 182 hPa (±36, N = 7), which was much higher than a standard setting of 150 hPa in pronuclear microinjection. A total of 7 vibratory microinjections out of 65 cases (10.8%) achieved a piercing time of zero, whereas no ordinary microinjections out of 61 cases did.

In the case of self-made micropipettes, the difference in piercing time between vibratory and ordinary microinjections was increased: 1.52 ± 5.98 s (N = 410) in the VM2 group and 10.87 ± 11.09 s (N = 396) at an average injection pressure of 151 hPa (±30; N = 38) in the OM2 group ($p < 0.0001$, Student's *t*-test). In other words, the vibratory microinjection achieved a piercing speed that was almost seven times faster. The piercing time in the VM2 group ranged from 0 to 60.23 s, but 353 out of 410 eggs (86.1%) showed a piercing time of zero, whereas none of the 396 eggs in the OM2 group achieved a piercing time of zero. The piercing times of the OM2 group ranged from 0.17 to 90.50 s. The longest piercing time of the VM2 group was observed on 29 October 2021. Only one set was conducted, and we could not find any good vibration conditions, changing it 5 times from the initial setting while vibratory microinjection was performed on only 14 eggs. More specifically, the voltage was changed from 3 Vpp to 5, 7, 6, 5, and 7 Vpp at a frequency of 33 kHz. In addition, the second and third worst piercing times were also observed on the same day: 52.73 and 47.33 s, respectively. As a result, the experiment on this day showed only one exception in which the average piercing time of vibratory microinjection was longer than that of ordinary microinjection: 14.41 ± 22.07 s (N = 14) vs. 12.32 ± 9.63 s (N = 14), respectively (NS, Student's *t*-test).

We summarize the comparison of piercing times between two types of vibratory microinjections and the comparison between two types of ordinary microinjections in Table 3. In the case of ordinary microinjections, the piercing times were almost equal, suggesting that the two different types of micropipettes, Femtotip and the self-made micropipette, shared similar piercing properties. In contrast, a statistically significant difference in piercing time was observed between the VM1 and VM2 groups (3.62 ± 3.72 vs. 1.52 ± 5.98 s, $p = 0.0062$), suggesting that the tube joint used in the VM2 group was capable of propagating the ultrasonic vibration developed at the piezoelectric actuators much more efficiently to the micropipettes than the screw joint because the remaining parts in both VMS6_1 and VMS6_2 were almost identical. The results obtained from the VM2 group suggested that VMS is approaching the level of 'instantaneous piercing' because 86.1% of vibratory microinjections achieved a piercing time of zero.

- Survival after microinjection

We summarize survival rates after microinjection and compare them between vibratory and ordinary microinjections (Table 4) and between the two types of micropipettes (Table 5). The survival rate was significantly better for vibratory microinjection than for ordinary microinjection, irrespective of the difference in micropipette type (Table 4). However, the survival rates for the VM1 and VM2 groups were almost equal, and the comparison between the OM1 and OM2 groups was not statistically significant (Table 5), although the survival rate in the OM2 group was slightly higher than in the OM1 group.

Table 4. Comparison of survival after cytoplasmic microinjection between vibratory and ordinary microinjections in each joint structure as well as beyond the difference in joint structure.

Joint Structure	Injection Mechanism	Vibration Propagation Structure	Group	Total No. of Eggs	Survival		*p* (Pearson's χ^2 Test)
					No. of Eggs	Rate (%)	
Screw + O-ring (Femtotip)	Vibration	Screw	VM1	65	59	90.8	0.0208
	Air pressure	---	OM1	61	46	75.4	
Silicone rubber tube (Self-made)	Vibration	Tube	VM2	410	370	90.2	0.0020
	Air pressure	---	OM2	396	328	82.8	
Femtotip + Self-made	Vibration	Screw or Tube	VM1 + VM2	475	429	90.3	0.0002
	Air pressure	---	OM1 + OM2	457	374	81.8	

Table 5. Comparison of survival after cytoplasmic microinjection between two joint structures in each microinjection.

