Userguide

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Piezo-actuated Mouse ICSI (intracytoplasmic sperm injection) using the Eppendorf PiezoXpert®

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Abstract

ICSI (intracytoplasmic sperm injection) is an important and commonly used assisted reproductive technology in both, humans and animals. The Mouse is one of the most common model organisms of choice to study mammalian fertilization. However, the ability to fertilize mouse eggs successfully by sperm injection has been hard to achieve due to the fact that the metaphase II mouse oocytes are extremely sensitive and convential ICSI gives low survival rates [1]. This problem can be solved with piezo-actuated micromanipulation where the capillary advances a very short distance at a very high speed. This enables the capillary to penetrate the cell membrane with minimum distortion of the cell and yields to high survival rates. The microinjection workstation required for this technique is very similar to standard ICSI, but with the addition of a piezo-assisted unit attached to the capillary holder. In this Userguide, the use of the Eppendorf PiezoXpert in combination with the Eppendorf TransferMan® NK 2 workstation is shown and parameter settings as well as optimization of the piezo-actuated microinjection procedure itself are discussed.

Introduction

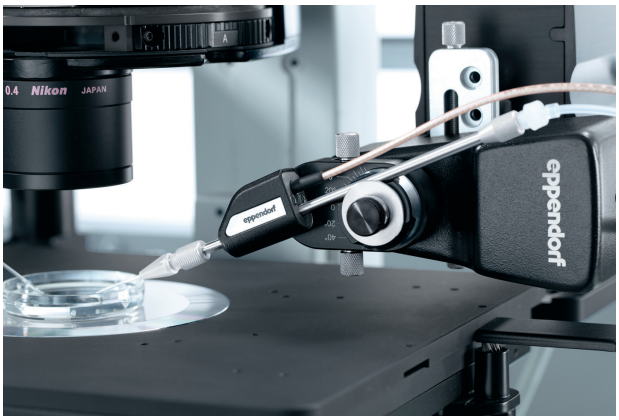

Fig. 1: Actuator of PiezoXpert mounted onto the right arm of the manipulator (Eppendorf TransferMan NK 2).

Intracytoplasmic sperm injection (ICSI) is a technique that involves the direct transfer of a single sperm into the oocyte cytoplam via a glass capillary with a spike.

While this conventional ICSI technique has been very successful in humans, it has proven unsuccessful in mice [2]. This is due to a lower viscosity of the ooplasm. Thus wound healing capacity of mouse oocytes is inferior to that of human oocytes. Furthermore, the oolemma of mouse oocytes is much more elastic than that of human oocytes. Successful ICSI in mice was first demonstrated by Kimura and Yanagimachi [3] using piezo-actuated micromanipulation, which is far less traumatic than the conventional method. This method proved to increase survival as well as fertilization rates of oocytes after sperm injection [3].

Eppendorf has a long tradition in the area of conventional ICSI. In particular, the Eppendorf TransferMan NK 2 system is an electronic micromanipulation system that offers a number of useful features for ICSI. In combination with the piezo impact unit PiezoXpert, Eppendorf is offering a complete system for both, conventional ICSI and piezo-actuated ICSI (Figure 1, 2).

Fig. 2: Eppendorf piezo-actuated mouse ICSI system. Workstation with 2 TransferMan NK 2, PiezoXpert, mounted onto a Nikon Eclipse TE 2000 microscope and incubator GALAXY 14 S.

Materials and Equipment

Animals, media, consumables and devices were used as described previously [4] with the following modifications:
Media:
CZB-HEPES (CZB-H)
CZB
CZB-HEPES with 12 % polyvinylpyrrolidone (PVP)
Density gradient (e.g. Percoll)
Fluorinert C-77 (FC-77), Fluorinert C-770 (FC-770)
Consumables:
100 µL pipette tips
Piezo impact unit:
Eppendorf PiezoXpert

Methods

1 Preparation of spermatozoa

Mouse sperms (fresh or frozen-thawed) are prepared based on the method of sperm head isolation.
If the sonication method is used, sperms are prepared by centrifugation and subsequent sonication to isolate sperm heads by diluting a small sample of sperms with buffer followed by repeated sonication (e.g. 4 x 15 seconds) [5].
If the piezo assisted method is used, described at 5.2.1, sperms are prepared using mini swim-up [6] or density gradient centrifugation.

