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Mercury- and Fluorinert® – free piezo-assisted transfer of ES cells into mouse embryos using the Eppendorf PiezoXpert®

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Abstract

Mutant mouse lines derived from injection of genetically modified embryonic stem cells are important and very powerful tools to analyze gene function. A standard method to combine these cells with mouse embryos to generate chimeric offspring is the injection of ES cells into host blastocysts. This is a highly efficient procedure if 129 derived ES cells are injected into C57BL/6 embryos. More challenging and less efficient is the injection of C57BL/6 derived ES cells into BALB/c donor blastocysts. Here we show that injection of ES cells into eight-cell stage mouse embryos or morulae can be achieved with great success using the Eppendorf PiezoXpert without mercury or Fluorinert filled injection capillaries. Additional advantages of the Eppendorf PiezoXpert are also discussed.

Figure 1: Chimeric male pups generated by the injection of C57BL/6 ES cells (black coat color) into eight-cell stage BALB/c embryos (white coat color).

Introduction

Genetically modified embryonic stem (ES) cells have the ability to contribute to embryonic development when combined with preimplantation embryos. For a long time the standard and technically least challenging method to generate modified mice is to inject 129 derived ES cells into the blastocoel of 3.5 dpc C57BL/6 (B6) mouse embryos [1]. In recent years, various methods have been developed to inject eight-cell stage embryos or morulae and the derivation of completely ES cell derived mice using the latter methods has been reported [2]. With the development of B6 derived ES cell lines and their use by the International Knockout Mouse Consortium (IKMC; http://www.knockoutmouse.org/) generation of germ line transmitting chimeras has become more challenging.

Injection of B6 ES cells into standard B6 blastocysts both encoding for black coat color does not allow the identification of the rate of chimerism by reading the coat color. For this reason, our facility switched to white BALB/c mice as blastocyst donors. However, these mice have one major disadvantage when being used as donors: since the embryonic development is slower than, for instance, C57BL/6 mice, often only morulae or very small blastocysts can be retrieved at the time point of recovery.

To circumvent further incubation of the retrieved embryos, we decided to solely inject into morulae and eight-cell stage embryos. The performance of this technique with a standard micromanipulation set up requires more experience and time as the risk of potential damage to the embryo by penetration of the zona pellucida is significant. Additional support by either cost-intensive infrared laser or by piezo-impact technology can ease the procedure and minimize the risk of damaging the embryo [3-4]. In our lab routine, a clear disadvantage of piezo-assisted injection is the common use of blunt end needles in combination with a heavy liquid like mercury or Fluorinert. We therefore successfully tested the new Eppendorf PiezoXpert using our standard beveled capillaries without the needs for these reagents. This way, it was possible to inject blastocysts, morulae and eight-cell stage embryos with the same injection capillary.

Material and Equipment

Micromanipulation set up (see Figure 2)

> Olympus® inverted microscope IX70 with DIC and optics up to 40x
> TransferMan® NK 2 micromanipulator (holding side) (Eppendorf®)
> PatchMan™ NP 2 micromanipulator (injection side) (Eppendorf)
> CellTram® Air microinjector (Eppendorf)
> CellTram® vario microinjector (Eppendorf)
> Eppendorf PiezoXpert (Eppendorf)
> Cooling stage (self-made, water-cooling) usual recovery time point.

Material

> ES cell medium (as recommended for the ES cell line to be injected) (self-made of components by Life Technologies®)
> PBS (PAA®)
> Trypsin (as recommended for the ES cell line to be injected) (Life Technologies)
> KSOM (self-made, components by Sigma-Aldrich®)
> KSOM/HEPES (self-made, components by Sigma-Aldrich)
> Mineral oil (Sigma® M8470)
> Mineral oil equilibrated with sterile water (mineral oil/H_2O)
> Cell culture dishes (Greiner Bio-One®)
> Transfer pipette (self-made and BioMedical Instruments®)
> Holding capillary (BioMedical Instruments)
> Injection capillary (beveled, size 36, BA = 20°, no spike or size 37, BA = 20°, spike, BioMedical Instruments)
Injection chamber (self-made, thin brass plate with round glass insert)