Injection Mechanism	Joint Structure	Vibration Propagation Structure	Group	Total No. of Eggs	Survival		p (Pearson's χ^2 Test)
					No. of Eggs	Rate (%)	
Vibration	Screw + O-ring (Femtotip)	Screw	VM1	65	59	90.8	NS (0.8942)
	Silicone rubber tube (Self-made)	Tube	VM2	410	370	90.2	
Air pressure	Screw + O-ring (Femtotip)	---	OM1	61	46	75.4	NS (0.1618)
	Silicone rubber tube (Self-made)	---	OM2	396	328	82.8	

NS: non significant

4. Discussion

'Instantaneous piercing' was almost achieved in the VM2 group, in which vibratory microinjections with self-made micropipettes were driven by VMS6_2. When the idea of VMS occurred to the first author of this paper, he aimed at and expected a function of instantaneous piercing. However, at the first stage of development of the second version of VMS, we did not have any experience with microinjection and requested all the microinjections from a research group in RIKEN. Since they used Femtotips as micropipettes, we then designed and developed the second version of the vibrator for Femtotip. Thereafter, we continued to develop successive versions of the vibrator for Femtotips because the continuous use of Femtotips could enable both evaluation and comparison among several versions of the vibrator and also for the already-mentioned reason that the product control by Eppendorf might ensure a fair comparison between vibratory and ordinary microinjections.

When we decided to change from Femtotips to self-made micropipettes, we decided to modify only the structure related to connecting or holding a micropipette in the vibrator of VMS6_1 and also determined as a first step to use commercially available products developed for a standard injection holder: a silicone rubber tube and a screw cap with a collar (Figure 3b). The 6 mm long silicone rubber tube was already established as an effective gasket to seal as high as 7000 hPa, but we were not confident that the tube was long enough to allow good vibration propagation, which mainly depends on the contact area firmly joining together both the shaft of the micropipette and the inner surface of the air passage of the vibrator. However, this concern was dispelled by the results of the 'propagation rate' (Table 1) because they indicated that the tube-type joint propagated vibrations with slight attenuation or slight amplification from the vibrator level to the tip level over the entire frequency range of 30 to 50 kHz, although the vibration amplitudes of the micropipette tip were not those of the bare tip but of the 2 mm lightweight cube attached to it. On the other hand, the screw-type joint extremely amplified the propagating vibration in some frequencies. Slight attenuation or amplification in vibration propagation was expected, but such a large amplification was neither expected nor designed. In the case of the screw-type joint, the contact strength of the male and female screws might vary from place to place and be not necessarily axisymmetric. This may result in stronger lateral vibration in some frequencies. In contrast, in the case of the tube-type joint, since the micropipette is held uniformly by the surface, the vibration is considered to be transmitted to the micropipette uniformly and axisymmetrically.

In microinjection experiments, the piercing times obtained from the VM2 group (tube-type joint) were satisfactory because the VM2 group's piercing times were much shorter than not only those of two types of ordinary microinjections but also those of the VM1 group (screw-type joint), and, furthermore, because 86.1% of the VM2 group achieved a piercing

time of zero, while only 10.8% of the VM1 group did. With respect to survival rate, there was no difference at all between the two VM groups, but there was a significant difference between the VM and OM groups, with the VM group having a significantly higher survival rate. The cause of the difference in survival rates in this study is unknown because the survival rates between the vibratory and ordinary microinjections in previous studies were not significantly different [5,6]. The present study differed from our previous studies, which employed (1) pronuclear microinjection instead of cytoplasmic microinjection and (2) disuse of injection pressure in the case of ordinary microinjections. In the previous study [6], we actually achieved the ordinary pronuclear microinjections on all 210 fertilized eggs without any application of injection pressure. In the case of cytoplasmic microinjection, however, we found that ordinary cytoplasmic microinjections needed the application of injection pressure to penetrate the cell membrane and inject a sufficient amount of DNA into the cytoplasm because the cytoplasmic microinjection without any injection pressure was rarely completed within two minutes.

The difference between the pronuclear and cytoplasmic microinjections is as follows. In the case of pronuclear microinjection, both the cell membrane and the pronuclear membrane must be penetrated simultaneously in order to inject the DNA solution into the pronucleus. In the case of cytoplasmic microinjection, on the other hand, only the cell membrane must be penetrated. The cytoplasm is mainly composed of colloidal substances and the physical property of the cell membrane is closer to liquid rather than solid. Therefore, when a micropipette pushes only the cell membrane, the membrane tends to be deeply indented. On the other hand, in the case of pronuclear microinjection, the cell membrane pushed by the tip of a micropipette reaches the target pronucleus, and the cell membrane and the pronuclear membrane come into contact. Because the pronucleus is a large mass and does not easily move when pushed by the tip of the micropipette, the cell membrane, with the pronucleus immediately behind it, does not move backward any further. Thereby, either the cell membrane and pronuclear membrane are penetrated simultaneously, or they are not. These differences between cytoplasmic and pronuclear microinjections probably explain our findings that the pronuclear membrane was easily pierced together with the cell membrane, although the cell membrane itself was not so easy.

