Piezo-actuated mouse intracytoplasmic sperm injection (ICSI)

Naoko Yoshida & Anthony CF Perry

Laboratory of Mammalian Molecular Embryology, RIKEN Center for Developmental Biology, 2-2-3 Minatojima Minamimachi, Chuo-ku, Kobe 650-0047, Japan. Correspondence should be addressed to A.C.F.P. (tony@cdb.riken.jp).

Published online 1 March 2007; doi:10.1038/nprot.2007.7

The mouse is a genetically tractable model organism widely used to study mammalian development and disease. However, mouse metaphase II (mII) oocytes are exquisitely sensitive and intracytoplasmic sperm injection (ICSI) with conventional pipettes generally kills them. This problem can be solved with piezo-actuated micromanipulation, in which the piezo-electric effect (crystal deformation in response to an externally applied voltage) propels a microinjection needle tip forward in a precise and rapid movement. Piezo-actuated micromanipulation enhances the penetration of membranes and matrices, and mouse ICSI is a major application. Here we describe a comprehensive, step-by-step mouse piezo ICSI protocol for non-specialists that can be completed in 2–4 h. The protocol is a basic prelude to multiple applications, including nuclear transfer cloning, spermatid injection, blastocyst injection, mII transgenesis, and streamlining micromanipulation in primates and livestock. Moreover, piezo ICSI can be used to obtain offspring from 'dead' (non-motile) sperm, enabling trivial sperm freezing protocols for mouse strain storage and shipment.

INTRODUCTION

Piezo-actuated micromanipulation harnesses the piezo-electric effect to transmit a small crystal lattice distortion to the tip of a pipette, driving it forward in a precise and controlled manner. Piezo-actuated micromanipulation has multiple applications in the study and engineering of gametes and embryos. It enabled the first intracytoplasmic sperm injection (ICSI) to produce mice[1], the first nuclear transfer cloning of mice[2] and pigs[3], the first productive frozen[4] and freeze-dried sperm injections[5], and the first production of nuclear transfer embryonic stem (ES) cells[6]. Piezo was utilized to generate the first transgenic offspring by injecting unfertilized oocytes in metaphase II (mII) transgenesis[7] and has been extended to the delivery of artificial chromosome transgenes[8,9]. Piezo has been employed for RNA interference (RNAi) in mII oocytes[10]; it enhances blastocyst injection with ES cells[11] and the manipulation of gamete precursors[12] and facilitates the renaissance of stem cell biology[13].

Piezo-actuated micromanipulation was developed by Atsushi Mimatsu and colleagues, and its application as a biological research tool was demonstrated in the mouse by Dr. Yasuyuki Kimura, who used it to generate the first mouse offspring by ICSI[1]. Piezo was necessary because in the mouse, oocyte plasma membranes are exquisitely sensitive and survival rates following ICSI with conventional (i.e., manual, non-piezo) microinjection rarely exceed 50% (ref. 14). Contrastingly, piezo ICSI can achieve mouse oocyte survival rates of 100%, with 90% development to morula/blastocyst stages *in vitro*. The efficacy of piezo is highly desirable, not only in ICSI but also where development following microinjection is less efficient, such as in nuclear transfer. Most mouse nuclear transfer clones have been generated via piezo-actuated delivery of donor nuclei into recipient oocytes.

Piezo displaces the microinjection needle ≥ 0.1 μm at ≤ 40 μm S^{-1} and can introduce precise, non-lethal plasma membrane breaks even with tips of relatively large outer diameter (>10 μm); indeed, piezo can successfully drive still wider 'pipes' (up to ~ 30 μm outer diameter) for other applications. The capacity to drive wide tips for non-lethal injection augments the range of applications possible with piezo; conventional microinjection is typically restricted to tip diameters of 1–2 μm—far too small for functional injection of eukaryotic cells. In addition, and again in contrast to conventional micromanipulation, piezo permits multiple procedures to be performed with a single needle. Thus, although many oocytes (such as those of humans, cattle, pigs and rabbits) can be injected conventionally, the relative speed and ease of piezo means that it is advantageous even in these species[3,15–20]. Ease of pipette fabrication (beveled tips are not required) and speed also make piezo suitable for the injection of blastocysts with ES cells[11], and the method additionally allows partial ablation of the inner cell mass (i.e., the removal of potentially competing embryonic cells).

The present protocol describes piezo for mouse ICSI, which is a preferred place to learn piezo before attempting other applications, such as nuclear transfer. Piezo ICSI shares basic steps with other piezo applications and controls for user technique (both micromanipulation and embryological); injected oocytes should develop efficiently *in vitro*, and *in vivo* following embryo transfer, enabling beginners to ascertain the quality of their piezo technique.

MATERIALS

REAGENTS

- Mice of the strain B6D2F_1, female C57BL/6 × male DBA/2 F_1 hybrid (suppliers include Charles River Laboratories and Shimizu Laboratory Supplies). **! CAUTION** Experimental procedures involving animals must be carried out according to national and institutional regulations.
- Kalium simplex optimized medium (KSOM)
- Nuclear isolation medium (NIM)
- Embryo-tested bovine testis hyaluronidase (Sigma, cat. no. H-4272)
- 12% (w/v) polyvinylpyrrolidone (PVP_{360}, average $M_r \approx 360{,}000$), dissolved in sterile, high quality distilled water
- Mineral oil (Shire or Nakalai Tesque)

- Elemental mercury (Hg^0), optional ! CAUTION Hg^0 is a cumulative neurotoxin absorbed through the skin. Handle with gloves and dispense waste according to local institutional guidelines.
- Fluorinert (3M Specialty Materials), optional. On a scale of 0 to 10 on which Hg^0 scores 10 in piezo, Fluorinert FC-77 scores 8 (acceptable), while FC-43 (score = 4) and FC-70 (score = 3) have higher viscosity and are more 'sticky'
- Hormones for superovulation. Consult your institutional animal facility for reliable sources of human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG), also known as equine chorionic gonadotropin (eCG)