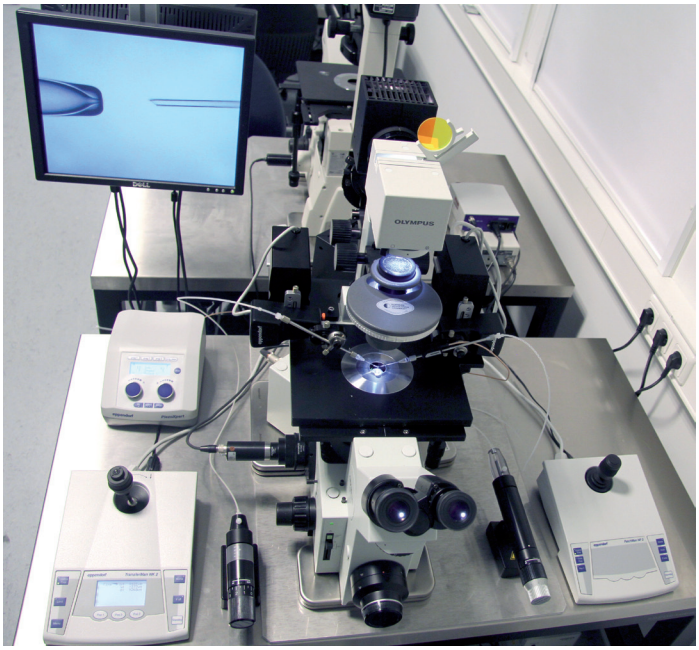

Figure 2: The microinjection set up. The injection chamber on a cooling stage containing the embryos and ES cells, the actuator of the PiezoXpert and the foot control.

Preparation of ES cells for injection into embryos
ES cells are thawed and plated on mitotically inactivated mouse embryonic fibroblasts (MEF) in the medium recommended for the cell line and are fed daily. After 2-3 days they are harvested by trypsinization and plated once again at a density which allow them to reach ~ 50 % confluence on the day of injection. Cells for injection have to be fed at least 2 h (R1, E14) or 3 h (JM) before trypsinization. This time is used to recover the embryos.
To harvest the cells the medium is aspirated, the cells are washed with PBS at least once before trypsin is added according to recommendations for the well size and the cell line used. After incubation at 37 °C for 3-5 min (depending on the cell line), cells are dispensed by pipetting them gently up and down and transferred to a 15 mL tube containing 5 mL HEPES buffered ES medium minus LIF. After centrifugation at 400 x g for 4 min the medium is aspirated and the pellet is resuspended by gentle flicking. Then 0.5 mL of the HEPES-buffered medium is added and the tube is placed on ice for at least 30 min before injection. During this incubation time the microinjection set up and the cooling stage are switched on and prepared for injection.

Recovery of eight-cell embryos and morulae
Embryos are recovered according to standard procedures. KSOM/HEPES is used as medium and embryos are washed by transferring two drops of equilibrated KSOM medium. Embryos are incubated in KSOM under mineral oil equilibrated with H_2O set up in 60 mm dishes at 37 °C, 5 % CO_2.

Equipment set up
The cooling stage is adjusted to 12 °C. The holding pipette is mounted onto the TranferMan NK 2. For equilibration of the capillary it is lowered into a PBS filled lid of a 35 mm dish and washed at least twice with PBS, once with 70 % ethanol, and finally filled to 2/3 with PBS before it is moved into a parking position above the dish by pressing the home function key of the TransferMan NK 2 control panel. The holding pipette can be reused for extended periods if it is properly cleaned first with PBS, followed by 70 % ethanol after each use. The Eppendorf PiezoXpert actuator is mounted onto the PatchMan NP 2 and connected to the CellTram vario. CellTram, pressure tube and capillary holder are filled completely with mineral oil and the injection capillary is inserted while a small amount of oil is dripping out of the capillary holder. It is extremely important to avoid any air bubbles. The capillary is lowered into the PBS filled dish. To test if the system is free of any bubbles and the capillary fits tightly a strong piezo impulse ("clean", setting of the Eppendorf PiezoXpert see below) is applied. The capillary should not vibrate or move. The capillary is then rinsed up to its bend as described for the holding pipette and incubated in PBS for at least 15 min.

Every time the capillary is removed from the medium, pressure must be applied by turning the wheel of the CellTram vario clockwise to fill it completely with oil. A small drop of oil should always be visible at the tip of the capillary.

Eppendorf PiezoXpert parameters
Pre-installation of Eppendorf PiezoXpert parameters (intensity, speed and number of piezo impulses) can significantly speed up the ES transfer procedure and at the same time shorten the time-period during which the embryos are exposed to stressful conditions, thus improving the final results. Up to 3 sets of optimized programs can be stored in the Eppendorf PiezoXpert. Each program consists of parameter sets A and B, see Figure 5. Usually, parameter set A is used for the penetration of the zona pellucida. In our case, set B is used subsequently for cleaning the needle in between injections. Both sets A and B can be triggered via either the button on the control unit or the foot control. To finally remove cell debris or oil from the injection capillary the 'clean function' of the Eppendorf PiezoXpert is used, which can be triggered via the 'clean' button.

