Piezo-actuated Mouse ICSI (Intracytoplasmic sperm injection)

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Abstract

Intracytoplasmic sperm injection (ICSI) is not only used in human assisted reproduction (ART), but it is also widely employed for veterinary in vitro fertilization during rare species preservation, germ-line rescue of transgenic animals or for any veterinary assisted conception. Piezo-assisted ICSI, which was first described in 1995 [1], can be employed for assisted conception in animals in which standard ICSI fails, such as mice. The microinjection workstation required for this technique is very similar to standard ICSI, but with the addition of a piezo impact unit attached to the capillary holder. This Userguide focuses on the piezo-assisted microinjection procedure itself.

Introduction

Intracytoplasmic sperm injection, the direct injection of a single sperm into the cytoplasm of an egg, was first described in hamsters [2] and has been successfully applied to humans, but also other species, like mice [1,3]. ICSI performed in animal models is an optimal tool to investigate the direct fertilization process as well as causes of infertility. Especially in the area of biomedical research, ICSI may also be used as a gene transfer technique when sperm are co-injected or coated with exogenous DNA. Furthermore, mouse ICSI is often applied when transgene expression or mutations compromise male or female mouse viability or fertility. In these cases, piezo-assisted ICSI can be one mean to rescue and maintain a very valuable mouse strain, since normal ICSI has been proven to be difficult in mice [1, 4-6].

Several studies have shown that sperm injection via a piezo-driven microcapillary is far less traumatic to the mouse egg than the conventional method. Moreover, piezo-assisted ICSI has been shown to increase fertilization success rates dramatically [1].

Eppendorf has a long tradition in the area of conventional ICSI. In general, the Eppendorf TransferMan® NK 2 system has a number of special features. The storage of up to three positions helps to speed up the procedure. In combination with the Prime Tech PMM 150-FU Piezo Impact Unit it can also satisfy all demands for piezo-assisted ICSI. The PMM 150-FU Piezo Impact Unit can easily be mounted on the Eppendorf micromanipulation system (see Figure 1). Here, we describe the mounting of the Prime Tech device on the manipulators as well as the experimental procedure itself.

Figure 1: Piezo-assisted ICSI workstation. The PMM 150-FU Piezo Impact Unit is mounted on the TransferMan NK 2 micromanipulator. The picture was kindly provided by PrimeTech, Ibaraki, Japan.
Materials and Equipment

1. Animals
Egg donor female mice (4-8 weeks) or frozen ovaries
Sperm donor male mouse or frozen-thawed sperm
Recipient female mice (pseudopregnant)

2. Media
- Whitten’s/Hepes/PVA: Whitten’s medium (no BSA) containing 7 mM NaHCO₃, 15 mM Hepes and 0.01% polyvinylalcohol (PVA) [7]
- Whitten’s/PVA: Whitten’s medium containing 0.01% PVA
- NIM/PVA: mMIM (modified nuclear isolation medium): 123 mM KCl, 2.6 mM NaCl, 7.8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 3 mM EDTA (disodium salt); pH value is adjusted to 7.2 by addition of 1 M KOH + 1 % PVA
- KSOM
- Mineral Oil
- Mercury or Fluorinert C-77 (FC-77)

3. Consumables
- Transfer pipettes
- 35, 60 mm culture dishes
- Culture tubes
- Micro centrifuge tubes
- Shallow Petri dishes for Microinjection, e.g. 60 mm ‘In vitro fertilisation dish’ (BecktonDickinson), or 100 mm dishes
- ICSI microcapillary, e.g. PiezoDrill Tips (Mouse ICSI), Eppendorf
- Holding microcapillary, e.g. VacuTips, Eppendorf

4. Devices
- Inverted microscope with appropriate contrasts (HMC or DIC) and long distance objectives with magnification up to 40x
- 2x TransferMan NK 2 micromanipulators (Eppendorf)
- CellTram® Air microinjector (Eppendorf)
- CellTram vario microinjector (Eppendorf)
- PMM 150-FU Piezo Impact Unit (PrimeTech, Ibaraki, Japan)

Methods

1. Collection and preparation of eggs and sperm
Freshly collected or frozen-thawed sperm can be prepared according to the mini-swim-up method [8] or by centrifugation and subsequent sonication to isolate sperm heads [9]. By using the first method, the sperm tail may be cut by applying a Piezo Drill impulse directly during the microinjection procedure. Sperm immobilization immediately before ICSI increases the rate of successful fertilization.

The metaphase II eggs are collected from superovulated females and further treated as described elsewhere [1, 10]. The cumulus-free eggs are transferred to a culture dish containing microdrops of Whitten’s/PVA covered with light paraffin oil. The dish is placed in a humidified incubator containing 5 % CO₂ in air at 37 °C until use.

2. Preparation of the microinjection dish
The arrangement of the drops in the microinjection dish depends on personal preferences; an example is shown in Figure 2. Here, a 5 µl flat drop of sperm suspension is positioned at the center of a flat Petri dish. 3 x 5 µl drops of NIM/PVA are positioned at one end of the dish along the midline. In addition, 2 x 5 µl injection drops of Whittens/Hepes/PVA are placed at the other end of the dish along the midline. Add approximately five eggs in one of the injection droplets. Cover with mineral oil.

Figure 2: Microinjection dish: black circles: droplets with wash medium (NIM/PVA); orange circles: droplets with eggs in Whitten’s/Hepes/PVA; blue ellipse: sperm reservoir
3. Mounting of the Prime Tech Piezo Device onto the universal capillary holder

Before starting the ICSI procedure, the Prime Tech Piezo Device has to be mounted onto the universal capillary holder. The Eppendorf universal capillary holder has a sliding knurled metal fitting at the front end. The knurled metal fitting at the front end accepts a capillary grip head for holding microinjection capillaries. This front fitting slides and rotates on the shaft of the universal capillary holder because it is connected to it by a small metal clip referred to as an ‘e-clip’ (see Figure 3a and enlargement, d).