2 Preparation of oocytes

The metaphase II oocytes are collected from superovulated females and further treated as described previously [4]. The cumulus-free oocytes are transferred to a culture dish [4].

3 Preparation of the microinjection dish

The arrangement of the drops in the microinjection dish depends on personal preferences. Examples for different sperm head separation methods (sonication method and piezo-assisted method) are shown as below.

ICSI dish with sonicated sperm
An example with sperm heads that are separated using sonication is shown in Figure 3 [4]. Here, a 5 µL flat drop of sperm suspension is positioned in the center of a flat Petri dish. 3 x 5 µL drops of CZB-HEPES with 12 % PVP are positioned at one end of the dish along the midline. In addition, 2 x 5 µL injection drops of CZB-HEPES are placed at the other end of the dish along the midline. Add approx. five eggs in one of the injection droplets. Cover with mineral oil.

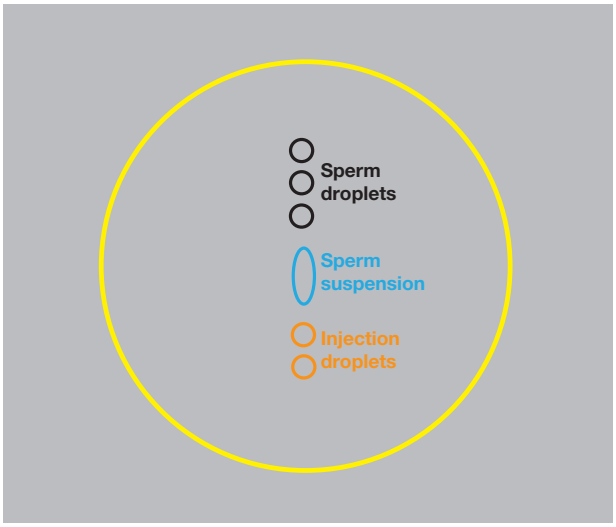

Fig. 3: ICSI dish with sonicated sperm. Image adapted from [4].

ICSI dish for sperm head isolation using piezo-assisted method
2 dishes (A; B) are prepared as shown in Figure 4. In dish A 2 x 5 µL flat droplets of CZB-HEPES with 12 % PVP are placed in the center of the dish. One droplet for the sperm suspension and the other one for the isolated sperm heads collection. In dish B, a 5 µL droplet of CZB-HEPES with 12 % PVP is placed at the center of the dish and 6 x 5 µL injection drops of CZB-HEPES are positioned adjacent to the center. Add approx. five eggs; one in each of the injection droplets. (One drop is left without cells for cleaning the capillary.)
First of all sperms are cut in the 'sperm suspension droplet' (dish A). Then the sperm heads are transferred to the 'sperm heads collection droplet'. Collect as many sperm heads as possible before transferring them to dish B, where the injection takes place.
The advantage of this preparation is to minimize the exposure time of oocytes out of the CO_2 incubator and thus improve the survival rate of oocytes.