EQUIPMENT

- Dumostar #5 fine biological forceps (Electron Microscopy Sciences, cat. no. 72705-01)
- Transfer pipette
- Holding pipettes (Eppendorf)
- Borosilicate glass capillaries (Sutter, cat. no. B100-75-100), 10 cm × 1 mm (outer diameter), 0.75 mm (inner diameter)
- Precision pipette, such as the P200 Pipetman (Gilson, Inc.)
- Pipette puller, such as the Flaming/Brown P97/IVF micropipette puller (Sutter Instrument Co.)
- Microforge (manufacturers include Narishige and De Fonbrune)
- Stereomicroscope, such as the SZX12 (Olympus)
- Stereomicroscope stage heated platform, such as the MATS-55SZX Thermo Plate (Tokai Hit Co.) or equivalent
- Workstation comprising inverted microscope, such as IX71 (Olympus) equipped with MO-202U micromanipulators, IM-5 and/or -6 injectors (Narishige), heated stage (MATS-55R30 Thermo Plate; Tokai Hit Co.) and Hoffman modulation contrast optics (4× and 20× objectives) or equivalent
- Air-cushioned table or platform (suppliers include Technical Manufacturing Corporation and Meiritsu, respectively). The need for insulation against vibration depends on the location of the workstation
- Piezo-actuated micromanipulator, such as the PMAS-CT150 (Prime Tech)
- Humidified CO_2 (5% (v/v) in air) incubator (suppliers include Sanyo and Heraeus)
- Falcon 10-cm (100 × 20 mm) Optilux petri dishes (Becton Dickinson, cat. no. 353003) or equivalent, bottoms are suitable for oocyte/embryo collection and lids for micromanipulation (compatible with Hoffman modulation optics)
- Falcon 3.5-cm (35 × 10 mm) dishes (Becton Dickinson, cat. no. 351008), used for short term embryo culture (~24 h)
- Falcon 6-cm (60 × 15 mm) dishes (Becton Dickinson, cat. no. 351007), used for longer culture
- Kimwipe delicate task wipers (Kimberly-Clark, cat. nos. 34155 (11.4 × 21.3 cm; small) and 34133 (30 × 30 cm)) or equivalent low-lint tissues
- 1-ml syringe
- 26 G needle
- Microinjection pipette (also referred to as microinjection needle)

REAGENT SETUP

Chatot, Ziomek, Bavister medium (CZB) Contains 81.6 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.1 mM EDTA.Na_2, 31 mM Na.lactate, 5.6 mM D-glucose, 25 mM $NaHCO_3$, 0.3 mM Na.pyruvate, 1 mM D-glutamine, 10 μg ml^{-1} phenol red (0.5% (w/v) in Dulbecco's phosphate-buffered saline (DPBS); Sigma, cat. no. P-0290), 1 mg ml^{-1} BSA; pH 7.4; ref. 21. Prepare with double-distilled or higher purity water. For embryo culture, CZB should be 0.22 μm sterile-filtered. Store and use as for KSOM. The source of BSA can effect development, but embryo-tested Fraction V powder (Sigma, cat. no. A-3311) is commonly used.

CZB·HEPES (CZB·H) This is essentially HEPES-buffered CZB, and contains 20 mM HEPES, 81.6 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.1 mM EDTA.Na_2, 31 mM Na.lactate, 5 mM $NaHCO_3$, 0.3 mM Na.pyruvate, 0.1 mg ml^{-1} polyvinyl alcohol, 10 μg ml^{-1} phenol red (0.5% (w/v) in DPBS); pH 7.4; ref. 22. It is used for micromanipulation, 0.22 μm sterile-filtered. Store in 5-ml polypropylene bottles at 4 °C for up to a month. ▲ CRITICAL Use a fresh bottle of medium per experimental day. ▲ CRITICAL Any marked departure from the osmolarity of CZB • H can induce oocyte lysis.

KSOM This is a frequently-used alternative to CZB for embryo culture. KSOM+AA (Specialty, cat. no. MR-107-D) contains amino acids plus 5.6 mM D-glucose, and should be further supplemented with 20 μl of phenol red solution (0.5% (w/v) in DPBS) and 1 mg ml^{-1} BSA (Sigma, cat. no. A-3311). ▲ CRITICAL Store in 5-ml polypropylene bottles (Perfector Scientific, cat. no. 2000-S) at 4 °C for up to a month; not all storage vessels are appropriate, as toxins may leach out over prolonged periods. Use a fresh bottle of medium each experimental day.

NIM Contains 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and 3.0 mM EDTA (pH 7.0) and is sterilized by autoclaving for use in sperm preparation[23].

PVP solution Dissolve 1.2 g PVP_{360} in ~8 ml high-quality, sterile water in a 50-ml Falcon tube on a roller (such as the Low Profile Roller of Stovall Life Science, Inc.) at room temperature (22–28 °C). The PVP_{360} takes ~1 h to dissolve. Make the final volume to 10 ml, 0.45 μm filter and store in 0.2–1.0 ml aliquots at −20 °C (storage may be for up to months or probably years). Different sources of PVP_{360} can affect oocyte activation and produce different developmental outcomes following piezo; ICN (recently rebranded 'Valeant') and Kanto (cat. no. 32285-20) have given good results. The 'in-use' aliquot of PVP_{360} solution is useable for several weeks stored at room temperature.

Embryo-tested bovine testis hyaluronidase stock Dissolve 30 mg of bovine testis hyaluronidase (2.25–4.5 × 10^4 U) in 3 ml CZB·H (to give 10 mg ml^{-1}). Store in 50 μl aliquots at −20 °C (storage may be for at least several months). It can be repeatedly thawed and refrozen. Use at a final concentration of 300–800 μg ml^{-1}.

Mineral oil The mineral oil overlay of culture droplets is dispensed from a wash bottle. To draw out plasticizers and other potential toxins from the wash bottle, fill the bottle with oil and allow it to stand for two weeks or more (discard this oil) prior to its first use. Sterilization of the oil is not necessary.

PMSG and hCG for superovulation Dissolve PMSG (sometimes called eCG) and hCG separately at 50 IU ml^{-1} in 0.9% (w/v) NaCl solution (not PBS). 0.22 μm-filter and store in 1 ml aliquots at −20 °C for 1–2 months (possibly longer depending on the batch, although the hormones gradually lose activity) or for 1–3 d at 4 °C. Do not refreeze.