Figure 3: Preparation of the universal capillary holder (a) for the assembly with the PrimeTech Piezo Impact Unit. b. The red arrow indicates in which direction the knurled screw has to be moved in order to remove the e-clip (d). c. Removing of the e-clip. d. The red arrow shows the magnification of e-clip in front and side view. e. After removing the e-clip in the first step, the knurled screw can be moved of the universal capillary holder. Please take good care of these two small pieces; they have to be replaced later on!

Before the universal capillary holder can be inserted into the PrimeTech Piezo Impact Unit, the e-clip and knurled metal fitting must be removed from the front end.

The universal capillary holder can then be inserted into the Impact Unit, as shown in Figure 4, and the fitting and e-clip can be replaced. For detailed description of the Prime Tech device please also visit http://www.primetech-jp.com.

The device vibrates the injecting microcapillary axially and drills its way into the egg. This unit, as a result of the piezoelectric effect, can advance the capillary holder a very short distance (e.g., 0.5 µm) at a very high speed. A stabbing, punctuate movement of an attached capillary punctures the cell membrane with minimum distortion of the egg.

4. Preparation of microinjection capillaries

The microcapillaries have to be fitted, aligned and equilibrated before starting the injection procedure.

First, the microcapillary have to be fitted into the universal capillary holder, which is connected to the microinjector CellTram via a tube. Gently push the capillaries past the sealing rings inside the tool holder. The injection angle can be adjusted independently via the knurled screws and the angle mark on the X-head of the TransferMan NK 2. The angle should be adjusted as shallow as possible.
Note: When substances heavier than water are used in the injection capillary, the efficiency of drilling improves significantly. Therefore, it has to be noted that the presence of Fluorinert C-77 (FC-77) near the tip of the microinjection capillary increases the penetrating capability of the capillary through the zona pellucida and oolemma. The filling with FC-77 should be performed from the back rear of the capillary with the help of Microloaders. After the capillary is inserted into the grip head of the capillary holder, Fluorinert will drop out of the tip. Leave the capillary until the flow out stops (approximately 5-10 minutes). Then proceed with the procedure. For equilibration of the injection capillary, place the capillary in one of the NIM/PVA drops. By rotating the CellTram regulator counter clockwise, draw in NIM/PVA.

5. Piezo assisted ICSI

The injection capillary and the holding capillary are directed at the focal plane and the positions are stored. The TransferMan NK 2 can store up to 3 positions. The capillary can be moved easily in any direction with the help of a single joystick. By pressing the joystick button twice (double-click) the capillary can be returned to a pre-set position. Usually one position is set as a “parking” position above a medium droplet, while another one serves as the “working” position, parallel to the bottom of the petri dish. The injection microcapillary is moved into the sperm drop and by slight rotation of the CellTram vario fine regulator counter clockwise, a single sperm head is aspirated. Once this sperm head moves slightly upwards in the capillary, another head can be aspirated. Usually 3-5 sperm heads are aspirated into the capillary.

Note: If the sperm are not sonicated (see above), the tail has to be removed by piezo pulses. Therefore position the sperm in a way that the junction between head and tail is at the opening of the capillary; then apply a few pulses to this junction area to separate head and tail. For initial attempts with the piezo unit, start with low intensity, and first gradually increase the frequency; as a general guide in piezo, use the lowest settings that work. The head and tail should separate.

Push the tail out of the capillary and proceed in the same way with the next 3-5 sperm.

Move the stage to place the sperm containing capillary into the injection droplet and place the holding capillary into the injection drop by recalling the previously stored position. The holding capillary and the egg are sharply focused at a magnification of 20x or 40x. Hold the egg so that the metaphase II spindle is at 6 or 12 o’clock, and the egg is touching the dish. Bring the injection capillary to the zona pellucida (ZP) and press the egg slightly to ensure that the capillary is in the center of the egg (Z axis). By moving the joystick slightly in fine mode, position the injection capillary at 3 o’clock carefully touching the zona pellucida (but without pushing) and advance the capillary as the zona is drilled by applying Piezo Drill pulses (see Figure 5 A).

Advance the sperm head to the tip of the capillary and move the capillary forward into the cortex of the egg on the side opposite to the entry site. The egg should be pricked in the middle and the oolemma membrane is broken gently by applying one Piezo Drill pulse while watching for relaxation of the oolemma (usually lower piezo settings are required than for breaking the zona). The sperm head is then injected (see Figure 5 B). In order to introduce only minimal volume of the medium into the cytoplasm, or none at all, the injection capillary is withdrawn gently after the head of the sperm has left the capillary tip.

A

Figure 5: ICSI using piezo-driven capillary. Sperm injection capillary is placed in the middle of the egg and the zona pellucida is penetrated by application of piezo drill pulses (A). Then, the oolemma is broken by application of piezo drill pulses and the capillary is inserted deeply into the oocyte where the spermatozoon is injected (B). These pictures were kindly provided by Frank Zimmermann, Biotechnology Laboratory, IBF, University Heidelberg, Germany.

B
6. Embryo Transfer

After the eggs have undergone ICSI they are placed in KSOM under mineral oil in the humidified incubator (5 % CO₂ in air). After reaching the 2-cell stage, the embryos are transferred to the oviduct of a pseudopregnant mother (day 0.5 p.c.) or are further cultured to the blastocyst stage in KSOM before they are transferred to the uterus of a pseudopregnant female mouse (day 2.5 p.c.).

References


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