APPLICATION NOTE No. 395

Intracytoplasmic Sperm Injection (ICSI) in the Mouse with the Eppendorf PiezoXpert®: How to Increase Oocyte Survival Rates After Injection

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Abstract

Intracytoplasmic sperm injection (ICSI) is a powerful technique that has been successfully used to inseminate metaphase II (MII) oocytes. While very efficient in terms of fertilization rates in humans, good results in mouse oocytes have been always difficult to achieve, as most of these lyse soon after injection. This problem has been mainly attributed to the higher fragility of the mouse oocyte compared to other species. In the 90's, the group led of Prof. Yanagimachi produced a breakthrough in the field with the introduction of the piezo-actuated micromanipulation technique, which allows to perform mouse ICSI much more efficiently. Despite the improvements, many laboratories today still struggle to have acceptable oocyte survival rates after sperm injection even using the piezo-actuated ICSI technique, as this is a technically demanding procedure that depends on many factors. In this Application Note, we describe a modified piezo-actuated ICSI procedure using the Eppendorf PiezoXpert, allowing even less experienced operators to increase the survival rates up to around 100%.

Introduction

The conventional intracytoplasmic sperm injection (ICSI) technique is based on the direct injection of a single sperm into a metaphase II (MII) oocyte using a glass beveled microcapillary. While, very successful in humans, this conventional ICSI technique has proven unsuccessful in the mouse. This problem is mainly attributed to the fragility of the mouse oocyte with low cytoplasm viscosity and an oolemma much more elastic and sensitive than oocytes of human or other species. Consequently, survival rates of mouse oocytes following ICSI with the conventional (i.e., manual, non-piezo) microinjection procedure rarely exceed 50% [1-2].

To overcome the low survival rates after ICSI in the mouse with conventional injection, Kimura and Yanagimachi [1] proposed the piezo-actuated micromanipulation. In this method, a piezo-electric effect (crystal deformation in response to an externally applied voltage) propels a microcapillary forward in a precise and rapid movement that allows the membrane to be penetrated [1-2]. Although technically demanding, this procedure is less traumatic than the conventional ICSI, and thus higher survival rates were described [1-3]. The efficacy of the piezo-drill is highly desirable, not only in ICSI procedures, but also for other challenging microinjection applications, such as somatic cell nuclear transfer (SCNT) or injection of highly-concentrated CRISPR complexes [2-4]. Unfortunately, many laboratories working with mouse-ICSI struggle for survival rates over 90%, even with piezo-actuated micromanipulation. Several factors may contribute to the observed lack of consistent results in survival rates, including the size of injection capillary; laboratory conditions (e.g., room temperature and humidity), skill level of the operator and, most importantly, the settings of the piezo-device. The piezo impulses generated by the PiezoXpert have shown to be effective and free of lateral oscillations, which are known to be traumatic to the oocyte's oolemma.

In this Application Note, we describe a modified piezo-actuated ICSI technique with the PiezoXpert that is based on withdrawing the microcapillary and applying rapid suction

simultaneously just after the sperm head was deposited in the oocyte cytoplasm. This rapid and simultaneous action allows the hole created in the oocyte membrane as a consequence of the injection impulse to seal immediately, thus increasing survival chances dramatically. With practice, this procedure enables even less experienced operators to increase the percentage of oocytes surviving the piezo-injection up to nearly 100%.

Materials and Methods

List of Equipment

> Stereo microscope (e.g., SZH, Olympus®, Japan)
> Inverted microscope with up to 40x objective (e.g., IX73, Olympus, Japan)
> Adaptive Electronic Condenser™ (MTG, Germany)
> CellTram® 4r Air microinjector for holding the MII oocytes (Eppendorf, Germany)
> CellTram® 4r Oil microinjector for sperm capture and injection (Eppendorf, Germany)
> Two TransferMan® 4r micromanipulators, one for positioning the holding micropipette, one for controlling the ICSI micropipette (Eppendorf, Germany)
> Eppendorf PiezoXpert® (Eppendorf, Germany)
> Antivibration pads (Eppendorf, Germany)
> CO_2 incubator Galaxy® 48 R (Eppendorf, Germany)

An example of a Piezo-assisted ICSI microinjection workstation is illustrated in Figure 1.