We discuss bubble formation because it may explain the lower survival rate of the OM groups, which almost always exhibited bubble formation. When a micropipette penetrates the cell membrane, a bubble of DNA solution usually forms and becomes visible around the tip of the micropipette, as shown in Figure 11c, because the DNA solution is pushed from behind by injection pressure. Therefore, the longer the injection pressure is applied, the larger the size of the bubble tends to be, although there appears to be a limit to its size because the DNA solution within the bubble flows out along the shaft of the micropipette. The mechanism for the higher mortality in the OM groups may be as follows. The bubbles burst abruptly, so even if a micropipette is withdrawn quickly, the DNA solution in the bubble is explosively injected into the cytoplasm. The larger the bubble, the greater the injection volume, which may increase the mortality rate of an egg. We did not precisely check either the presence or the size of the bubble in each microinjection, but the bubble of DNA solution not only formed much more frequently but also became larger when injection pressure was applied than when vibration was applied. A piece of evidence supporting the above-mentioned impression might be the results of the piercing time. Because the piercing time of the ordinary microinjection was much longer than that of the vibratory microinjection, and because none of the 396 eggs for the ordinary microinjection achieved a piercing time of zero, the ordinary microinjections probably developed larger bubbles of DNA solution before the cell membrane was punctured, and forced larger amount of DNA solution into the cytoplasm when the cell membrane was punctured. In addition, a larger bubble may also cause more damage to the cell membrane because a large hole may be opened in the cell membrane corresponding to the size of the bubble. In contrast, vibratory microinjection frequently resulted in instantaneous piercing of the cell membrane (86.1%), thereby not allowing enough time for the formation of a bubble. Therefore, the formation

of a large bubble might be one of the reasons why the ordinary cytoplasmic microinjection with injection pressure had a significantly higher mortality rate.

We have already mentioned that cytoplasmic microinjections need the assistance of either injection pressure or vibration, but there were some exceptions. In this study, 17.1% (26/152) of the eggs pushed by Femtotips and 5.1% (43/849) of the eggs pushed by self-made micropipettes were pierced before either injection pressure or vibration was applied. Although the injected volume of DNA solution was much smaller than that with the assistance of either injection pressure or vibration, 28 eggs out of the 69 (= 26 + 43) eggs died immediately after microinjection. Since the mortality rate of those eggs was as high as 40.6%, their cell membranes were considered to be very "fragile". When not "fragile", as shown in the remaining 932 eggs analyzed in this study, their cell membranes needed assistance, such as vibration or pressure, to penetrate. Despite impacts such as vibration and pressure, as well as the injection of larger amounts of DNA solution, the 932 eggs showed a mortality rate as low as 13.8%.

As another application of the VMS, we believe that the VMS may also be useful for genome editing, such as the CRISPR/Cas9 system, because Cas9 had been mainly introduced into the cytoplasm as mRNA until purified protein was supplied [16,17]. Since purified Cas9 proteins have become commercially available, they appear to be microinjected into the pronucleus as RNPs (RNP: a complex of Cas9 protein and gRNA) to produce knock-in mice [18]. However, in a study that aimed at generating knockout mice, Cas9 mRNA and single guide RNA (sgRNA) were compared with three different transduction methods: cytoplasm only, pronucleus only, and both cytoplasm and pronucleus, and it was reported that cytoplasm only was the most efficient [19]. Therefore, cytoplasmic microinjection is still used when nucleases and nickases for the base-editing or prime-editing are introduced as mRNAs, suggesting that vibratory cytoplasmic microinjection may be helpful.

For the pronuclear microinjection with DNA constructs as small as 1.67 kb, the VMS (version 3) significantly reduced the incidence of the "pulling-out" event compared to the non-vibratory microinjection [6]. Using BAC DNA and self-made micropipettes, we are currently investigating the incidence of the "pulling-out" event between vibratory pronuclear microinjection (using the VMS6_2) and non-vibratory pronuclear microinjection (with injection pressure). So far, "pulling-out" events are rarely observed when the VMS6_2 is used, but are more likely to occur when pressure is applied. In the case of the vibratory microinjection, even when the micropipette was advanced into the pronucleus without avoiding the nucleolus, it was frequently observed that the nucleolus moved rather as if it was escaping. Therefore, the VMS may be useful for genome editing using pronuclear microinjection as well as cytoplasmic microinjection.