EQUIPMENT SETUP

Microinjection needle preparation Borosilicate glass capillaries are pulled on a micropipette puller. Consult a local representative for instrument guidelines because several factors can affect performance, including the model, unit-to-unit variation and humidity. Pipettes may be pulled on a Flaming/Brown P97/IVF micropipette puller equipped with a 4.5 mm box filament, and should give a gradual taper. Suggested initial settings in this case are: heat = 830; pull = 75; velocity = 130; time = 100. Pulls which produce tapers beginning ~2.6 cm from the shoulder of the pull to the resultant whisp-like fiber tip are acceptable, but successful parameters vary. Flush-ended needles are generated from each pulled capillary on a microforge. Working with the 10× objective, introduce a ball of glass (300–500 μm diameter) onto the 'V' of the platinum-iridium filament of the microforge and bring it into sharp focus. Move the horizontal capillary so that it is just above the sharply defined edge of the glass ball in the same focal plane. Lower it to touch the ball where the capillary external diameter is 6.5–8.5 μm (**Fig. 1a**); the point of contact is where the break will occur. Apply a brief heat (current) pulse via the microforge foot pedal, ensuring that sufficient current flows through the filament to cause it to glow brightly. The filament expands and the capillary locally fuses with the glass of the ball, causing it to break when the filament contracts, producing a flush (flat) end (**Fig. 1b,c**). The required level of heat is determined empirically. ▲ CRITICAL Good needles are essential for success in piezo ICSI. The needle tip should appear regular and flush-ended. Next, move the needle upwards so that it is 5–10 μm above the glass ball and forwards ~3 mm (**Fig. 1d**). Starting at zero, gradually increase the heat to introduce a bend in the needle ~28 (±2)° from the horizontal (**Fig. 1e**). Remove the needle and, if appropriate, introduce ~2–3 mm Hg^0 into its wide, unworked end from a disposable 1-ml syringe fitted with a 26 G needle. Microinjection pipettes can be stored on rolled parafilm in a 10-cm Petri dish in ambient conditions for weeks. ● TIMING 10 pipettes take ~20 min to prepare.

Preparation of the microscope for micromanipulation A piezo workstation is shown in **Figure 2**. Locating the workstation on an air-cushioned table or platform helps to insulate against vibration (e.g., caused by freezers, elevators, air-conditioning or traffic), which otherwise can make micromanipulation unfeasible. A single holding pipette may be used for many sessions (over months to years) without changing it. At least one change of microinjection needle is recommended per session. Mount the microinjection needle firmly and use the injector to apply positive pressure and advance the front (e.g., Fluorinert or Hg^0) so that it approaches the needle tip. Lower the assembly into a droplet containing PVP_{360} solution and examine it at 200× magnification. The oil (holding pipette) and Fluorinert or Hg^0 (microinjection pipette) fronts should be static unless caused to move by the injector. If either holding or

microinjection pipette fronts recede autonomously, there is a leak in the corresponding line, which must be repaired. Good microinjection needles have a smooth-looking exterior; there is no evidence of external bubble nucleation, which is caused by grease or colloidal, residual aggregated particles. The needle tip ideally appears straight with a flush tip although slight curvature is tolerated. Draw some of the PVP_{360} solution into the pipette and expel several drops of Hg^0 or Fluorinert; repeat this process to wash and lubricate the interior. To wash the needle exterior, rub it against a globule of Hg^0 in PVP_{360} solution or (later) on the internal aperture of the holding pipette in the microinjection droplet. If Hg^0 is used, fill the microinjection pipette line with water. Alternatively, fill the line with Fluorinert FC-77 and omit Hg^0. **! CAUTION** Elemental mercury (Hg^0) is a cumulative neurotoxin. Handle with gloves and dispose of it with care according to local institutional guidelines. It is emphasized that Hg^0 should be avoided for human ICSI. ● **TIMING** ~2 min.

PROCEDURE

Preparation and incubation of oocytes ● TIMING 30–45 min

1| To obtain relatively large numbers of oocytes (25–35/ $B6D2F_1$), superovulate by serial intraperitoneal injection (26 G needle) of 5 IU PMSG followed 45–54 h later by 5 IU hCG (i.e., inject ~0.1 ml of each stock per female). Typically (with standard mouse room light/dark cycles), administer hormone injections at 6–7 pm and avoid administering them after 10 pm. Females of 8–10 weeks give good yields, but the optimum varies. If the females have been shipped, allow 1–7 d for recovery prior to superovulation (i.e., PMSG injection). We recommend the strain $B6D2F_1$ because they breed and superovulate efficiently, their oocytes are relatively easy to micromanipulate, and resultant embryos develop robustly *in vitro* and *in vivo*.

2| 12–15 h post-hCG injection, sacrifice the mice (3–5 per experiment usually suffices), collect their oviducts and place them in CZB•H. If micromanipulation and animal facilities are not adjacent, transport the oviducts at room temperature (i.e., avoid exposure to cold) in a 1.5-ml Eppendorf tube containing 100–200 µl CZB•H. Transfer the first oviduct in a 200 µl drop of CZB•H containing 6–15 µl bovine testis hyaluronidase stock solution.

Figure 2 | Piezo workstation. Abbreviations: lem, left electric micromanipulator; rem, right electric micromanipulator; lmm, left manual micromanipulator; rmm, right manual micromanipulator; sb, supporting block; hpi, holding pipette injector; mni, microinjection needle injector; mb, metal base; dc, digital camera (optional); air, air-cushioned platform; im, inverted microscope; st, microscope stage; hp, holding pipette; mn, microinjection needle; p, piezo.

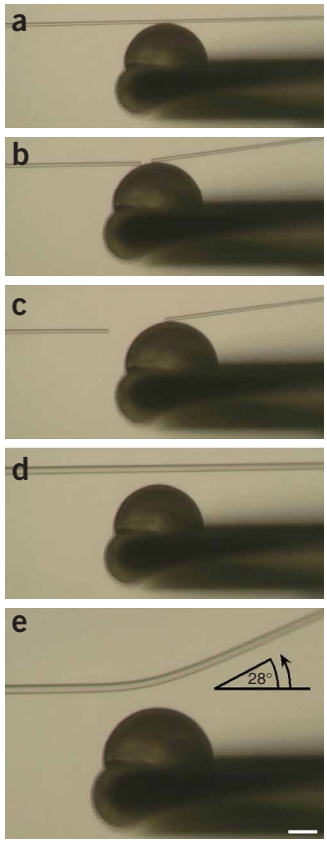

Figure 1 | Piezo microinjection pipette fabrication. Crafting a piezo needle from a pulled capillary on a microforge. The capillary is lowered so that it kisses the glass ball (**a**). Following a brief heat pulse, the capillary fractures (**b**) and is raised above the glass ball (**c**) before being advanced ~3 mm (**d**). A gradual increment of heat (starting at 0) is then applied to the ball, causing the newly-fashioned microinjection needle steadily to deviate upwards. Heating is stopped when the needle has deflected by 28° (**e**). See 'Microinjection needle preparation' for detailed description. Scale bar = 100 µm.