List of Materials

> VacuTip I holding capillary with inner diameter (ID): 15 µm, angle: 35° (Eppendorf, Germany)
> Piezo-ICSI capillary (e.g., Piezo Drill Tip Mouse ICSI capillary with blunt end tip, ID: 6 µm, angle: 25° (Eppendorf, Germany) or Piezo Drill Micropipets (Origio/CooperSurgical, U.S.A.)
> Plastic dishes with low rim for micromanipulation
> Mineral oil, PVP (e.g., LifeGlobal®, U.S.A.) and culture media used should be embryo-tested
> Cytochalasin B (C6762, Sigma-Aldrich, U.S.A.)
> Hyaluronidase (e.g., LifeGlobal, U.S.A.)
> Fluorinert™ (e.g., FC-40, Sigma-Aldrich®, U.S.A.) or mercury (215477, Sigma- Aldrich, U.S.A.)
> Eppendorf Microloader™ for backfilling of the ICSI capillary (Eppendorf, Germany)

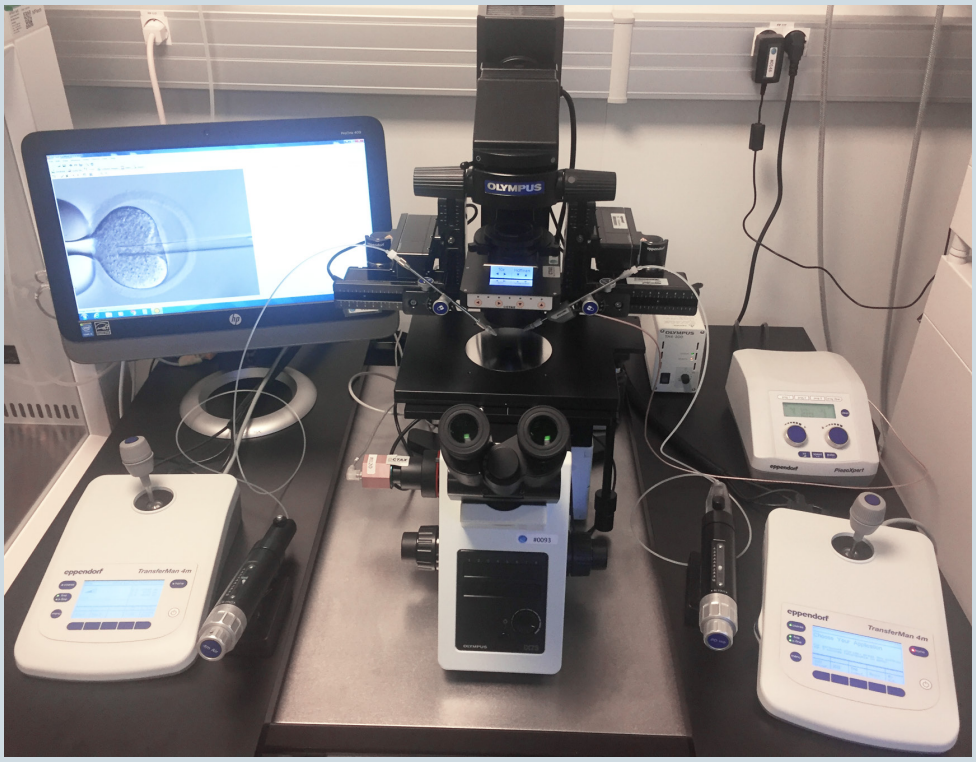

Figure 1: Workstation for piezo-assisted mouse ICSI. The inverted microscope is equipped with two TransferMan 4r for holding and injection, the manual microinjectors CellTram 4r Air & Oil, a PiezoXpert mounted at the injection side and a camera/monitor system for documentation.

Micromanipulation dish

The micromanipulation dish should be prepared with small droplets of manipulation medium (e.g., Hepes-buffered) supplemented with 5 µg/mL cytochalasin B for oocyte microinjection. Next to the droplets set 10% (v/v) PVP droplets for washing the piezo-actuated ICSI pipette and for performing the isolation of sperm heads. The arrangement of the manipulation medium and PVP droplets in the microinjection dish depends on personal preferences and protocols. Detailed instructions are described elsewhere [5-7].