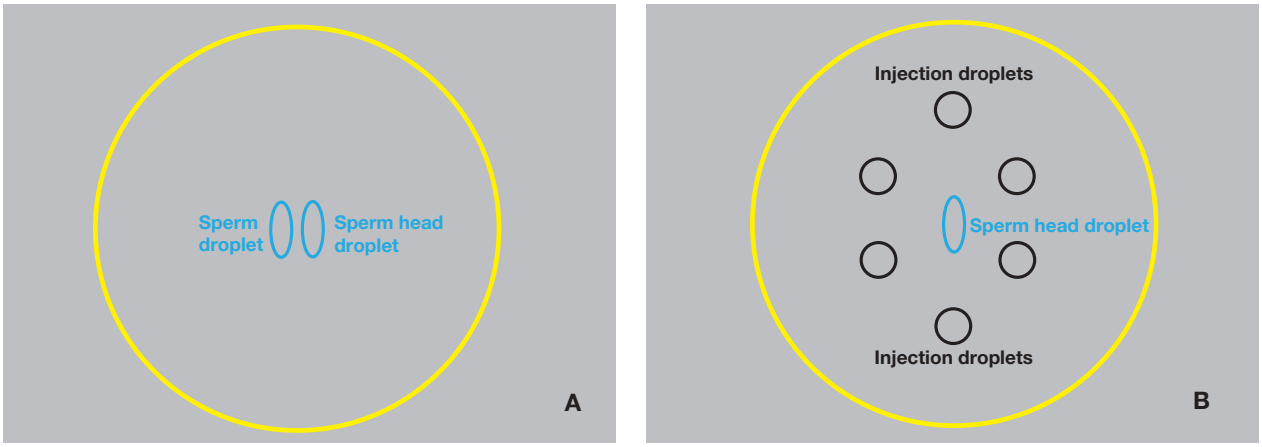

Fig. 4: ICSI dishes for sperm head isolation (A) and injection (B) using piezo-assisted method

4. Equipment setup

4. 1 Installation of PiezoXpert onto TransferMan NK 2

Loosen the M3x12 cheese head screw and remove the X head (Figure 5A). Rotate the X head 180°.
Tighten the X head again using the M3x12 cheese head screw.
Loosen the knurled screw (Figure 5B). Remove the knurled screw and the pressure plate from the X head.
Place the provided spacer plate on the bore of the X head.
Secure the spacer plate using the knurled screw and pressure plate.

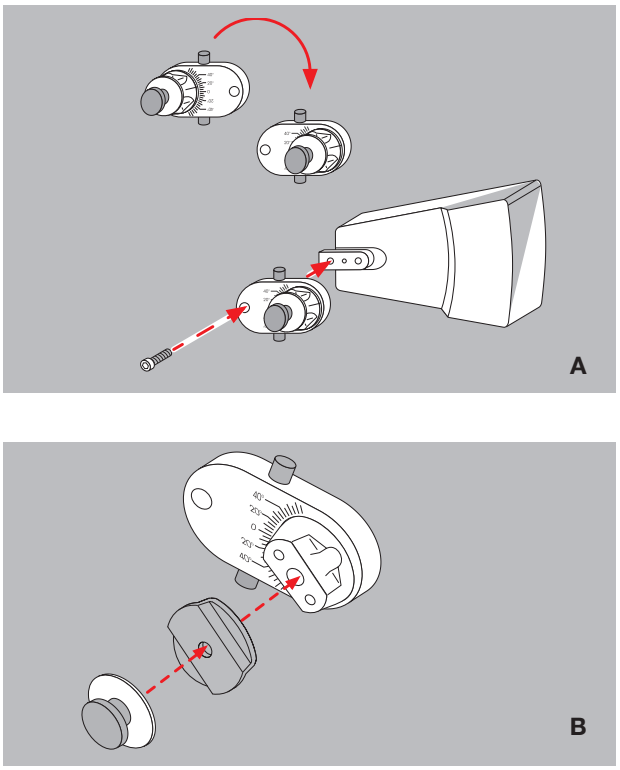

Fig. 5: Installation of spacer plate onto TransferMan NK 2

Place the actuator in the upper (see Figure 6A) or lower (see Figure 6B) groove of the distance plate. Tighten the knurled screw to secure the actuator between the distance plate and pressure plate.
The flatter the angle of the capillary, the more direct the effect of the piezo impulses. Most people use straight or low angled capillaries for piezo-actuated injection.

