

Biopsy of Blastomeres from Cleavage-stage Mouse Embryos with Eppendorf PiezoXpert®

Norhazlin Jusoh Mohd. Yusoff and Nor Ashikin Mohamed Noor Khan, Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, Universiti Teknologi Mara, Malaysia
Ko Kwan Mor, Eppendorf Asia Pacific Headquarters, Malaysia

Abstract

Embryo biopsy is the removal of one or more cells from the preimplantation embryo. For biopsy the 8-cell stage embryo is prevalently used because of its mitotic index and totipotency of the individual cells (1). The biopsy procedure requires an opening in the zona pellucida (ZP), which is usually performed chemically,

mechanically or using laser systems. This Application Note introduces a piezo-assisted mouse embryo biopsy technique using the Eppendorf PiezoXpert. The survival of biopsied embryos was verified at the morula and blastocyst stages.

Introduction

Preimplantation genetic diagnosis (PGD) combines the assisted reproduction techniques of *in vitro* fertilization (IVF) and micromanipulation with embryo biopsy and DNA analysis of blastomeres for genetic screening of embryos. Basically there are three stages at which cells can be removed from an embryo for pre-implantation genetic diagnosis: removal of the first and second polar bodies (polar body biopsy), removal of trophoctoderm cells from the blastocyst (blastocyst biopsy) and removal of one or two blastomeres from the 8-cell embryo (cleavage-stage embryo biopsy) (2).

Polar body biopsy can only be applied to the diagnosis of maternally inherited diseases whereas blastocyst biopsy, on the other hand, is limited by the small number of embryos which progress to the blastocyst stage *in vitro*. Cleavage-stage embryo biopsy is the more favorable biopsy method as the removal of one or two blastomeres from embryo permits the analysis of the genetic constitution resulting from both the paternal and maternal contribution. Moreover, removal of up to two cells from an 8-cell embryo does not have any detrimental effect on its metabolism (3).

Since the zona pellucida can be dissolved in a low-pH solution, acidic Tyrode's has been used in embryology for decades to remove the ZP in animal systems such as that of the mouse (4). The main disadvantage of using the acidic Tyrode's method is its toxicity and cytoplasmic acidification which can lead, in worst case, to cytoplasmic degeneration (5).

Laser systems are an alternative for the purpose of puncturing the ZP. During the zona drilling, the laser generates heat in a well defined area which lyses the zona proteins, thus creating a slit along the ZP.

In this Application Note, we show that the Eppendorf PiezoXpert can be used as a good alternative to the acidic Tyrode's method as well as laser systems for cleavage-stage mouse embryo biopsy.



Figure 1: The piezo-assisted micromanipulation system consists of an inverted microscope, two TransferMan® NK 2 micromanipulators, a CellTram® vario microinjector, a CellTram Oil microinjector and an Eppendorf PiezoXpert piezo-assisted device.

Material and Equipment

- > Imprinting Control Region (ICR) mice (6-10 weeks old)
- > Culture media: M16 with 5% Bovine Serum Albumin (Sigma-Aldrich®)
- > Handling media: M2 media (Sigma-Aldrich)
- > Embryo biopsy media (Irvine Scientific®)
- > Mineral oil, embryo tested (Sigma-Aldrich)
- > 35 mm & 60 mm dish (Nunc®)
- > Incubator 37 °C in 5% CO₂ with 95% humidity (Memmert®)
- > 29-gauge flushing needle for collection of 2-cell embryos from oviducts
- > Pregnant Mare Serum Gonadotrophin (PMSG) Hormone (Intervet®)
- > Human Chorionic Gonadotrophin (hCG) Hormone (Intervet)
- > Inverted microscope with up to 40x, modulation contrast (DMIRB, Leica®)
- > 2x Micromanipulators (TransferMan® NK 2, Eppendorf)
- > Microinjector (CellTram® Oil, Eppendorf)
- > Microinjector (CellTram® vario, Eppendorf)
- > Piezo-drill unit (Eppendorf PiezoXpert®, Eppendorf)
- > Anti-vibration pads (Eppendorf)
- > VacuTip I (ID 15 µM, tip angle 35°, Eppendorf)
- > Biopsy capillary, Biopsy Tip I (ID 19 µM, tip angle 35°, Eppendorf)
- > Microloader™ (Eppendorf)
- > 3M® Fluorinert® FC-770 3M®

Methods

Superovulation

Female mice were injected with PMSG hormone between 6 and 7 p.m. After 48 hours, hCG hormone was given by intraperitoneal administration. Subsequently, the females were cohabitated with males at ratio of 1:1 male to female. The doses of PMSG and hCG were 10 I.U., respectively.