Setting up of the micropipettes and adjustment of the piezo-settings

The ICSI microcapillaries used in piezo-actuated micromanipulation have typically a long blunt end tip, with a 20-25° angle, that is flat and has an inner diameter of around 5.5-6 µm. The microinjection pipettes are usually backfilled with either mercury (approx. 2 µL) or Fluorinert (approx. 10-20 µL) to enhance the transmission of piezo impulses and the drilling efficiency [5-6]. The piezo impulse settings (intensity, speed and pulse number) usually depend on individual laboratory conditions and thus should be optimized for your laboratory set-up and then adjusted for each experiment. A description on how to setup the parameters for the PiezoXpert are described in previous ICSI Eppendorf Application Notes [5-6].

In general, the penetration of the zona pellucida requires a stronger piezo impulse (e.g., intensity: 10-40, speed: 5-7, pulse: ∞, set at the PiezoXpert in Channel A) whereas the elastic oolemma should be treated with a softer impulse (e.g., intensity: 1-10, speed: 1-7, pulse: 1, set at the PiezoXpert in Channel B). The sperm immobilization and head/tail separation is performed at higher piezo impulses (e.g., intensity: 10-40; speed: 5-10, pulse: ∞). The sperm decapitation parameters can be stored in program 2 of the PiezoXpert. Using mercury, the settings of the parameters are lower compared to the settings when using Fluorinert.

Oocyte collection

MII oocytes can be collected from superovulated females, as described elsewhere [8]. After denudation in hyaluronidase, the cumulus-free oocytes should be extensively washed and transferred to a culture dish and put in an incubator at 37°C and optimal % CO_2 and low % O_2 concentration until use [8].

Sperm collection and sperm head isolation with piezo impulses

Mouse sperms can be collected from cauda epididymis taken from an adult male in a microdroplet of culture medium, and cultured for 10-15 min at 37°C and optimal % CO_2 and % O_2 concentration [8]. After incubation, 2-3 µL of the sperm concentration should be taken with a pipette and further diluted into a 200-300 µL droplet of culture medium. For the isolation of the sperm heads, sperms should be transferred into a droplet of embryo-tested PVP 10 % (v/v), previously prepared in the micromanipulation dish. Sperm heads can then be isolated and loaded in the ICSI microcapillary, as described elsewhere [2-3]. Briefly, single, motile spermatozoa should be selected and aspirated through the tail into the microinjection capillary (Figure 2A). Then, a single sperm is positioned so that the neck is placed at the opening of the capillary (Figure 2B). Once at this point, a few stronger piezo impulses can be applied to separate the head from the tail. The procedure should be repeated individually until having around 5 to 6 isolated sperm heads, which can then be loaded in the capillary.

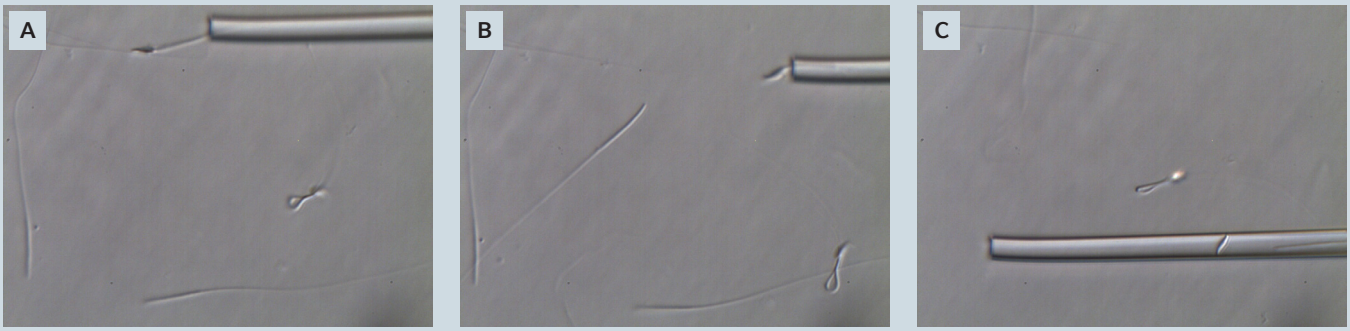

Figure 2: Piezo-assisted decapitation of the mouse sperm
A: The sperm is aspirated into the piezo ICSI microcapillary with the tail first
B: The sperm is positioned to the tip end so that the head of the sperm just looks out.
C: A stronger piezo impulse is applied causing the separation of the head from the tail at the neck of the sperm