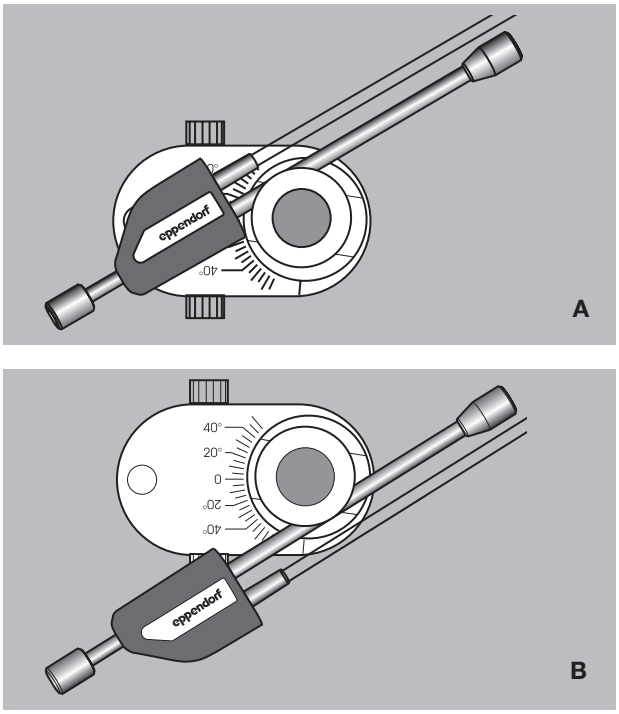

Fig. 6: Installation of piezo actuator onto TransferMan NK 2

Connect the tubing from CellTram vario to the rear end of the capillary holder of the actuator.
Dispense the oil by rotating the CellTram vario knob to the right until it is dripping from the opening of the grip head.

4. 2 Preparation of microinjection capillary

If Fluorinert FC-77 or FC-770 is used, back-fill the capillary using a Microloader. Approx. 1/3 of the capillary is filled with Fluorinert.

If mercury is used, back-fill the capillary with approx. 2 µL of mercury (i.e. 4 mm column) using a Hamilton syringe under a fume hood.

Mount the capillary in the actuator grip head.

Make sure that the capillary goes deep enough and touches the stopper of the capillary holder.

Absorb a small amount of medium via the front capillary opening, so that the samples will not get in contact with Fluorinert or mercury (depending on substance in use), but the heavy liquid is still as close as possible to the front opening. This is important to ensure an optimal performance for the drilling.

Focus on the periphery of the PVP drop, bring the capillaries into the same focus so that the periphery of the drop and capillaries are both in focus. Set Position 1 for both capillaries. Optionally, the Z-Limit (special function of the TransferMan NK 2) can be set using the periphery as a guideline to prevent capillary breakage.

Raise both capillaries, so that they are just above the medium droplet. The capillaries should remain in the mineral oil to prevent evaporation and capillary blockage. Then Position 2 is set.

The parameters (intensity, speed and number of pulses) of the PiezoXpert can be preinstalled. Up to 3 sets of optimized

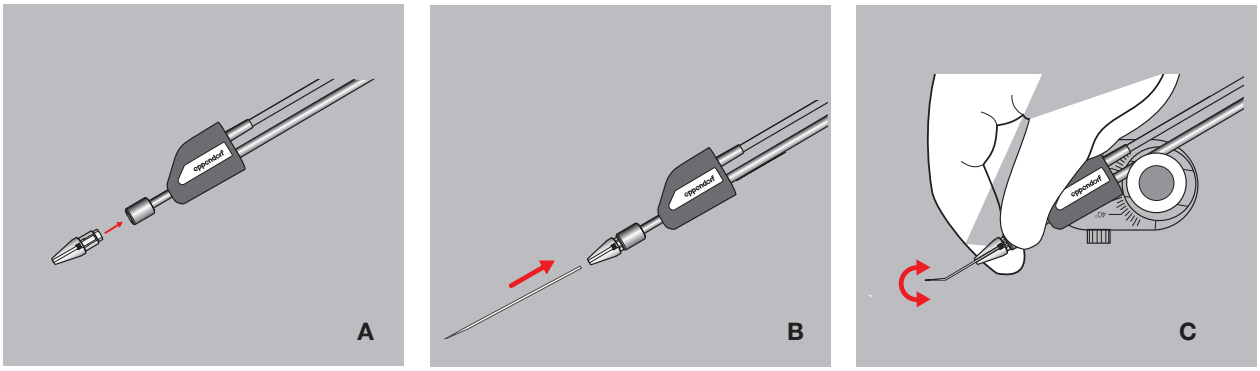

Figure 7: Preparation of microinjection capillary. Loosely screw the grip head into the front knurled screw of the actuator (A). Carefully push the capillary into the grip head until it touches the stopper (B). Tighten the grip head (C) and rotate the front knurled screw to align the angled capillary.