Collection of embryos

48 h after hCG administration and mating, the females were euthanized by cervical dislocation and oviducts were removed. 2-cell stage embryos were collected from the oviducts by flushing the oviducts with M2 medium using a 29-gauge flushing needle with M2 media under the stereo-microscope. The embryos were then washed 2 times in droplets of M16 media. The embryos were cultured in droplets of M16 media with 5% bovine serum albumin (BSA), overlaid with mineral oil and incubated at 37 °C, in 5% CO₂ with 95% humidity until they developed to the 8-cell stage.

Incubation time before biopsy

The day after 2-cell embryos were cultured (Day 3 after mating) 8-cell embryos were formed. Only the morphologically normal 8-cell embryos were chosen for biopsy. All embryos were then incubated in biopsy medium (Ca²⁺ and Mg²⁺ free) for 10 to 30 minutes in the incubator before starting the biopsy procedure.

Preparation of the biopsy dish

5 droplets of 5 µL calcium-magnesium free embryo biopsy media were placed in the center of the lid of a 35 mm culture dish. The droplets were covered by pre-equilibrated mineral oil to prevent evaporation. Five embryos were then transferred into the droplets (one embryo in each droplet).

Micromanipulation setup

The Eppendorf PiezoXpert was mounted onto the micromanipulator TransferMan NK 2 which is used for controlling the biopsy capillary. CellTram Oil and CellTram vario were set up as microinjectors for embryo holding and biopsy, respectively. An example of a piezo-assisted microinjection workstation is illustrated in Figure 1. For a detailed description on how to install the Eppendorf PiezoXpert onto the TransferMan NK 2 micromanipulator and on the CellTram vario microinjector (see Figure 2), please refer to the Eppendorf Userguide No. 037 (6).



Figure 2: Actuator of Eppendorf PiezoXpert mounted onto the Eppendorf micromanipulator TransferMan NK 2.

Preparation of microcapillaries

The Biopsy Tip I was back-filled with FC-770 using a Micro-loader. The whole capillary was filled with Fluorinert.

The Biopsy Tip I was inserted into the capillary holder of the Eppendorf PiezoXpert deep enough to touch the internal stopper of the capillary holder. This capillary mounting is important to ensure an optimal performance for the drilling.

Each TransferMan NK 2 can store up to 3 positions. When a position is stored, the capillary can be moved back to this position automatically simply by pressing the position button or double-clicking on the joystick button.

Procedure of storing positions for biopsy (Figure 3):

A "working" position in the focal plane was chosen for both the holding and the biopsy side (position 1). The biopsy capillary and the holding capillary were directed at the focal plane, and the positions were stored as Pos. 1. In addition a "parking" position for both capillaries was stored slightly above the droplet so that the pipettes do not interfere with the embryos when the dish is moved around the stage. The positions in the overlay medium were defined as Pos. 2.

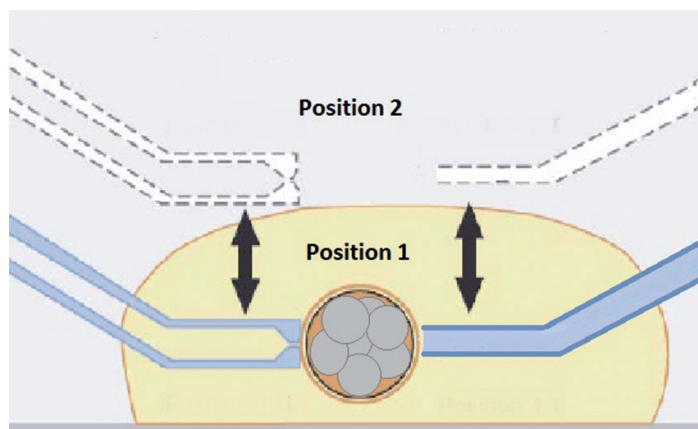


Figure 3: Storing of positions for the blastomere biopsy procedure. The biopsy droplet is overlaid with oil. Positions of the holding and the biopsy capillaries at the working level are stored as Position 1. Positions of both capillaries above the biopsy droplets are stored as Position 2 for changing to another biopsy droplet.