Modified piezo-actuated ICSI with cytoplasm aspiration to reduce lysis

Matured oocytes are transferred to the micromanipulation dish to the droplets of manipulation medium (supplemented with cytochalasin B, a microfilament disrupter that relaxes the oolemma) and incubated at 37°C for at least 5 min before starting the ICSI procedure. Having loaded the sperm heads into the ICSI capillary, as described above, both the holding and ICSI capillaries are moved to the droplet containing the oocytes and both capillaries should be positioned next to them. An oocyte is then fixed firmly at the holding capillary and the focus of the inverted microscope is aligned to the equatorial plane of the oocyte. The microinjection capillary loaded with the sperm heads is then placed in focus with the equatorial plane of the oocyte (Figure 3A). Afterwards, the zona pellucida is penetrated using the stronger piezo impulses stored at Channel A of the PiezoXpert (Figure 3B-C). A sperm head is then positioned close to the tip end of the ICSI capillary. The ICSI capillary is pushed into the oocyte almost up the opposite side of the oocyte, close to the holding pipette, making a deep indentation in the oocyte (Figure 3D-E). At this point, a small suction is applied into the ICSI capillary, a single soft piezo impulse is triggered (set in Channel B of the PiezoXpert) to break the oolemma and then the sperm head is carefully ejected into the cytoplasm of the oocyte (Figure 3F). Afterwards, the ICSI capillary tip is rapidly withdrawn from the oocyte while aspirating at the right end of the indentation a bit of the oolemma into the capillary tip end (Figure 3G). By simultaneously withdrawing the micropipette and aspirating, it is possible to close the hole within the elastic oolemma (Figure 3H-I). Injected oocytes must then be washed thoroughly in culture medium droplets to eliminate the traces of the Hepes-buffer and cytochalasin B present in the manipulation medium and can then be cultured under appropriate conditions.