5 Piezo-assisted intracytoplasmic sperm injection (ICSI)

5. 1 System optimization prior to ICSI

The TransferMan NK 2 can store up to 3 positions. When a position is stored, the capillary can be recalled to the position automatically simply by pressing the position button or a double-click on the joystick button. Preinstallation of positions can significantly speed up the injection process and reduce the time of cells out of the CO_2 incubator and eventually improve the survival rate of oocytes.

For mouse ICSI, usually two position storages are used:

Position 1 - Position to perform injection or sperm head separation

Position 2 – Parking position above the droplets for moving the plate from sperm droplet to oocytes droplets or vice versa

Figure 8: Setting of parameter sets A and B

programs can be stored. Each program consists of parameter set A and B. Usually, parameter set A is used for the penetration of the zona pellucida and set B is used for the penetration of the oolemma. Both sets A and B can be triggered via either the button on the control unit or the foot control.

Optimization of parameters:

1. Set the parameters for the speed and number of pulses to 1.
2. Gradually increase the value for intensity (starting from 1) until the piezo impulses are strong enough to penetrate the membranes.
3. Fine tune the speed and pulse parameters.
4. Use the lowest parameter settings that work.

Usually, the parameter settings for Fluorinert are slightly higher than for mercury.

A

	Parameter set A (zona penetration)	Parameter set B (oolemma penetration)
Intensity	10	1
Speed	1	1
Pulse	∞	1

B

	Parameter set A (zona penetration)	Parameter set B (oolemma penetration)
Intensity	20	5
Speed	1	1
Pulse	∞	1

Table 1: Parameter settings for mouse ICSI using mercury (A) and Fluorinert (B).

NOTE: Users should optimize the parameters for their own experiments as the settings always depend on individual laboratory protocols. As a guideline, if no cryopreserved oocytes are used, we recommend the parameters for both mercury and Fluorinert respectively, shown in Table 1.

For sperm head separation using the PiezoXpert, parameters with higher intensity and speed can be used. These parameters can be saved e.g. in program 2.

5.2 During ICSI

5.2.1 Isolation of sperm head:

When using the sonication method, refer to 1.

For applying the piezo-assisted method: First aspirate a single, motile sperm (head first) into the injection capillary (using the ICSI dish for sperm head isolation (Figure 4A).

Position the sperm so that its neck is at the opening of the capillary (Figure 9A).

Then apply a few pulses to separate the head from the tail. Aspirate the head into the injection capillary (Figure 9B). Repeat the procedure with the next sperm. Separate as many sperm heads as possible

Transfer the separated sperm heads into the ICSI dish before injection (see Figure 4B).

5.2.2 Microinjection of sperm heads into oocytes:

Aspirate up to five sperm heads into the capillary. This way is more efficient and less time consuming than treating one sperm at a time. Make sure the sperm heads are not directly lined up adjacent to each other within the capillary to avoid accidentally injecting more than one sperm head at a time into an oocyte.

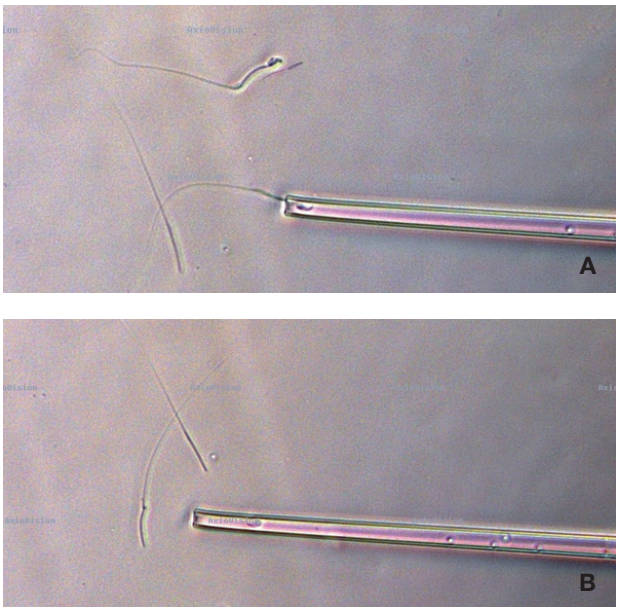

Fig. 9: Immobilization of a sperm using PiezoXpert (A, B)