3| Working under the stereomicroscope at 15–20× magnification, hold the oviduct wall with one pair of forceps and tear it with another pair of forceps where the oocyte-cumulus complex makes a bulge. Tease the oocyte-cumulus complex from the oviduct; it should emerge as a single mass. Discard the oviductal remnant and repeat the process for the remaining oviducts in the same CZB•H/hyaluronidase droplet.

4| Leave the oocyte-cumulus masses in the same dish for 5–30 min to allow the hyaluronidase to digest the intercellular matrix. Cells fall away from the complexes, leaving the oocytes on a cumulus cell carpet. This step is optionally accelerated by placing the dish on a stage heated at 37 °C for up to ~20 min.

5| Remove oocytes with a transfer pipette and place them in a drop (~200 µl) of fresh CZB•H (no hyaluronidase). Wash the oocytes by pipetting them 4–6 times. Repeat this washing procedure 3–4 times, each time in a fresh droplet (~200 µl) of CZB•H.

6| Transfer the oocytes to a drop of equilibrated culture medium (e.g., KSOM) under mineral oil in a 3.5-cm dish, washing

once to remove CZB•H. Overlay embryo culture and micromanipulation (but not collection) droplets (5–20 μl) with mineral oil to prevent evaporation. Place the 3.5-cm dish containing the oocytes in an incubator containing humidified 5% (v/v) CO_2/air mix at 37 °C for at least 15 min until required. From oviduct to incubator, oocyte isolation should take less than 60 min.
! CAUTION HEPES is mildly toxic, more so at 37 °C, so minimize exposure of the oocytes to CZB•H.
▲ CRITICAL STEP Equilibrate culture medium in humidified 5% (v/v) CO_2/air mix at 37 °C for at least 15 min prior to use.
■ PAUSE POINT Oocytes may be incubated in humidified 5% (v/v) CO_2/air mix at 37 °C for several hours (until they are >20 h post-hCG) prior to micromanipulation, but they are more typically injected prior to 18 h post-hCG; oocytes efficiently retain their fertilization capacity at least 22 h post-hCG *in vivo*[24]. Following oocyte collection, spermatozoa may be prepared as follows.

Preparation of spermatozoa ● TIMING 5–10 min

7| Collect sperm from the cauda epididymides of males that are at least 10 weeks old, yielding $\sim 5 \times 10^6$–10^7 sperm per epididymis. The cauda epididymidis is a distinctive yellow pouch of convoluted tubules surrounded by fat and muscle; it is smaller, smoother and whiter in younger animals, and generally contains fewer sperm. Sperm may also be removed from caput (testis proximal) epididymides, although such sperm are non-motile and need to be isolated by dicing (Step 9B). Sperm isolation is otherwise similar for caput and cauda epididymides, although it is now described as it pertains to the cauda epididymidis.

8| Place epididymidal tissue on a clean piece of Kimwipe (or equivalent) paper soaked in PBS or CZB•H in a 3.5-cm dish to keep it moist until ready for processing. Each epididymis is surrounded by a layer of muscle—the tunica. Place the dissected epididymis on a clean, dry Kimwipe and holding the tunica with one pair of forceps, use another to pull the epididymidal tissue out. Cut epididymis-tunica adhesions and trim away fat pads from the cauda before further blotting it with the Kimwipe to remove residual blood and fat.
▲ CRITICAL STEP Do not allow the epididymidal tissue to dry out, as this will markedly reduce sperm yield. Work swiftly, processing a maximum of two epididymides at a time for option A and six for option B (Step 9).

9| Isolate sperm by simple epididymal puncture (option A) or by dicing epididymides with fine scissors (option B), the method best suited to larger scale isolation.

(A) Epididymal puncture

(i) Pinch the cauda epididymidis between thumb and forefinger so that the cauda bulges, and gently squeeze. Carefully prick the bloated cauda with a pair of fine, sharp forceps and pinch gently so that sperm ooze out onto the surface of the epididymis.
(ii) Remove the sperm by pinching the suspension between forceps that have been dunked in oil (to reduce sperm sticking to them).
(iii) Wash the forceps by agitating them in a dish containing CZB or KSOM (equilibrated in the CO_2 incubator at 37 °C for ≥15 min) under oil and return the dish to the incubator.
(iv) The drop becomes milky as the sperm actively disperse. This takes about 5 min.

(B) Dicing epididymides

(i) Mince epididymides with fine scissors in a 3.5-cm dish containing ~500 μl of buffer (e.g., NIM[23]) or medium (e.g., CZB•H) at room temperature (23–28 °C) for 2–5 min. Displace sperm from the tissue by brief, gentle pipetting.
(ii) Filter the suspension through a double-folded Kimwipe into a clean (sterile) 10-ml beaker, to eliminate tissue debris. Expel residual suspension by gently squeezing the tissue (without breaking it) between thumb and forefinger over the funnel.
■ PAUSE POINT Sperm may be stored at −20 °C or −70 °C for at least several months without elaborate cryopreservation; place them directly into the freezer in medium or buffer. Storage of non-motile, 'dead' sperm or sperm heads for mouse ICSI and subsequent production of live offspring has been described for freeze-thaw[4,25] and freeze-dry[5] sperm microinjection.

Piezo-actuated intracytoplasmic sperm injection (ICSI) ● TIMING 1–2 h

10| Mix sperm suspensions with PVP_{360} solution so that the final concentration of PVP_{360} is 5–15% (w/v). Mix using a pipette, such as a Gilson Pipetman P200 or equivalent, equipped with a sterile P200 (yellow) tip truncated ('cut off') ~5 mm from the end. PVP_{360} acts as a lubricant and retards live sperm, making them easier to collect. With a transfer pipette, remove mII oocytes from the CO_2 incubator and place them into a microinjection droplet (CZB•H) on the microscope stage. **Figure 3** shows dispensation of mineral oil and a working arrangement of drops in a micromanipulation dish.
▲ CRITICAL STEP Sperm in PVP_{360} suspensions at room temperature often lose their ability to support full development after ~1 h. If good development is desired, make fresh sperm-PVP_{360} suspensions every 1 h. However, spermatozoa retain their ability to induce meiotic resumption for many hours, so a single sperm-PVP_{360} is sufficient for beginners.