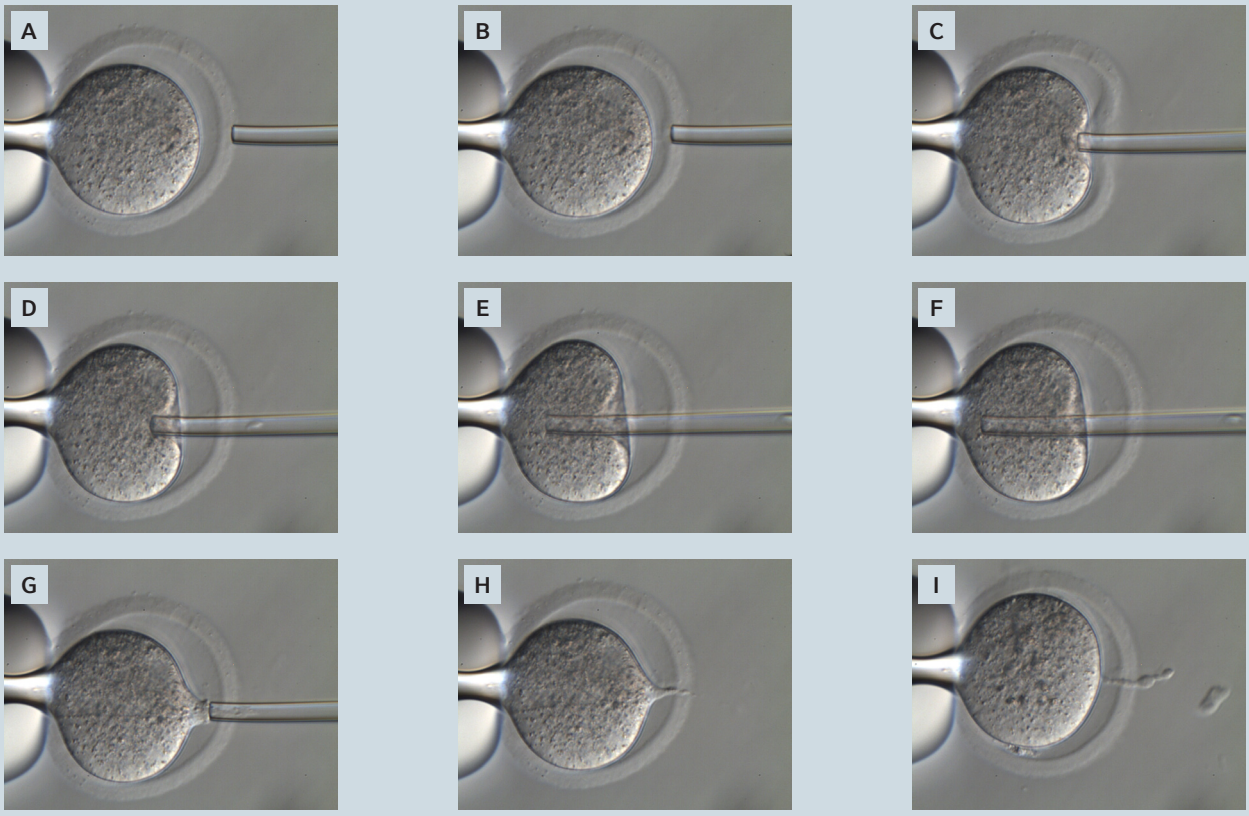

Figure 3: Piezo-actuated mouse ICSI using the modified technique. (A–C) The equatorial plane of the oocyte and the piezo ICSI capillary are aligned in microscopic focus. A stronger piezo impulse is applied to ICSI capillary to penetrate the zona pellucida. (D–F) The ICSI capillary, loaded with the sperm head, is then moved deep into the oocyte. Minimal suction is applied into the ICSI capillary and with a single soft piezo impulse the oolemma is penetrated and the sperm is released into the cytoplasm. (G–I) When withdrawing the ICSI capillary out of the injected oocyte, just at the right end of the indentation a short suction is applied into the capillary. This causes a closing of the penetrated oolemma to close.

Results and Discussion

The method described here was adapted from the original technique developed in the group of Prof. Yanagimachi [1] with the intent to reduce the percentage of lysed oocytes following piezo-actuated ICSI in the mouse.

The penetration of the zona pellucida and oolemma should be performed with a gentle piezo impulse which just allows to succeed in drilling but not to stress the cell by lateral oscillations. As the piezo impulse settings usually do not only depend on the individual set-up but also on experimental conditions, settings need to be adjusted for each experiment.

The closing of the oolemma by a short suction of this penetrated cell region into the ICSI capillary when just leaving the oocyte minimizes the risk of cell lysis, which is often observed at this sensitive MII stage. It is recommended to perform this procedure in a manipulation medium supplemented with cytochalasin B, as this microfilament disruptor agent relaxes the oocyte membrane and thus allows the membrane to seal easily immediately after the sperm into the cytoplasm. With some practice and the optimal set-up of the micromanipulation workstation and the experimental conditions, which are detailed in the Application Note, this modified technique should enhance the survival rate of injected oocytes to nearly 100%.

Commonly, in hybrid mouse strains (e.g., B6CBAF1) around 90% or more of the injected oocytes should yield the two-cell stage on the next day. These can be cultured up to the blastocyst stage and produce offspring in up to ~50% of cases following embryo transfer (20-30% is more common), depending on how skillfully the ICSI and the embryo transfer is performed.

Literature

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Ordering information

Description	Order no. International	Order no. North America
TransferMan® 4r, micromanipulator with DualSpeed™ joystick for direct and dynamic movement control	5193 000.012	5193000020
Eppendorf PiezoXpert®, for piezo-assisted micromanipulation, incl. actuator and foot control	5194 000.016	5194000024
CellTram® 4r Air, manual pneumatic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000.013	5196000013
CellTram® 4r Oil, manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000.030	5196000030
Microscope Adapter, for mounting onto different inverse microscopes	Available on request	Available on request
Piezo Drill Tip ES, capillary for piezo-assisted mouse ES cell transfer, 25° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.095	5195000095
VacuTip I, holding capillary, 35° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.036	5195000036
Eppendorf Microloader™, tip for backfilling of microcapillaries, set of 2x 96 pcs	5242 956.003	930001007
Galaxy® 48 R, CO_2 incubator, 48 L, 230 V/50/60 Hz, High-Temp Disinfection, 1-19 % O_2 Control	CO48 330.001	CO48230005

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