Preparation of the Eppendorf PiezoXpert

The parameters (intensity, speed and number of pulses) of the PiezoXpert can be pre-installed. Up to 3 sets of optimized programs can be stored. Each program allows setting of parameter sets A and B. Usually parameter set A is used for the penetration of the zona pellucida. Set B is used afterwards in the case when cells to be biopsied cannot be isolated from the embryos by gentle suction with the biopsy capillary. In these rare cases, additional gentle piezo impulses can help achieve successful cell isolation. Both sets A and B can be triggered via the button on the control unit or the foot control.



Figure 4: Control panel of the PiezoXpert.

Optimization of parameters:

1. The parameters for the speed and number of pulses were set to 1.
2. The value for intensity (starting from 1) was gradually increased until the piezo impulses were strong enough to penetrate the membranes.
3. The speed and pulse parameters were fine tuned.

NOTE: Users should optimize the parameters for their own experiments as the settings always depend on individual laboratory protocols. As a guideline, if non-cryopreserved embryos are used, the Eppendorf PiezoXpert parameters using Fluorinert recommended for 8-cell embryos are shown in Table 1. Parameter set A is for zona drilling. Parameter set B is for disconnection of blastomeres (when necessary). These parameters can be saved in the Eppendorf PiezoXpert control unit as different programs according to the embryonic developmental stage of the embryos to be biopsied.

Table 1: Parameter settings for 8-cell embryo-biopsy using Fluorinert (FC-770)

| | Parameter set A | Parameter set B |
|-----------|-------------------|-----------------|
| Intensity | 22 - 26 | 3 |
| Speed | 3 - 5 | 1 |
| Pulse | ∞ (1 or 2) | 1 |

Embryo biopsy procedure

An 8-cell embryo was held in place with the holding capillary by suction using CellTram Oil.

The embryo was positioned so that subzonal space was adjacent to the biopsy capillary (Figure 5A). This is an important step to prevent cell lysis of the blastomere adjacent to the biopsy capillary when piezo impulses are applied. The biopsy capillary was briefly pushed against the zona pellucida before zona penetration. A slight suction is also helpful to ensure full contact of zona pellucida and biopsy capillary (Figure 5B).

By triggering the piezo impulses stored in parameter set A of the Eppendorf PiezoXpert the zona pellucida was perforated by the biopsy capillary.

A blastomere was then aspirated gently but uniformly out of the 8-cell embryo into the biopsy capillary using the fine tuning knob of the CellTram vario. This step has to be done carefully to avoid all components of the embryo being

aspirated into the biopsy capillary (Figure 5C-D). In some cases, when there are tight connections between the eight blastomeres and simple aspiration with the biopsy capillary is not sufficient to isolate one blastomere from the embryo, a few weak piezo impulses (parameter set B) can be applied to detach the blastomere from the embryo.

Thereafter, having controlled the presence of an intact nucleus in the aspirated blastomere by gently expelling it from the biopsy capillary, the blastomere was transferred into the margin of the droplet. When the biopsies were completed, the embryos were transferred to the culture dish and kept inside the incubator. The biopsied blastomeres remained in the biopsy dish and were subsequently prepared for genetic analysis.