11| If the sperm is intact (i.e., head, midpiece and tail are joined) decapitate it (**Supplementary Video 1** online) to allow injection of the head alone. Bring a sperm into sharp focus (200× magnification) and draw its head into the microinjection pipette (inner tip diameter, 5.5–7.5 μm; outer tip diameter, 6.5–8.5 μm) so that the pipette lip touches the head-midpiece boundary. Apply several pulses with the piezo unit foot pedal. For initial attempts with the PMAS-CT150 (or equivalent) piezo unit, try intensity = 3, frequency = 6; as a general guide in piezo, use the lowest settings that work. The head and tail should separate. Many preparation protocols (e.g., freeze-thawing) decapitate sperm before the sperm are placed on the stage. Injection of a head alone minimizes the volume introduced into the oocyte.
? TROUBLESHOOTING

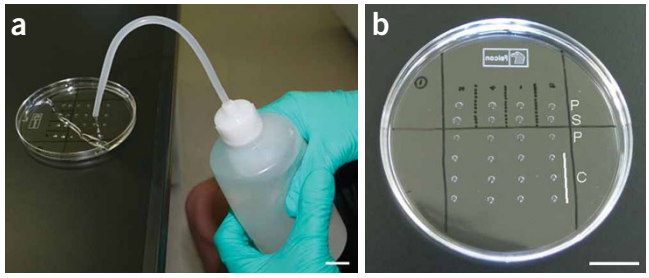

Figure 3 | Microinjection dish setup. As soon as the medium and sample drops (5–15 μl each) are placed on the dish lid they are overlaid with mineral oil from a wash bottle (**a**) to prevent evaporation. A suggested arrangement of the PVP, medium and sample drops is shown in (**b**). Abbreviations: P, PVP_{360} solution; S, sample; C, CZB•H. Scale bars = 2 cm.

12| Draw one or more sperm heads into the microinjection pipette. With experience, multiple (5–10 or more) heads can be accumulated within a pipette at intervals ~100 μm; although only a single head is typically injected per oocyte, collecting multiple samples removes the need to return to the sperm-PVP_{360} droplet each time between injections. Working at 40× magnification, move the microscope stage so that the pipette is in the microinjection droplet (CZB•H) containing 10–15 oocytes (transferred from the batch of Step 6 in the CO_2 incubator), optionally first washing it in a CZB•H droplet to remove PVP_{360} solution from the needle exterior.
? TROUBLESHOOTING

13| Lower the holding pipette into the same droplet and position the holding and microinjection pipettes so that their tips are on either side (usually with the holding pipette on the left) of a selected oocyte at the center of the field (**Fig. 4a,b**). Use the microinjection needle to orientate the oocyte so that its mII plate is located straight up or down along the *y*-axis (i.e., 12 o'clock or 6 o'clock). Either orientation minimizes the risk of damaging the mII plate during microinjection; even slight mechanical damage to the spindle (i.e., mII plate) prevents normal development. Select an orientation (6 o'clock in **Fig. 4b**) that gives the largest space between the plasma membrane and the zona pellucida (perivitelline space) on the side of the microinjection pipette.
? TROUBLESHOOTING

14| At 200× magnification, bring the oocyte plasma membrane into sharp focus. Use the fine *z*-axis control to move the pipettes up or down so that their ends are in focus. Steps 14–24 are demonstrated in **Supplementary Video 2** online.
▲ **CRITICAL STEP** Accurate focus is crucial for several steps in oocyte micromanipulation.

15| Applying gentle suction within the holding pipette, push the oocyte against the holding pipette aperture with the microinjection pipette (**Fig. 4c,d**). Pushing causes the oocyte zona and plasma membrane to engage the holding pipette aperture and cover it, anchoring the oocyte and making it easier to inject (**Fig. 4e**).

16| Move the most advanced sperm head so that it is 50–100 μm from the pipette tip. Check the focus and touch the zona periphery with the tip (**Fig. 4e**).

17| Introduce a small negative pressure within the microinjection pipette and apply piezo pulses (start with intensity = 3, frequency = 6) while gently pushing the microinjection pipette toward the holding pipette. The tip should rapidly pass through the zona, emerging into the perivitelline space (**Fig. 4f,g**). Stop pushing as soon as you see this. Failure to control the tip risks subjecting the plasma membrane to high intensity piezo, resulting in rapid cell lysis.
? TROUBLESHOOTING

18| The zona core is just visible (it is difficult to see without experience) entering the pipette as the tip penetrates into the perivitteline space. Although the oocyte cytoplasm can tolerate the presence of a zona plug, the plug can interfere with injection. Eject the zona plug prior to injection by placing the pipette tip against the plasma membrane (without piezo pulses) and gently increasing positive pressure (**Fig. 4g**). When the core emerges from the pipette, it produces an impression ('dent') in the plasma membrane. Dislodge the protruding core by wiping the tip against the oocyte (gentle movement mainly along the y-axis). Sustain positive pressure until the end-most sperm is 10–50 μm from the tip (**Fig. 4h**).

19| Ensure that the plasma membrane is in sharp focus and fine-adjust the tip (*z*-axis) so that it is also in focus. Avoid the first polar body (Pb_1), which can be displaced; it is not usually attached to the oocyte. Maintain a marginal positive pressure within the pipette.

20| Steadily advance the tip towards the opposite side of the oocyte, where it is being anchored by the holding pipette (**Fig. 4i–k**). Stop when the tip has advanced ~95% of the oocyte diameter. This produces a deep invagination in the (remarkably elastic) oocyte plasma membrane, which is now stretched around the microinjection needle. Advance of the needle tip takes 2–5 sec.
? TROUBLESHOOTING

21| Change the piezo pre-set channel (foot switch) to a gentler setting (e.g., intensity = 1, frequency = 1). While maintaining zero net pressure (or a very small negative one) within the microinjection pipette, apply a single piezo pulse.
▲ CRITICAL STEP Ensure that the oocyte plasma membrane visibly relaxes along the shaft of the needle (**Fig. 4l**). This is an indispensable indication that the membrane has been punctured.
? TROUBLESHOOTING

22| Deposit the sperm head in the cytoplasm (**Fig. 4m**) with the application of small positive pressure in the microinjection pipette. Introduce the minimum extraneous medium and do not suck cytoplasm into the pipette.
? TROUBLESHOOTING

23| As soon as the sperm has been deposited, withdraw the needle smoothly from the oocyte (**Fig. 4n–p**). The membrane should return to its original position and seal. If the plasma membrane clings to the pipette, pause briefly during withdrawal. From zona penetration to needle withdrawal takes 25–35 sec.
? TROUBLESHOOTING