5. Conclusions

In the sixth-version vibratory microinjection system, we developed two types of vibrators, one (VMS6_1) connecting a Femtotip, a commercially available micropipette, and the other (VMS6_2) connecting a self-made micropipette, and compared both types of vibrators. The two types of vibrators were designed with different micropipette connections; the main parts of the vibrators remained unchanged. The tube-type joint used for self-made micropipettes transmitted vibrator-level vibration to the tip of the micropipette with only slight attenuation or amplification, but the screw-type joint did not. The two were also compared in the cytoplasmic microinjection of fertilized eggs using a high-concentration solution of high molecular weight BAC DNA. VMS6_2 significantly shortened the time taken to pierce the cell membrane of fertilized eggs and achieved 'instantaneous piercing' of the cell membrane in approximately 86% of the allocated fertilized eggs. Therefore, we concluded that the good propagation of vibration due to the tube connection of the vibrator and micropipette was verified and confirmed in cytoplasmic microinjection. Furthermore, when cytoplasmic microinjection was compared between vibration (vibratory microinjection) and pressurization (non-vibratory microinjection with injection pressure), not only was the cell membrane piercing time significantly longer with pressurization than

with the two types of vibration, but the mortality rate immediately after injection was also significantly higher with pressurization. This suggests that the addition of vibration during microinjection may be more useful not only for pronuclear microinjection but also for cytoplasmic microinjection than pressure.

Supplementary Materials: The following supporting information can be viewed at: https://www.youtube.com/playlist?list=PLIbaIJN5kq1ajmhzDr-TXc-Cx1tFJMn17, Video S1: Screw OM; Video S2: Screw VM; Video S3: Tube OM; Video S4: Tube VM.

Author Contributions: F.M. performed and evaluated the microinjection experiments, and J.H. developed and evaluated the vibrators. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are contained within the article and supplementary materials.

Acknowledgments: The authors wish to thank Mutsuko Onoue for assistance with microinjections and also wish to thank Matthew Day for English proofreading.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. List of date of experiment, set number, type of microinjections, vibration condition or injection pressure, number of eggs allotted in each set, number of alive or dead eggs.

Date/Pipette	Set No.	Group	Vibration Condition Injection Pressure	No. of Eggs	No. of Alive	No. of Dead
6 November 2020 Femtotip	1	VM1	42 kHz, 4.5 Vpp	12	10	2
		OM1	182 hPa	11	6	5
	2	VM1	42 kHz, 4.5 Vpp	10	9	1
		OM1	189 hPa	9	8	1
13 November 2020 Femtotip	1	VM1	39 kHz, 12 Vpp	11	11	0
		OM1	156 hPa	11	10	1
	2	VM1	39 kHz, 12 Vpp	9	9	0
		OM1	154 hPa	8	8	0
20 November 2020 Femtotip	1	VM1	39 kHz, 10 Vpp	10	9	1
		OM1	137 hPa	10	6	4
4 December 2020 Femtotip	1	VM1	45 kHz, 13 Vpp	12	11	1
		OM1	226 hPa	10	7	3
	2	VM1	45 kHz, 13 Vpp	2	1	1
		OM1	231 hPa	2	1	1
25 June 2021 Self-made	1	VM2	45 kHz, 3 Vpp	14	13	1
		OM2	177 hPa	13	11	2
	2	VM2	45 kHz, 3 Vpp	12	10	2
		OM2	188 hPa	12	8	4
30 July 2021 Self-made	1	VM2	30 kHz, 3 Vpp	14	12	2
		OM2	140 hPa	13	12	1
3 September 2021 Self-made	1	VM2	39 kHz, 7 Vpp	12	12	0
		OM2	138 hPa	12	10	2
	2	VM2	39 kHz, 7 Vpp	14	10	4
		OM2	142 hPa	14	13	1

