Applications

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Somatic cell nuclear transfer (SCNT) into mouse oocytes using Eppendorf PiezoXpert®

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Abstract

Since the birth of the first cloned animal, Dolly, the world famous sheep [1], mammalian cloning by somatic cell nuclear transfer (SCNT) has been successfully performed in many species. Compared to farm species, cloning of rodents is harder to achieve, since the oocytes of rodent species and particularly of mouse are smaller and more fragile. By allowing the transfer of a nucleus without rupturing the oocyte, the use of piezo-driven, mercury-filled micropipettes made mouse SCNT more successful. But this technique still has some drawbacks. Due to lateral oscillation of the piezo device, the number of successfully micromanipulated oocytes can be severely reduced. In this Application Note we report on our experience with mouse SCNT using the Eppendorf PiezoXpert, the latest advance in piezo drilling largely free of lateral oscillations.

Introduction

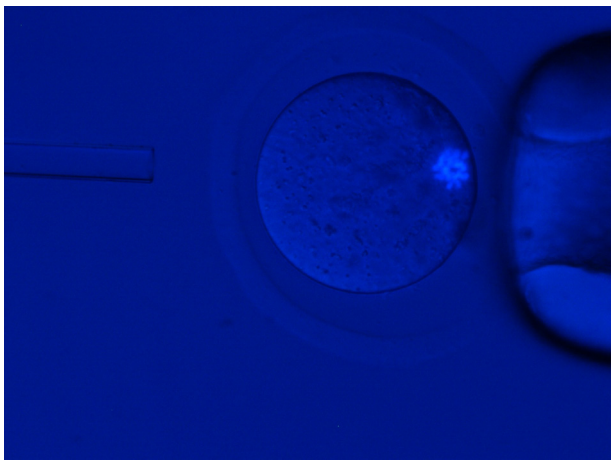

Figure 1: Enucleation of mouse oocyte preloaded with Hoechst 33342 (5µg/mL) in order to visualize the chromosomes of the metaphase II meiotic spindle. Use of Hoechst for for visualization purposes only. Original magnification 40x objective.

After fertilization the mammalian oocyte can give rise to up to 10^{15} body cells of over 200 different types. Besides this natural ability to incorporate the sperm nucleus, the oocyte can also 'reprogram' the somatic nucleus after somatic cell nuclear transfer (SCNT), which is better known to laypeople as 'cloning'. The term 'reprogramming' indicates the activation of a broad repertoire of gene functions that are normally silent in the somatic nucleus, whereby the nucleus-transplanted oocyte achieves a totipotential or pluripotential state that is almost indistinguishable from that seen after fertilization. Among other goals, cloning promises to enable new approaches to endangered species preservation, livestock propagation, development of new genetic models, development of animals producing valuable biopharmaceutical, as well as stem cell-based methods for treating disease and injury. Instrumental to these goals, the oocyte is the only natural means to reprogram somatic nuclei, that is, the only cell existing in nature endowed with such ability.

SCNT was theorized by Hans Spemann in the 1930s and pioneered by Robert Briggs, Thomas King and John Gurdon in the 1950s and '60s in the context of amphibian species, as experimental means to test if the total genetic information of the somatic nucleus was preserved and if epigenetic changes undergone by the nucleus were reversible after cell differentiation. Despite reproducible success in amphibian species, cloning of mammals from somatic nuclei proved so much tougher to achieve that the task was even suggested to be biologically impossible. This attitude changed in 1997, when Dolly the sheep was cloned, followed by Cumulina the cloned mouse [1, 2]. To date, about 20 mammalian species or hybrids thereof have been cloned. The cloning of Dolly was accomplished by electrofusing 277 enucleated sheep oocytes with udder cells, followed by transfer to genital tract of 29 cloned embryos, of which 1 was born as healthy lamb - Dolly. Cumulina was the product of 62 mouse oocytes that were stripped of their meiotic chromosomes (see Figure 1 as example) and then microinjected with the nuclei of ovarian cumulus cells, followed by cloned embryo transfer to recipient females. It is thus apparent that two distinct SCNT methods were used for Dolly and Cumulina - cell fusion vs nucleus microinjection. Compared to farm species, oocytes of rodent species and particularly of mouse are smaller and more fragile, thereby proving also more difficult to micromanipulate. Two major innovations that were pivotal in making mouse SCNT more amenable to succeed, were: 1) the use of a piezo-driven, mercury-filled micropipette, which facilitated nucleus microinjection into the mouse oocyte without its rupturing; and 2) the use of Strontium chloride as an improved means of oocyte activation after SCNT [3].