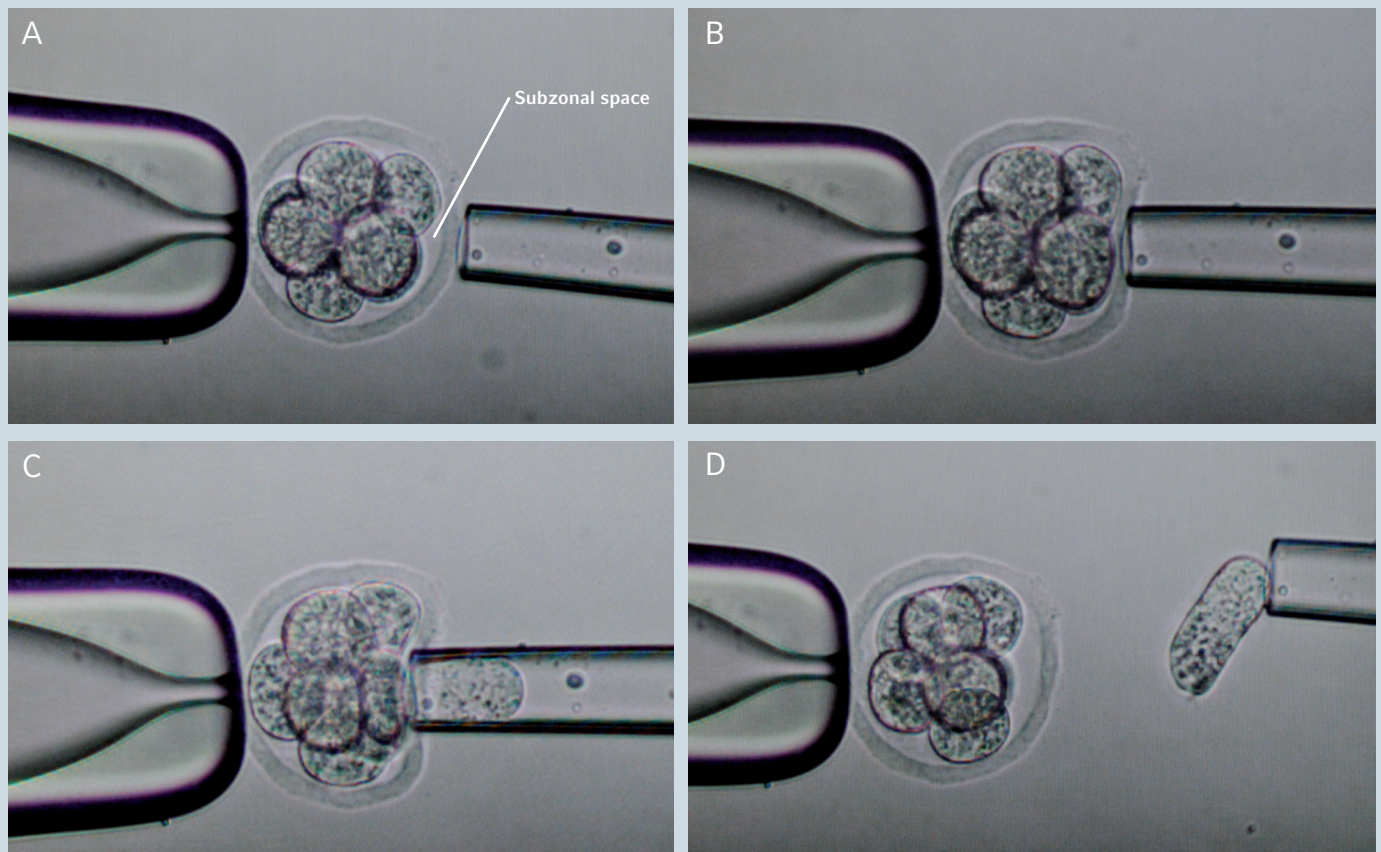


Figure 5: Steps of embryo biopsy. A) The embryo is positioned so that subzonal space is adjacent to the biopsy capillary. B) A slight suction is applied to ensure full contact of zona pellucida and biopsy capillary. C) Piezo impulses are applied and a blastomere is slowly aspirated. D) A blastomere is removed.

Culture after biopsy

After the biopsy procedure, all biopsied embryos were cultured in M16 media, covered with mineral oil, for a further four days. The biopsied embryos were observed

under an inverted microscope every day, with emphasis on the development of morula and blastocyst stages.

Results and Discussion

Survival rates after biopsy with the Eppendorf PiezoXpert were checked at morula and blastocyst stage (Table 2).