Table A1. *Cont.*

Date/Pipette	Set No.	Group	Vibration Condition Injection Pressure	No. of Eggs	No. of Alive	No. of Dead
1 October 2021 Self-made	1	VM2	6 variations *	13	11	2
		OM2	183 hPa	13	8	5
8 October 2021 Self-made	1	VM2	45 kHz, Vpp (3.5, 3.8, 4.0, 3.9)	11	11	0
		OM2	179 hPa	11	11	0
	2	VM2	45 kHz, 4 Vpp; 39 kHz Vpp (4, 5, 6)	6	3	3
		OM2	183 hPa	6	4	2
15 October 2021 Self-made	1	VM2	31 kHz, Vpp (8, 7.5)	15	12	3
		OM2	157, 140 hPa	15	11	4
	2	VM2	31 kHz, Vpp (7.5, 7.0)	11	8	3
		OM2	125 hPa	11	9	2
29 October 2021 Self-made	1	VM2	33 kHz, Vpp (3, 5, 7, 6, 5, 7)	14	12	2
		OM2	119 hPa	14	12	2
5 November 2021 Self-made	1	VM2	37 kHz, 7 Vpp	14	14	0
		OM2	157 hPa	14	12	2
	2	VM2	37 kHz, Vpp (7.5, 8.0)	14	14	0
		OM2	158 hPa	11	11	0
12 November 2021 Self-made	1	VM2	49 kHz, 3 Vpp	10	10	0
		OM2	127 hPa	9	9	0
	2	VM2	49 kHz, 3 Vpp	12	12	0
		OM2	127 hPa	11	9	2
10 December 2021 Self-made	1	VM2	45 kHz, 8 Vpp	11	11	0
		OM2	185 hPa	11	11	0
17 December 2021 Self-made	1	VM2	41 kHz, 10 Vpp	13	9	4
		OM2	160 hPa	13	9	4
	2	VM2	41 kHz, 10 Vpp	10	9	1
		OM2	162 hPa	10	7	3
21 Juanuary 2022 Self-made	1	VM2	40 kHz, Vpp (7, 8)	15	15	0
		OM2	232 hPa	15	15	0
	2	VM2	40 kHz, Vpp (8, 9)	13	12	1
		OM2	243 hPa	11	11	0
15 April 2022 Self-made	1	VM2	31 kHz, Vpp (10, 11); 40 kHz, Vpp (6, 7, 8)	15	14	1
		OM2	164 hPa, 145 hPa	14	14	0
	2	VM2	40 kHz, 9 Vpp	7	7	0
		OM2	156 hPa	7	6	1
22 April 2022 Self-made	1	VM2	38 kHz, 10 Vpp	12	12	0
		OM2	145 hPa	13	11	2
	2	VM2	38 kHz, 10 Vpp	13	13	0
		OM2	149 hPa	13	10	3

Table A1. *Cont.*

Date/Pipette	Set No.	Group	Vibration Condition Injection Pressure	No. of Eggs	No. of Alive	No. of Dead
13 May 2022 Self-made	1	VM2	44 kHz, 6 Vpp	15	15	0
		OM2	136 hPa	15	14	1
	2	VM2	44 kHz, 6 Vpp	14	12	2
		OM2	142 hPa	14	12	2
27 May 2022 Self-made	1	VM2	42 kHz, 10 Vpp	15	14	1
		OM2	110 hPa	15	14	1
	2	VM2	42 kHz, Vpp (10, 9, 8)	9	8	1
		OM2	112 hPa	9	5	4
3 June 2022 Self-made	1	VM2	45 kHz, 8 Vpp	15	15	0
		OM2	137 hPa	14	12	2
	2	VM2	45 kHz, 8 Vpp	9	9	0
		OM2	147 hPa	9	7	2
17 June 2022 Self-made	1	VM2	42 kHz, 7 Vpp	13	10	3
		OM2	122 hPa	13	9	4
	2	VM2	42 kHz, 7 Vpp	8	6	2
		OM2	126 hPa	8	5	3
24 June 2022 Self-made	1	VM2	41 kHz, 6 Vpp	15	15	0
		OM2	154 hPa	14	8	6
	2	VM2	43 kHz, 10 Vpp	12	9	3
		OM2	138 hPa, 119 hPa	12	11	1

* (1) 37 kHz, 8 Vpp; (2) 37 kHz, 7.5 Vpp; (3) 37 kHz, 7 Vpp; (4) 38 kHz, 10 Vpp; (5) 39 kHz, 10 Vpp; (6) 40 kHz, 10 Vpp.

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