Now, fifteen years after Cumulina, and despite success rates of up to 48% ES cell derivation and 9% full-term development (reviewed in [4]), mouse SCNT remains a difficult procedure that is well established and works robustly only in a few laboratories worldwide. Two main hurdles are the micromanipulation per se, and the 'enigma' of finding culture conditions that allow cloned embryos to thrive. The mammalian oocyte is surrounded by a glycoprotein shell - the zona pellucida - and is bordered by a membrane - the oolemma. Both these natural barriers must be trespassed in order to place the somatic nucleus in the oocyte's cytoplasm (ooplasm). To this end, the piezo-driven, typically mercury-filled micropipette is used three times on the oocyte: first time to penetrate the zona just prior to aspirate the oocyte's own chromosomes ('enucleation'), second time to penetrate the zona just prior to nuclear transfer, and third time to make a hole in the oolemma and release the nucleus in the ooplasm. The piezo device is not essential, and conventional microinjection [5] can also lead to successful mouse cloning, although this approach has not become popular. Likewise, laser-assisted zona penetration can in part replace the use of piezo impact in mammalian cloning [6], but in fact its use is established only in the infertility clinic, where the value of human oocytes justifies additional support by this technology.

One problem of current piezo devices is that the micropipette oscillates laterally besides axially. Bringing this problem under control can be time-consuming and frustrating. Practically, this problem can reduce the number of successfully micromanipulated oocytes from over 100 to just a handful, per session. Here we report on our experience with mouse SCNT using the Eppendorf PiezoXpert, the latest advance in piezo drilling largely free of lateral oscillations. This application note is based on encouraging preliminary results obtained during the field test of the new Eppendorf PiezoXpert.

Materials and Methods

Equipment and reagents

- Nikon TE2000 inverted microscope, or equivalent, fitted with Nomarski optics e.g. ELWD 4x and 40x Nomarski objectives
- Micromanipulator (manual Narishige)
- Micromanipulation chamber (Figure 2A, B)
- Eppendorf PiezoXpert (Figure 5)
- Micrometer syringe 2 mL (Gilmont) for actuation and pressure control of the holding micropipette
- Eppendorf manual Celltram vario microinjector for nuclear transfer micropipettes
- Microforge (TPI de Fonbrune)
- Pipette puller P-97 (Sutter Instruments Co.)
- Borosilicate glass capillaries 0.78 mm inner diameter (ID) (Harvard Apparatus)
- Elemental mercury and microliter syringe for mercury (Hamilton type 701N)
- Stock solution polyvinylpyrrolidone 40 kDa (PVP, Calbiochem), 16% w/v
- Stock solution Cytochalasin B (5000 µg/mL in DMSO)
- CO_2 incubator set to 37 °C and 7% CO_2
- Culture media (Hepes-buffered CZB medium, α-MEM)
- Culture dishes (Corning, cat.no. 430588)
- 4 well dishes (Nunc, cat. No. 144444)
- Temperature of 28-29 °C in the whole room, or microscope stage fitted with Thermoplate (Tokai Hit)
- **CAUTION:** Mercury is toxic and must be stored and disposed according to local laws and regulations.
- **NOTE:** Mercury can be replaced with Perfluor (a.k.a. FC-770).

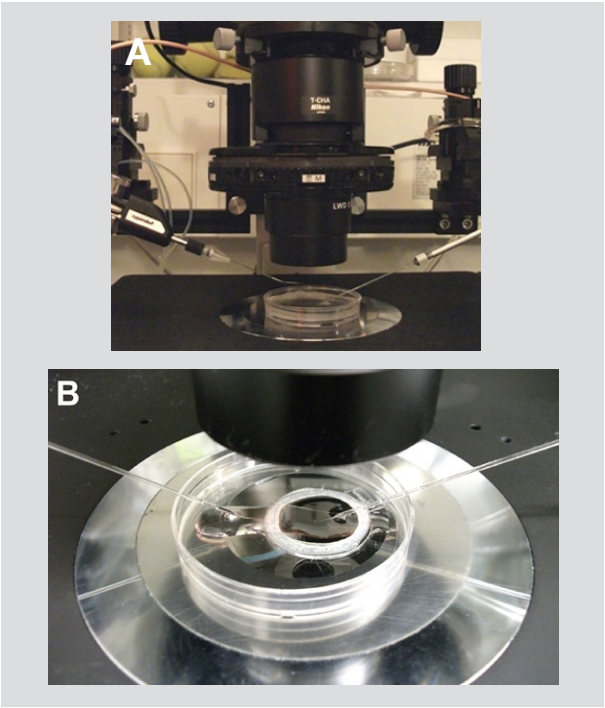

Figure 2: A Micromanipulation chamber on the stage of the inverted microscope, with micropipettes, **B** (left, enucleation or nuclear transfer micropipette; right, holding pipette).