Table 2: Survival rates of the biopsied embryos

| | n 8-cell embryos | n morulae | n blastocysts |
|----------------------|------------------|-----------|---------------|
| Eppendorf PiezoXpert | 219 | 213 | 195 |

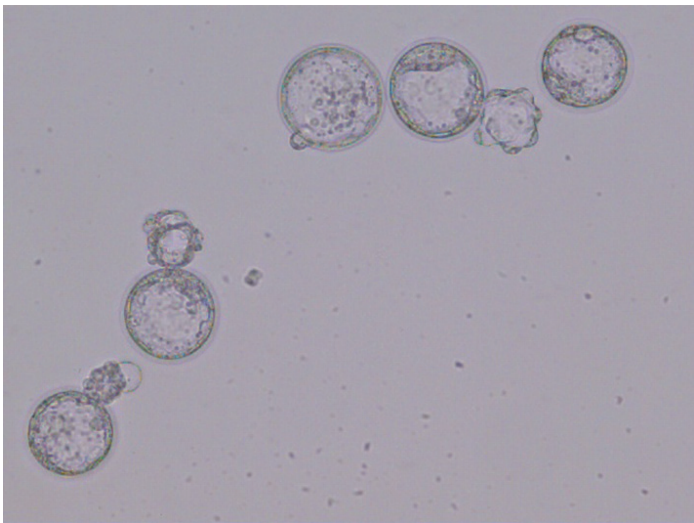


Figure 6: Expanded and hatched blastocysts.

In this experiment, 97.3% of biopsied embryos continued development to the morula stage and 89.0% to the blastocyst stage (see example in Figure 6). Since transplantation of blastocysts was not performed in this study, implantation and birth rates after piezo-assisted biopsy have yet to be verified.

Piezo-assisted micromanipulation has been widely used for mouse Intracytoplasmic Sperm Injection (ICSI), enucleation as well as somatic cell transfer (6-9). This mechanical method has been proven successful for the penetration of ZP and oolemma, and it also increases the survival rate of oocytes after injection (7, 8).

In our study we demonstrated that the Eppendorf PiezoXpert can also be used for isolation of a blastomere from an 8-cell embryo as an alternative technique to the conventional acidic Tyrode's and the laser-assisted micro-manipulation.

One of the main advantages of piezo-assisted micromanipulation is the fact that it does not require acidic Tyrode's which may have adverse effects to embryo development. In addition, this method is more convenient compared to the acidic Tyrode's method since the same microcapillary used for zona drilling can be used for blastomere aspiration. Acidic Tyrode's method usually incorporates the need for a double tool holder on the micromanipulator: one for drilling the ZP with acidic Tyrode's solution and the other one for blastomere aspiration.

The laser drilling system has the advantage of resulting in a lower rate of blastomere lysis during biopsy compared to acidic Tyrode's method (10). However, according to the observations of J. Harper and A. Doshi (11), longer exposure of laser irradiation may cause selective damage by photo-thermal energy to further embryonic development.

In conclusion, the piezo-assisted zona perforation and isolation of a blastomere from an 8-cell embryo with Eppendorf PiezoXpert delivers excellent results of embryo survival rate development to the blastocyst stage.

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Ordering information

| Description | Order no. International |
|---|-------------------------|
| Eppendorf PiezoXpert® , For piezo-assisted micromanipulation, incl. actuator II, foot control and grip head 4 size 0 | 5194 000.016 |
| TransferMan® 4r¹ , Micromanipulator with DualSpeed™ joystick for direct and dynamic movement control | 5193 000.012 |
| CellTram® 4r Air² , Manual pneumatic microinjector, with gears 1:1 and 1:10, for holding and injection | 5196 000.013 |
| CellTram® 4r Oil² , Manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection | 5196 000.030 |
| VacuTip I , Holding capillary, 35° tip angle, 15 µm inner diameter, 1 mm flange, sterile, set of 25 | 5195 000.036 |
| Biopsy Tip I , Capillary for laser-assisted biopsy of cells and organelles, 35° tip angle, 19 µm inner diameter, 1.9 mm flange, sterile, set of 25 | 5195 000.052 |
| Microscope Adapter , Adapter for micromanipulators, available for different inverse microscopes of major brands | Available on request |
| Microloader™ , Pipette tip for back-filling microcapillaries, set of 2 x 96 pcs | 5242 956.003 |

¹The TransferMan 4r replaces the TransferMan NK2.

²The CellTram 4r Air and CellTram 4r Oil replace the CellTram Oil and CellTram vario.

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