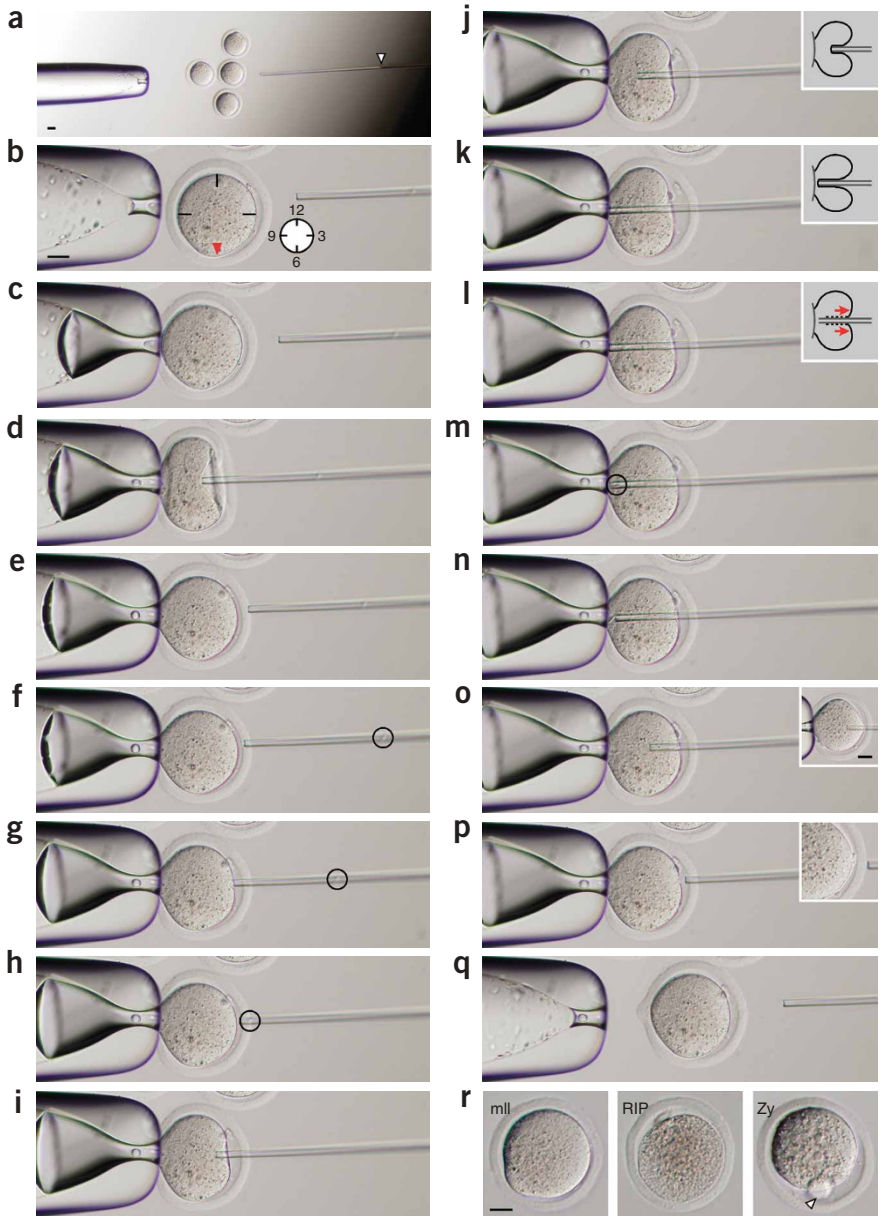

Figure 4 | Mouse piezo ICSI. Successive micromanipulation steps (**a–q**) are described in the text. Panel (**a**) was obtained at 40× magnification by the microscope (4× objective), while all subsequent panels were obtained at 200× magnification (20× objective). The Hg^0 front in (**a**) is marked with a white arrowhead. In (**b**), the oocyte orientation is visualized by the position of its mII bump (red arrowhead) on an imagined clock face (in this case, it is at 6 o'clock). Circles mark the position of the sperm head prior to injection in (**f**), (**g**) and (**h**) and immediately after it (**m**). Insets schematically represent membrane invagination (**j, k**) and piezo-induced puncture (**l**), and show evisceration of a different oocyte (**o**) and the resulting exit wound (**p**) caused when the membrane adheres to the withdrawing pipette (this oocyte died). In (**r**), panels show a metaphase II oocyte (mII), a dead oocyte (*requiescat in pace*, RIP), and a pronuclear zygote (Zy) 6 h after piezo ICSI with pronuclei and a Pb_2 (white arrowhead). Scale bars = 20 μm throughout.

24| Release the injected oocyte by gently applying positive pressure within the holding pipette (**Fig. 4q**). Any minor distortion caused by holding rapidly disappears. Allow oocytes at least 5 min (some workers prefer 10–30 min) to recover before being returned to the CO_2 incubator. Fatal mechanical trauma to the oocyte during micromanipulation usually results in the onset of lysis within 30 sec. Remove dead eggs (**Fig. 4r**) from the survivors during transfer to the CO_2 incubator. For embryo culture,

wash the oocytes ~4 times in equilibrated culture medium to remove CZB•H (HEPES is toxic at 37 °C) before incubation. For optimal development, limit the time the oocytes are out of the incubator; ≤40 min is a guideline. Experienced workers can successfully inject 15 oocytes in <10 min. When learning, one oocyte can take 30 min or much longer to inject. A breakdown of typical times taken to complete Steps 1–24 is shown in **Table 1**.

? TROUBLESHOOTING

25| Examine the outcomes of injections at 200× magnification, 5–8 h later. Oocyte activation produces a cytoplasm that is darker and more heterogeneous, and the plasma membrane is no longer smooth but irregular (**Fig. 4r**). The mII 'bump' will have disappeared and there will be a second polar body (Pb_2), the Pb_1 having degenerated, although a remnant often remains. Male and female pronuclei will be visible, sometimes containing multiple pronucleoli whose numbers decrease with time. Visible light damages these structures, so for further culture, examine zygotes rapidly, wash them 4–6 times in CO_2-equilibrated culture medium (e.g., KSOM) and return them to the CO_2 incubator.

? TROUBLESHOOTING

26| Next day (corresponding to embryonic day 1.5, E1.5), wash 2-cell embryos once in CO_2-equilibrated culture medium (removing dead cells) and return them to the CO_2 incubator to monitor *in vitro* development further or transfer them to a surrogate mother. Embryo development *in vitro* following piezo ICSI to ~E3.5 is shown in **Supplementary Video 3** online.