Collection and preparation of mouse oocytes and nucleus donor cells

- Oocytes are collected from the oviducts of 6-8 week-old B6C3F1 (C57BL/6J x C3H/HeN) mice after gonadotropin stimulation (10 IU each PMSG and hCG injected i.p. 48 h apart). Mice are sacrificed by cervical dislocation 15 h after hCG and the oviducts are removed. The oviductal bulge is teased and the cumulus-oocyte complexes are released in HCZB medium containing 50 U/mL hyaluronidase and 0.1% PVP (w/v) and incubated (RT) until the oocytes are free of cumulus cells. The naked oocytes are transferred to α-MEM in the incubator until micromanipulation (see Oocyte incubation and embryo culture).
- The cumulus-oocyte complexes make a convenient source of donor cells for nuclear transfer. Dispersed cumulus cells are kept in the refrigerator (4 °C) until use.
- **NOTE:** Resist temptation to speed up things by allowing the hyaluronidase digestion to take place in the incubator at 37 °C; while the enzymatic reaction of hyaluronidase will be faster at 37 °C, this temperature will also activate the contaminating proteases, causing damage to oocytes, unless the hyaluronidase is recombinant.
- **NOTE:** If the cumulus cells are not kept in the refrigerator they may attach to the culture dish.
- **NOTE:** Experimenters must comply with national regulations concerning welfare, care and use of animals (e.g. IACUC in the USA, LANUV in Germany).

Preparation of holding and microinjection pipettes

- In the holding pipette, inner diameter (ID) at the tip should be smaller than that of the oocyte, the outer diameter (OD) at the tip should be larger (e.g. OD 100 µm; ID, 20 µm). See also Figure 3.
- In the enucleation micropipette, ID should be 12-15 µm at the tip, which must be cut blunt at 90° using the microforge. The micropipette is bent with an angle of approx. 20° at a distance of 8-10 mm from the tip.
- In the NT micropipette, ID should be 7-8 µm at the tip, which must be cut blunt at 90° using the microforge. The micropipette is bent with an angle of approx. 20° at a distance of 8-10 mm from the tip.
- Back-load a small amount of mercury (about 2 µL i.e. 4mm column) into the micropipette using a Hamilton micrometer syringe.
- **NOTE:** If the OD of the holding pipette is too small it can cause the oocyte to 'jump' when the piezo-operated micropipette penetrates the zona pellucida (Figure 3B). If the ID opening of the holding pipette is too large it can cause excessive deformation of the oocyte, with possible consequences.
- **CAUTION:** Notched or jagged micropipette tips often kill the oocyte during injection.
- **CRITICAL STEP:** Polish the micropipette with hydrofluoric acid 20 % in water (v/v). Without this step, the pipette soils rapidly and needs to be changed.

Set up of the Eppendorf PiezoXpert

- Connect the Eppendorf PiezoXpert to a Gilmont syringe or Celltram vario. In the Boiani laboratory the enucleation and NT micropipettes are mounted on the left, the holding micropipette on the right side (Figure 2B). Pressure circuits are filled with water (enucleation and NT micropipette) and with oil (holding micropipette).
- Fit the capillary to the actuator of the Eppendorf PiezoXpert.
- Mount the enucleation or the NT micropipette in the shaft holder of the piezo unit.
- Push the mercury to the tip of the micropipette using the manual injector.
- Lower the micropipette into the HCZB medium of the micromanipulation chamber (Figure 2).
- Expel air and some mercury from the pipette in the HCZB droplet.
- **CRITICAL STEP:** Accurate preparation of the Piezo unit is very important for the success of NT. Spending time on the initial set-up is worth it, as it will save time later. If the pipette is adapted correctly with the piezo unit, then it will work very well even with lowest power settings.