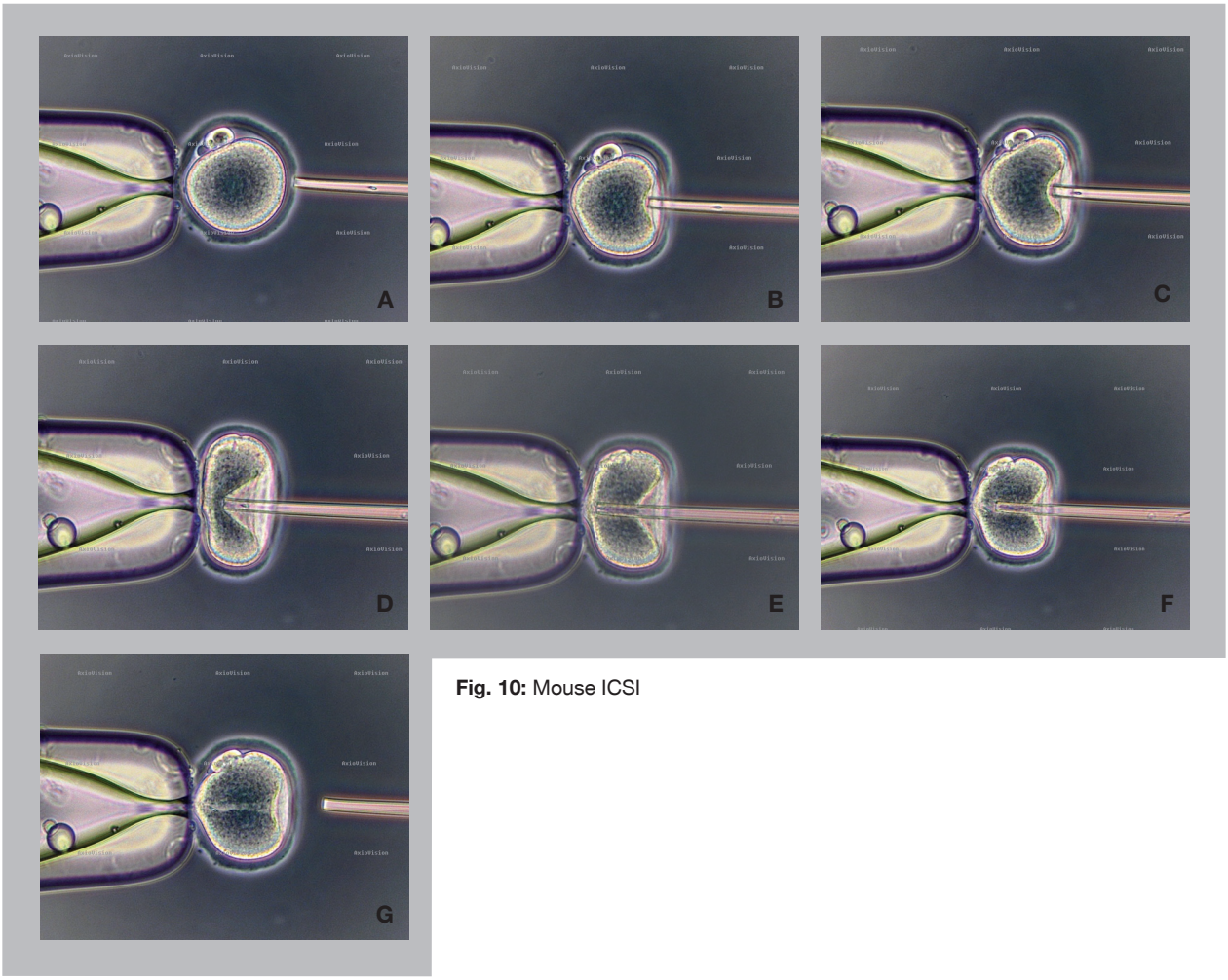

Fig. 10: Mouse ICSI

Move the stage to the injection droplet. Place the holding capillary using the previous stored positions. The oolemma of the oocyte should be sharply focused at 20x or 40x. Aspirate and hold the oocyte using the holding capillary and place the polar body at the 6 or 12 o'clock position. Use Position 1 to recall the injection capillary and bring the injection capillary to the zona pellucida (Figure 10 A); (Y-OFF function can be activated on TransferMan NK 2 control board to reduce the lateral movement of the capillary while penetrating the cell which may cause cell lysis). Advance the injection capillary while applying piezo impulse parameter set A via the foot control to penetrate the zona (Figure 10 B). Try to expel the zona plug into the perivitelline space (Figure 10 C). Subsequently, push the oolemma till a funnel shape is seen. Move one sperm head forward using the dispensing function of the CellTram vario until it is close to the tip of the capillary and advance the capillary until it almost reaches the opposite side (Figure 10 D). Then trigger piezo drilling parameter set B via the foot control until the relaxation of the oolemma is observed (Figure 10 E). The sperm head is then injected with a minimum of medium (Figure 10 F). Withdraw the capillary gently (Figure 10 G). Release the injected oocyte. Repeat the procedure for all other oocytes.

5.3 Post-ICSI

After injection, transfer the oocytes in KSOM under mineral oil in a humidified 5 % CO_2 incubator at 37 °C and further treat as described elsewhere [4].

References

[1] Yoshida N and Perry ACF. Piezo-actuated Mouse Intracytoplasmic Sperm Injection (ICSI). *Nature Protocols* 2007; Vol. 2, No. 2:296-304.

[2] Collas P and Barnes FL. Nuclear Transplantation by Microinjection of Inner Cell Mass and Granulosa-Cell Nuclei. *Mol Reprod Develop* 1994; 38:264-167.

[3] Kimura Y and Yanagimachi R. Intracytoplasmic Sperm Injection in the Mouse. *Biol Reprod* 1995; 52:709-720.