● TIMING

The first (PMSG) injection for superovulation is administered three days prior to piezo. For a piezo micromanipulation experiment on Monday (morning), mice should be injected with PMSG on Friday (afternoon or evening) and hCG on Sunday (afternoon or evening), for example. After collection (taking 30–60 min), oocytes are placed in CO_2-equilibrated medium in a CO_2 incubator, after which spermatozoa are collected (5–10 min) and then the micromanipulation dish setup (5 min). Experienced workers place 15–20 oocytes in an injection droplet and inject them sequentially, which takes 10–20 min. At least 5 min after injection of the last oocyte, the entire batch is washed in drops of equilibrated culture medium and returned to the CO_2 incubator. From oocyte collection to injection of the final sample, piezo ICSI typically takes 2–4 h for 40–120 oocytes. The timing of the entire procedure, including superovulation, is summarized in **Table 1**.

? TROUBLESHOOTING

Step 11: Sperm are difficult to collect

Selection of motile sperm from a freshly prepared suspension ensures that they were viable just prior to injection, maximizing developmental potential. However, if they are difficult to pick up, they can be immobilized by touching them and applying one or two high or low intensity piezo pulses.

Steps 11, 17 and 21: Piezo pulses have no effect

This common problem has multiple possible causes. (i) The needle is inadequate. If the tip is not flush-ended, only part of it contacts the sample. Critical flaws in pipette fabrication or damage during use may not be visible microscopically but they require that the needle is replaced. (ii) Hg^0 or Fluorinert is too far from the tip. Piezo power dissipates the further the Hg^0 or Fluorinert front is from the pipette tip. To remedy the problem, advance the front. As a guide, there should be sufficient power even when the Hg^0 front is outside the field (as a guide, this corresponds to ~500 μM from the tip at 200× magnification when the front is just outside the field). (iii) Air bubbles interrupt the Hg^0 or Fluorinert column. (iv) The pipette is inadequately secured within its housing and/or the housing itself is not tightly mounted onto the workstation (everything needs to be firmly secured). (v) Failure to engage the sample, often because the pipette tip is either above or below it; the plane of focus should pass through (or near) the oocyte center and the pipette tip should be in the same plane. To achieve this, bring the sample periphery (e.g., outer face of the zona pellucida or plasma membrane) into sharp focus and then adjust the tip

TABLE 1 | A timeline for mouse piezo ICSI.

Step 1		Steps 2–6	Steps 7–9	Steps 10–24	Step 25	Step 26	
Day −3	**Day −1**		**Day 0**			**Day 1**	**Day 3**
PMSG	hCG	Oocyte collection	Sperm collection	ICSI		Wash embryo Embryo transfer	
(6–7 pm)	(6–7 pm)	(7–9 am)	(9–9:15 am)	(am)			
					Zygote	2-cell	Morula (early) Blastocyst (later)
45–54 h	12–15 h		2–4 h	5–8 h	~12–24 h	48 h	

along the *z*-axis so that it falls sharply into the same focus. (vi) The pipette tip may not be in full contact because the sample surface is irregular, in which case a small negative pressure (suction) within the microinjection pipette may help the two to engage. However, high negative pressures and/or piezo settings should be unnecessary and are likely to damage samples. (vii) In some models it is possible to set piezo actuation in the wrong direction so that the needle is caused to move in reverse rather than forwards; flip the 'L to R' setting at the rear of the transformer. (viii) The piezo unit may be broken—it is sensitive to being knocked or dropped even a short distance. When working well with Hg^0, the lowest intensity setting may be adequate for all piezo steps.

Steps 11, 17 and 21: Piezo pulses violently displace the microinjection needle tip
If the needle tip and/or sample appear to 'jump' when piezo pulses are applied, either the setting is too high (use a lower setting), the assembly may not be fastened securely or the pipette has been filled with injection medium (not PVP_{360} solution) near its end. Relatively dense solutions are apparently needed near the pipette tip to add momentum and stability, ensuring that the piezo pulse is efficiently transmitted in a controlled manner.

Steps 12 and 22: Sperm heads stick to each other and the microinjection pipette interior
Samples stick to the pipette interior—particularly as they get nearer the tip—if the needle is too narrow; an internal diameter of ~6.5 μm should be comfortably within the acceptable range. If the problem persists, change the needle for one with a wider tip. Sample stickiness is affected by the preparation method. For example, extraction with some detergents (e.g., 1% (w/v) SDS) causes sperm stickiness whereas others (e.g., 1% (v/v) Triton X-100) tend not to. If sperm heads stick to each other, load the pipette with fewer, possibly only one head. Increase the concentration of PVP_{360} (next time). Heads that stick to the pipette can sometimes be dislodged with 1–3 low intensity piezo pulses. If this fails, apply slight suction and repeat the piezo pulse, before pushing, again with a piezo pulse. Alternatively, toggle back to the high (zona drilling) piezo intensity with the foot switch and apply a short burst of stronger pulses. Although higher intensity piezo applied to the plasma membrane typically kills the oocyte, once the pipette tip is within the cytoplasm, the oocyte can usually tolerate a higher intensity. If these remedies are unsuccessful the pipette interior may be dirty, so withdraw from the oocyte (which must be discarded) and wash the needle thoroughly by expelling several drops of Hg^0 or Fluorinert in PVP_{360} solution, or change the needle.

Step 13: The mII plate is not visible
When learning, we recommend the use of $B6D2F_1$ oocytes, in which the mII plate (containing the spindle and chromosomes) is a relatively discernible (compared with that of some other strains) translucent disc lying just under the plasma membrane, making a pronounced cortical bump. The disc is easier to see at higher temperatures because spindle assembly is more complete. However, stage temperatures above 30 °C cause oocyte lysis after microinjection. Injection works well at room temperatures in the range 16–28 °C.

Step 20: The oocyte plasma membrane breaks as the microinjection tip is advanced
Do not apply piezo pulses until the pipette tip is fully advanced to the opposite side of the oocyte. If breakage occurs to the membranes of more than one oocyte during or immediately after advance even without piezo, the tip may be damaged and the pipette should be replaced. Occasionally, batches of oocytes are idiopathically hypersensitive and have to be discarded.

Step 23: The oocyte plasma membrane sticks to the microinjection pipette
If this occurs, the membrane is pulled outwards as the pipette is withdrawn to produce a surface exit wound resembling an explosion, eviscerating the oocyte (**Fig. 4o,p** insets). Not surprisingly, these oocytes usually die. The problem can be reduced by positioning the sperm sufficiently far back prior to pipette advance (Step 20), and exerting a constant (but marginal) positive pressure during advance. This displaces injection media from the advancing tip to make a sleeve around the needle, reducing adherence between the needle and the plasma membrane. Additionally, needles can be purged between injections; interiors can be cleansed by displacing Hg^0 or Fluorinert in the PVP solution washing drop, and exteriors by rubbing the shafts on Hg^0 drops (in the PVP drop) or within the aperture of the holding pipette (in a drop of CZB•H). If stickiness persists, change the needle.