Removal of the chromosomal spindle from oocytes ('enucleation')

- Add 1 µg/mL Cytochalasin B to the HCZB medium
- Place about 10-30 oocytes in the medium in the south sector of the micromanipulation drop. The number of oocytes to be handled at one time depends on the operator's skill. Each group should be processed within 10 min.
- Pick a single oocyte with the holding pipette and roll it using the enucleation micropipette until the metaphase II chromosomal spindle is positioned directly in front of the opening of the holding pipette (3 o'clock position; Figure 3A).
- The spindle is recognizable without any DNA staining using Nomarski optics.
- While holding the oocyte from the right-hand side, approach the oocyte with the enucleation micropipette from the opposite side (Figure 3A).
- Neutralize the backpressure of the micropipette and make contact with the zona pellucida.
- Drill the zona pellucida (Figure 3B). See piezo settings in Table 1.
- Without applying any impulse push the enucleation micropipette through the hole (Figure 3C), traverse the ooplasm and reach the spindle at the 3 o'clock position (Figure 3D).
- Gently turning the knob of the manual injector, suck the spindle into the enucleation micropipette along with a minimal amount of cytoplasm (Figure 3E-F).
- Withdraw the micropipette fast but gently and pull out of the oocyte (Figure 3G-I), which now becomes an 'enucleated' oocyte or ooplast.
- **CAUTION**: To avoid damaging or lysing the oocyte, ensure there is sufficient perivitelline space between the zona pellucida and the oolemma; this space takes care that the residual energy of the micropipette after piercing through the zona is dampened, thereby not damaging the oolemma.
- **CRITICAL STEP:** Do not apply piezo impulses to cut the membrane during removal of the spindle.