The injection procedure
The precooled injection chamber is loaded with ~ 400 µL of cold KSOM/HEPES. To reduce the number of mouse embryonic fibroblasts (MEF) within the ES cell suspension when transferred to the injection chamber the cells are resuspended by gentle flicking and on ice while 6-10 embryos are placed in the upper third of the injection chamber using a mouth pipette. Approximately 40 µL of the resuspended ES cells are pipetted from the upper two thirds of the medium (MEF are much larger than ES cells and thus sediment faster than small ES cells) and are slowly added to the injection chamber. This chamber is then placed on the injection set up. The lowest magnification is used to focus on the embryos, the holding pipette is moved down to the embryo level and the position is stored at the TranferMan NK 2 as 'position 1'. With the TransferMan NK 2 up to three working positions can be stored and easily recalled by either pressing the 'position1-3' button or by 'double-clicking' the joystick button. The holding pipette is raised slightly (while remaining immersed in medium) and the injection needle is moved down. The oil is aspirated back a short distance and small, round, healthy-looking ES cells with clearly visible nuclei are collected like pearls on a string using the 20 x objective x 1.5. When the required amount of cells has been collected, an embryo is picked up with the holding pipette and moved to the middle of the injection chamber slightly above the bottom (stored as 'position 2'). In case cell debris or MEF attach to the injection capillary during collection of the cells they can be removed very conveniently by applying short piezo impulses (stored in B). One embryo is aspirated using the holding pipette and moved to the injection position ('position 2') in the middle of the slide. The injection capillary is brought in position and with the help of the foot control a piezo impulse is applied.

Depending on the capillary used, parameter settings on the Eppendorf PiezoXpert may vary slightly. By using beveled needles, the piezo impulse induces a small slit in the zona pellucida instead of a circular hole, through which the needle can be inserted easily (Figure 3). There is no need to manipulate or even push with the holding site to penetrate the zona. Four to nine ES cells are expelled and the injection capillary is retracted carefully, while allowing the slit to close again. This is also possible with spiked needles (see Figure 4). The injected embryo is placed on the right half of the upper side of the injection chamber (store as 'position 3'). The above described injection procedure is repeated until all embryos of a batch are injected. Each batch of embryos is transferred to a fresh drop of KSOM and stored in the incubator.
As soon as all batches of embryos have been injected they are washed by transferring them to at least one fresh drop of KSOM.

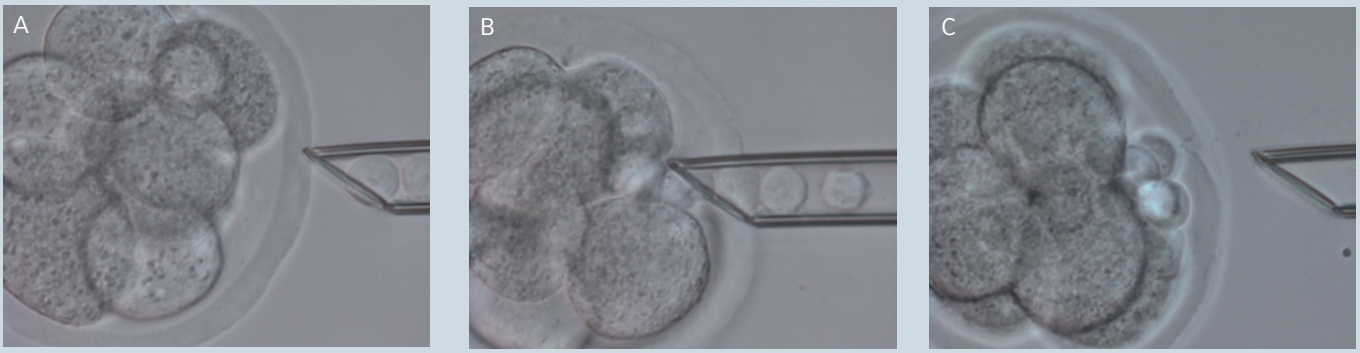

Figure 3: The injection of ES cells into eight cell embryos using the Eppendorf PiezoXpert.

The embryo attached to the holding capillary is brought into injection position slightly above the bottom of the injection chamber (a), the injection capillary is moved to the zona, a piezo impulse is applied via the foot control and cells are released between embryo and zona (b). The injection capillary is drawn back (c) and the small opening in the zona will close rapidly.