Step 24: Many oocytes are dead
This is normal for beginners. Improvement requires stamina built up through regular practice. One or two injection sessions per week are insufficient for most learners to achieve this; four or five times per week (without gaps between weeks) is more likely to yield success. Injection sessions are typically limited to one per day. A high proportion of manipulated oocyte deaths can occur even for experienced operators. This probably reflects submicroscopic needle damage or injectionist fatigue, which is why stamina is necessary—in particular with respect to optical focusing on samples. As an illustration of this, oocyte survival may

decline as the experiment proceeds, which should be controlled against where appropriate. The number of oocytes killed is also generally proportional to the needle tip diameter.

Step 25: Pronucleus formation has not occurred after 6 h

This is typically because the plasma membrane was not broken in Step 21. Also, oocyte activating capacity declines with sperm sample aging and is abolished by certain treatments, such as exposure to dimethyl sulphoxide or heating at 48 °C in NIM[23,26].

ANTICIPATED RESULTS

Survival rates 30 min after injection should approach 100%, but this value declines with increasing microinjection pipette tip diameter. Optimally, tip inner diameters are 5.5–7.5 μm for sperm injection. Oocyte activation following ICSI also promotes cell lysis independently of injection *per se;* lysis is more likely if the oocytes were activated by, or soon after, injection. However, it is not uncommon for 80% or more of injected oocytes to yield pronuclear zygotes and experienced operators can produce developmentally competent zygotes in excess of 90%. These can produce offspring in up to ~50% of cases following embryo transfer (20–30% is more common), depending on how skillfully the transfer is performed and to what host. ICR (CD-1) is a favored recipient strain because it supports pre- and post-term development even when litter sizes are small (as low as one).

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS Grant support from the RIKEN President's Fund is gratefully acknowledged.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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1. Kimura, Y. & Yanagimachi, R. Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52**, 709–720 (1995).
2. Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. & Yanagimachi, R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–374 (1998).
3. Onishi, A. *et al.* Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289**, 1188–1190 (2000).
4. Wakayama, T., Whittingham, D.G. & Yanagimachi, R. Production of normal offspring from mouse oocytes injected with spermatozoa cryopreserved with or without cryoprotection. *J. Reprod. Fertil.* **112**, 11–17 (1998).
5. Wakayama, T. & Yanagimachi, R. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotechnol.* **16**, 639–641 (1998).
6. Munsie, M.J. *et al.* Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. *Curr. Biol.* **10**, 989–992 (2000).
7. Perry, A.C.F. *et al.* Mammalian transgenesis by intracytoplasmic sperm injection. *Science* **284**, 1180–1183 (1999).
8. Perry, A.C.F. *et al.* Efficient metaphase II transgenesis with different transgene archetypes. *Nature Biotechnol.* **19**, 1071–1073 (2001).
9. Moreira, P.N. *et al.* Efficient generation of transgenic mice with intact yeast artificial chromosomes by intracytoplasmic sperm injection. *Biol. Reprod.* **71**, 1943–1947 (2004).
10. Shoji, S. *et al.* Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit *via* Cdc20. *EMBO J.* **25**, 834–845 (2006).
11. Kawase, Y. *et al.* Application of the piezo-micromanipulator for injection of embryonic stem cells into mouse blastocysts. *Contemp. Top. Lab. Anim. Sci.* **40**, 31–34 (2001).
12. Tanemura, K. *et al.* Birth of normal young by microinsemination with frozen-thawed round spermatids collected from aged azoospermic mice. *Lab. Anim. Sci.* **47**, 203–204 (1997).
13. Kanatsu-Shinohara, M. *et al.* Generation of pluripotent stem cells from neonatal mouse testis. *Cell* **119**, 1001–1012 (2004).
14. Rybouchkin, A., Dozortsev, D., de Sutter, P., Qian, C. & Dhont, M. Intracytoplasmic injection of human spermatozoa into mouse oocytes: a useful model to investigate the oocyte-activating capacity and the karyotype of human spermatozoa. *Hum. Reprod.* **10**, 1130–1135 (1995).
15. Huang, T., Kimura, Y. & Yanagimachi, R. The use of piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. *J. Assist. Reprod. Genet.* **13**, 320–328 (1996).
16. Ogura, A. *et al.* Recent advances in the microinsemination of laboratory animals. *Int. J. Androl.* **23** (Suppl 2): 60–62 (2000).
17. Inoue, K. *et al.* Improved postimplantation development of rabbit nuclear transfer embryos by activation with inositol 1,4,5-trisphosphate. *Cloning Stem Cells* **4**, 311–317 (2002).
18. Zou, X. *et al.* Production of cloned goats from enucleated oocytes injected with cumulus cell nuclei or fused with cumulus cells. *Cloning* **3**, 31–37 (2001).
19. Choi, Y.H. *et al.* Production of nuclear transfer horse embryos by piezo-driven injection of somatic cell nuclei and activation with stallion sperm cytosolic extract. *Biol. Reprod.* **67**, 561–567 (2002).
20. Hirabayashi, M. *et al.* Offspring derived from intracytoplasmic injection of transgenic rat sperm. *Transgenic Res.* **11**, 221–228 (2002).
21. Chatot, C.L. *et al.* An improved culture medium supports development of random-bred 1-cell mouse embryos. *J. Reprod. Fertil.* **86**, 679–688 (1989).
22. Lawitts, J.A. & Biggers, J.D. Culture of preimplantation embryos. *Methods Enzymol.* **225**, 153–164 (1993).
23. Perry, A.C.F., Wakayama, T. & Yanagimachi, R. A novel *trans*-complementation assay suggests full mammalian oocyte activation is coordinately initiated by multiple, submembrane sperm compartments. *Biol. Reprod.* **60**, 747–755 (1999).
24. Marston, J.H. & Chang, M.C. The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. *J. Exp. Zool.* **155**, 237–251 (1964).
25. Kuretake, S., Kimura, Y., Hoshi, K. & Yanagimachi, R. Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* **55**, 789–795 (1996).
26. Yoshida, N., Brahmajosyula, M., Shoji, S., Amanai, M. & Perry, A.C.F. Epigenetic discrimination by mouse metaphase II oocytes mediates asymmetric chromatin remodeling independently of meiotic exit. *Dev. Biol.* **301**, 464–477 (2007).