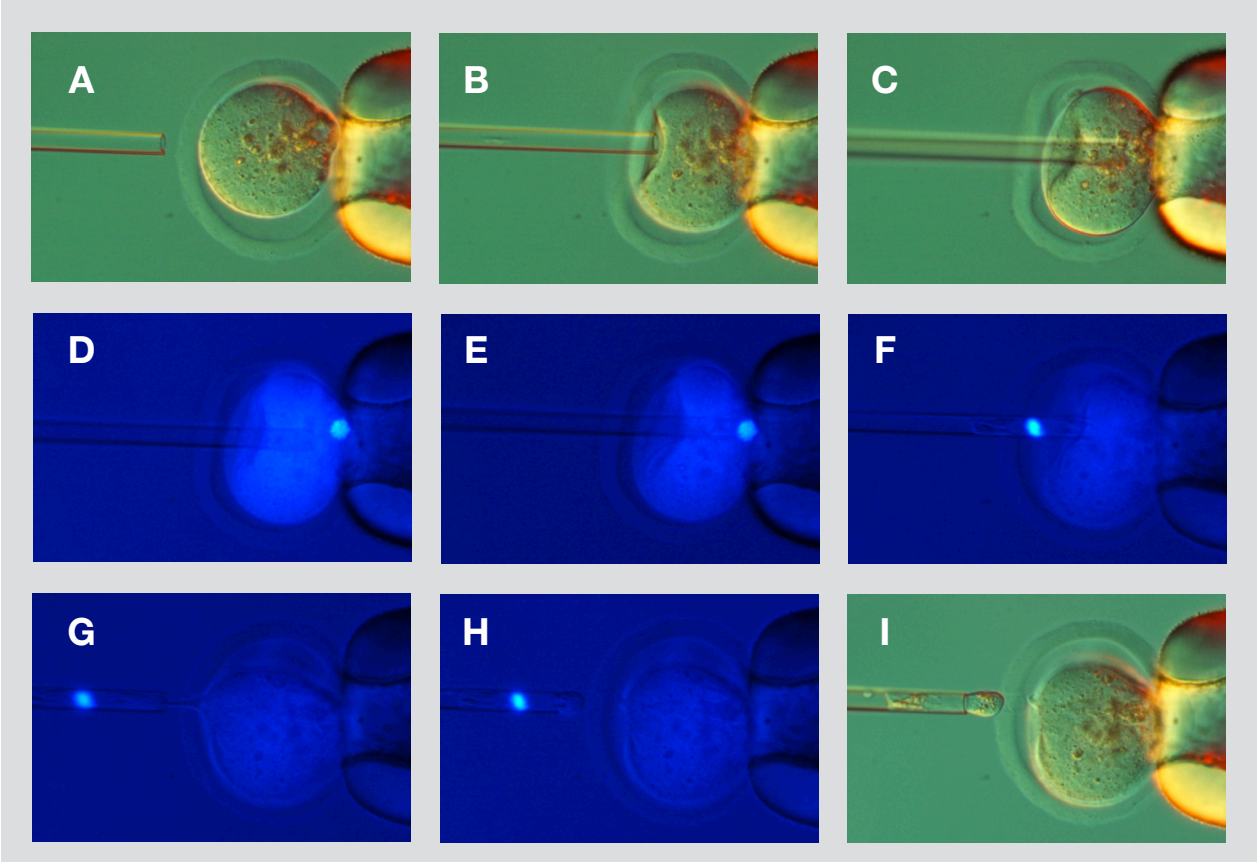

Figure 3: Steps of enucleation of mouse oocytes preloaded with Hoechst 33342 (5 µg/mL) in order to localize the chromosomes under UV excitation. Use of Hoechst 33342 is for visualization purposes only. Original magnification 40x objective.

Injection of single cumulus cell nuclei into enucleated oocytes

- Add 1% PVP to the HCZB medium, so as to reduce 'stickiness' of isolated nuclei during SCNT.
- Transfer approximately 10^3-10^4 cells in the north sector of the micromanipulation drop.
- Place about 10-30 oocytes in the HCZB medium in the south sector of the micromanipulation drop. The number of oocytes depends on the operator's skill level. Each group should be processed within 10 min.
- Turning the knob of the injector, aspirate about 50 cumulus cells into the micropipette while applying strong piezo impulses. While being aspirated into the micropipette, the cell membranes are broken and most of the cytoplasm is displaced.
- Pick a single oocyte with the holding pipette.
- Bring a single nucleus to a distance of 20-30 µm from the tip of the micropipette.
- While holding the oocyte from the right-hand side, approach the oocyte from the opposite side with the nuclear transfer micropipette (Figure 4A).
- Neutralize the backpressure of the micropipette and make contact with the zona pellucida.
- Apply 2-4 piezo impulse to the zona pellucida so as to drill the zona pellucida (Figure 4B). See piezo settings in Table 1.
- Without applying any impulse push the NT micropipette through the hole (Figure 4C), traverse the ooplasm (Figure 4D) and reach the opposite side (3 o'clock position; Figure 4E).
- Apply one single piezo impulse (see piezo settings in Table 1) to puncture the oolemma at the micropipette's tip, so as to make a hole. Rapid relaxation of the oolemma shows that the hole has indeed been made. Note that as the oolemma relaxes, the nucleus may move a bit backwards inside the micropipette.
- Release a single nucleus inside the oocyte (Figure 4G).
- Withdraw the micropipette fast but gently and pull out of the oocyte (Figure 4H-I).
- Allow the injected oocytes 5-10' recovery and then return them to culture medium (see Oocyte incubation, activation of reconstructed oocytes and embryo culture).