[4] Stein P and Schneider I. Piezo-actuated Mouse ICSI. Eppendorf Userguide 032. www.eppendorf.com.

[5] Nagy A, Gerstenstein M, Vintersten K, Behringer, B. Manipulating the Mouse Embryo: A Laboratory Manual (Third Edition). Cold Spring Harbor Laboratory Press; 2003.

[6] Deng M, Kishikawa H, Yanagimachi R, Kopf GS, Schultz RM, Williams CJ. Chromatin-mediated cortical granule redistribution is responsible for the formation of the cortical granule-free domain in mouse eggs. *Dev Biol* 2003; 257(1):166-76.

Ordering information

Product	Description	Order no. International	Order no. North America
Eppendorf PiezoXpert®	Basic device incl. Actuator, Foot control and spacer plate*	5194 000.016	5194000024
TransferMan® NK 2 **	Proportional micromanipulator for suspension cells	5188 000.012	920000011
CellTram® Air **	Manual pressure device for the reliable holding of suspended cells	5176 000.017	920002021
CellTram® Oil **	Manual pressure device for the reliable holding of suspended cells	5176 000.025	920002030
CellTram® vario **	Manual hydraulic microinjector, with gears 1:1 and 1:10	5176 000.033	920002111
VacuTip™ ** and ***	25 glass capillaries for holding large cells (e.g. eggs), sterilized, tip angle 35°	5175 108.000	930001015
PiezoDrill Tip Mouse ICSI™	25 glass capillaries for the transfer of mouse sperms, angle 25°	5175 220.005	930001091
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Microscope Adapter	Adapter for micromanipulators available for different inverse microscopes	Available on request	Available on request
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GALAXY® 14 S (120 V) ****	Incubator with 1-19 % O_2 control		CO14S-120-0200

* For mounting the PiezoXpert onto TransferMan NK 2 or PatchMan NP 2

** This product is registered in Europe as a medical device (according to Medical Device Directive MDD/93/42/EDD). This product is not registered in the U.S. as a medical device and does not have a 510(k) registration. For resarch use only. Not for use in human medical applications.

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