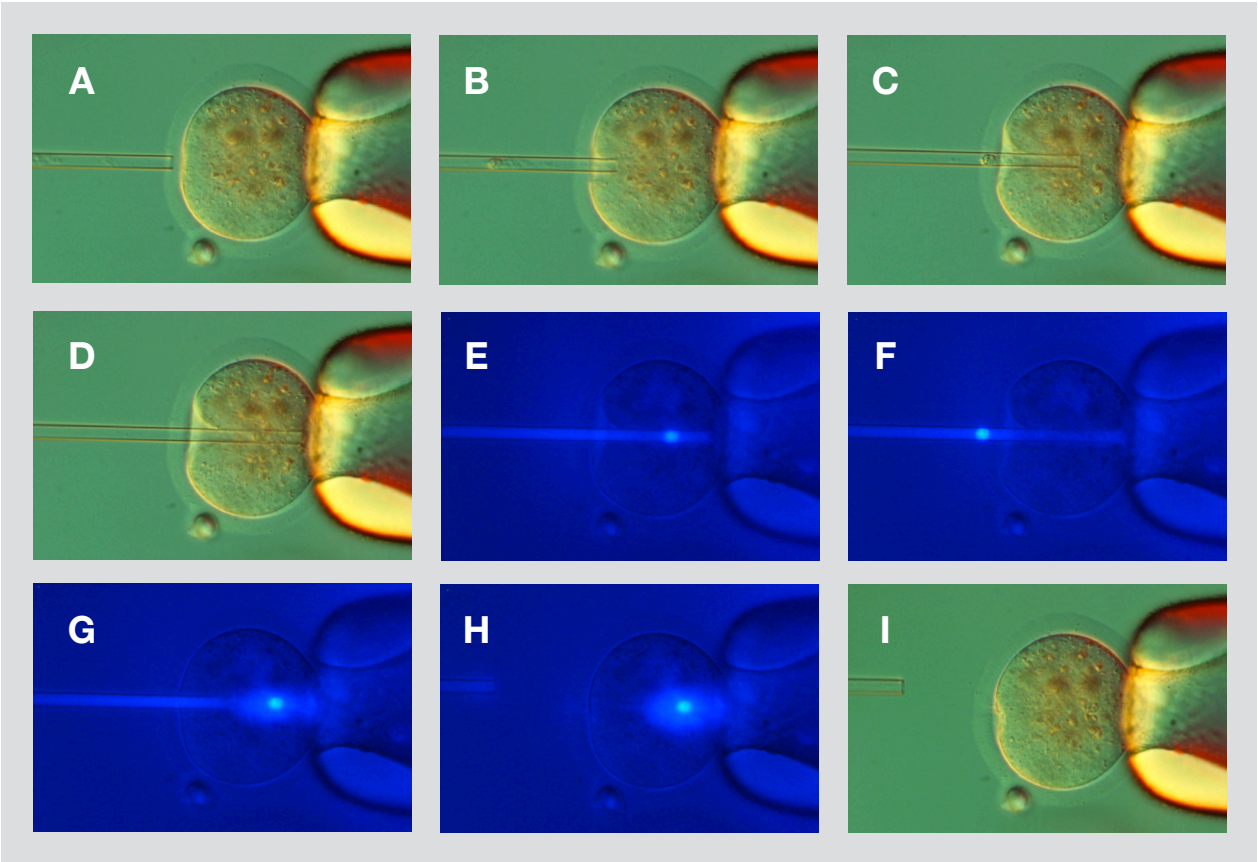

Figure 4: Steps of nuclear transfer in mouse oocytes. Cumulus cells were preloaded with Hoechst 33342 (5 µg/mL) prior to performing SCNT. Use of Hoechst 33342 is for visualization purposes only. Original magnification 40x objective.

Table 1: Values of the settings used for the Eppendorf PiezoXpert in the Boiani laboratory (values may vary depending on the tubing used to connect the syringe with the SCNT micropipette, on the fluid in the tubing, on the amount of mercury in the SCNT micropipette, etc.).

	Enucleation (zona drilling)			Nucleus pick-up			SCNT (zona drilling)			SCNT (oolemma)		
Eppendorf PiezoXpert	Intensity	Speed	Pulse	Intensity	Speed	Pulse	Intensity	Speed	Pulse	Intensity	Speed	Pulse
	18	3	∞	29	3	∞	28	3	∞	11	1	1

Table 2: Performance of the Eppendor PiezoXpert at mouse cloning in the Boiani laboratory.

	n oocytes	n survived	n blastocyts
Eppendorf PiezoXpert	111	93	28

- **CRITICAL STEP:** The nuclei must move without any friction inside the NT micropipette, and must be released in one shot without remaining attached to the micropipette opening's edge.
- **CRITICAL STEP:** Do not apply the piezo impulse until the pipette has reached the opposite side. If the piezo impulse is applied in the middle of the oocyte, the oocyte will die after injection.
- **CAUTION:** Keep the volume of medium coinjected with the nucleus to a minimum.
- **NOTE:** More than 90% of cumulus cells will be at the G0/G1 stage of the cell cycle, so you can use them without any imposed synchronization of the cell cycle.

Oocyte incubation, activation of reconstructed oocytes and embryo culture

- We routinely incubate oocytes and embryos in 500 µL α-MEM in 4-well plates without oil overlay (see incubator settings under „Equipment and reagents").
- The nucleus-transplanted oocytes are activated using Ca^{2+}-free α-MEM supplemented with 5 µg/mL Cytochalasin B and 10 mM Strontium chloride [3].
- Transfer the nucleus-transplanted oocytes to activation medium and incubate for 6 h in a 7% CO_2 incubator at 37 °C.
- Allow development in α-MEM, which is supplemented with 0.2% BSA and 50 mg/mL Gentamicin. Lack of oil overlay serves the purpose of letting ammonia (embryotoxic) diffuse in the air.
- **CRITICAL STEP:** Make the activation medium fresh the day before use using the stock solution, and incubate at 37 °C in a 7% CO_2 incubator.
- **CRITICAL STEP:** Add Cytochalasin B and Strontium chloride at least 60 min before use, and equilibrate at 37 °C in a 7% CO_2 incubator.

Results and discussion

As remarked in a previous User Guide [7], microinjection pipettes driven by the Eppendorf PiezoXpert had lower extent of lateral oscillation [8] compared to Primetech PMM. These oscillations are easily revealed by the ‚jumping' movement of the oocytes when in the process of being drilled. The use of high-speed microcinematography will document the lateral oscillations of the Eppendorf PiezoXpert conclusively in future studies.

Using the piezo settings listed in Table 1, survival rates after microinjection with the Eppendorf PiezoXpert were comparable to those afforded by the PMM piezo routinely used in the Boiani laboratory (Table 2). Cloned embryo development after SCNT with the Eppendorf PiezoXpert was successful in that the nucleus-transplanted mouse oocytes developed to blastocysts *in vitro*.

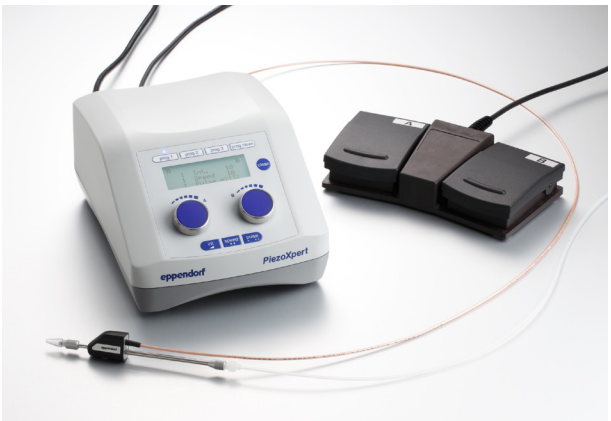

Figure 5: Eppendorf PiezoXpert with actuator in the front and foot control.

Although the amount of data as well as tested time period are not sufficient for statistical analysis, the Eppendorf PiezoXpert proved itself extremely user-friendly. In skillful hands, Eppendorf PiezoXpert supported up to 4 enucleations and 5 nuclear transfers per minute. This means that a skilled single operator could generate over 200 cloned mouse embryos in one session. It follows that by using an optimized culture protocol such as that of Wakayama (reviewed in [4]), 200 reconstructed oocytes could yield up to 140 blastocysts, 70 ES cell lines and 20 cloned mice. With these astounding numbers, alternatives to oocyte-mediated reprogramming, such as iPS cell technology [9], become less attractive. In fact, the sole real limitation to high-throughput oocyte-mediated reprogramming, is the source of oocytes, which are difficult and ethically problematic to obtain in primates. In conclusion, while both PMM and the Eppendorf PiezoXpert deliver good results in skillful hands, the Eppendorf PiezoXpert proved more efficient at zona piercing and oolemma penetration, compared to PMM, probably due to a lower extent of lateral oscillations. Our field test results justify further refinement and development of Eppendorf PiezoXpert technology for application as a reliable support to micromanipulation.

References

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Ordering information

Product	Description	Order no. International	Order no. North America
Eppendorf PiezoXpert®	Basic device incl. Actuator, Food pedal and Distance 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**, ***	25 glass capillaries for holding large cells (e.g. eggs), sterilized, tip angle 35°	5175 108.000	930001015
Microloader	Capillary tip for filling microinjection capillaries, set of 2x 96 pcs.	5242 956.003	930001007
Microscope Adapter	Adapter for any inverse microscopes	Available on request	Available on request
Galaxy 14 S (230 V) ****	"Personal" sized CO_2 incubator offering 14L of CO_2 Incubation with an LED display	CO14S-230-0000	
Galaxy 14 S (230 V) ****	Incubator with active 1-19 % O_2 control	CO14S-230-0200	

* For mounting the Eppendorf PiezoXpert onto TransferMan NK 2

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

*** This product is proven non cytotoxic by the mouse embryo development test.

**** New Brunswick CO2 Incubators have been designed for research use only. New Brunswick CO2 Incubators in general are not certified for any human IVF/medical application

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