Note: The same procedure and settings work for injection of ES cells into morulae; the only difference being the number of cells transferred (8 - 12).
To reuse the holding and the injection capillary, rinse as described above. To remove cell debris or oil from the injection capillary, the 'clean' function of the Eppendorf PiezoXpert is used. We changed the settings of the 'clean' function to maximum (see Table 1). Note: Do not use the 'clean' function immediately next to ES cells since the impact may have a negative effect in close proximity.

Figure 4: After ES cell injection into an eight-cell embryo using a spiked capillary with the Eppendorf PiezoXpert. The injection capillary is retracted and the small opening in the zona will close rapidly

Embryo transfer

Injected embryos are incubated overnight and developed to the blastocyst stage. These are then transferred to the uterus of 2.5 day pseudo-pregnant females (B6 x CBA) or if those are not available to the infundibulum of 0.5 day females. In general, in both cases implantation is performed bilaterally with 4-7 blastocysts placed on each side. Pups are born 17 or 19 days later, depending on the foster mothers used.

Table 1: Parameter settings A and B as well as clean function using a beveled capillary without a heavy liquid

Procedure	Intensity	Pulse	Speed	Remarks
Injection (A)	18 - 26	Infinite (approx..2)	5	Varies depending on capillary
Cleaning during Injection (B)	30	infinite	5	7.0
Clean function	86	infinite	20	Final cleaning of capillary (for subsequent use)

Results and discussion

Since 2010 the demand for C57BL/6 derived mice has increased steadily in our facility. While the excellent performance of 129 derived ES cells in terms of generating highly chimeric germ line transmitting males was met with great satisfaction, we were not as successful in obtaining those from C57BL/6 ES cells. It is common knowledge that C57BL/6 are not as efficient as 129 derived ES cells [1, 3, 5]. In comparison, we had to use up to eight foster mothers to receive a single clone, while we used at most two for 129-derived ES cells. Although we took great care during the injection process, and cell preparation, the outcome was disappointing. One of the reasons was the use of white blastocyst donor mice. Due to space limitations, we had to use BALB/c mice instead of establishing a colony of albino B6 mice. The embryos of BALB/c have a slightly delayed development compared to B6. As a consequence, we were often able to harvest only morulae or very small blastocysts and had to wait longer before we could start injection. Even then blastocoels were often so small that the insertion of a sufficient number of cells was not always possible. Another reason for the low efficiency was the unsatisfactory performance of C57BL/6 derived ES cells themselves, which became evident when we injected C57BL/6 derived ES cells harboring an intact agouti locus (JMA) into the standard C57BL/6 host blastocysts. Only one out of four C57BL/6 derived clones produced high chimeric males. This unacceptable performance, accompanied by the large amount of time invested, prompted us to attempt with the injection of morulae and eight-cell stage embryos. This procedure yields results without the assistance of a piezo device or a laser system but has the disadvantages that it is time consuming and has to be performed very carefully in order not to harm the embryo. We therefore started to use piezo-assisted micromanipulation. We intended to avoid the use of mercury mercury or Fluorinert filled capillaries due to the toxicity (mercury) and the additional step required. Furthermore, using these reagents hampers the reuse of the injection pipettes when performing non-assisted and assisted blastocyst injections on the same day.

Hence we attempted to use the Eppendorf PiezoXpert, without those reagents, with beveled or even spiked capillaries and were immediately successful (see Figure 3). We have been using the Eppendorf PiezoXpert for all injections of C57BL/6 derived ES cells for seven months. Altogether we injected 20 clones for 13 different constructs. We obtained highly chimeric mice (see Figure 1) for each construct yielding germ line transmission for eight of them. The outcome of the last five constructs injected is still pending. In the few cases where we injected agouti 129 derived ES cells into C57BL/6 blastocysts and eight-cell stage embryos on the same day we recovered more highly chimeric males with the piezo-assisted injection although the overall number of pups born was lower. Therefore, we now use the Eppendorf PiezoXpert for injecting C57BL/6 eight-cell embryos as well. In comparison with other users we are using higher intensity settings for the penetration of the zona pellucida, which appears to compensate for working without a heavy liquid inside the capillary. This effect can also be seen when comparing the use of mercury (liquid density of 13534 kg/m^3) and FC77 or FC 770 (~1940 kg/m^3). The heavier the liquid, the lower the impulse settings required for successful penetration of membranes [6,7]. Since we could not observe an increased lysis rate while using 'blank' piezo-assisted manipulation, we believe that the use of higher settings while abstaining from such liquids results in comparable impact to the embryos as the combination of mercury with a low intensity setting. At the moment, this observation is limited to ES cell transfer, with the 'weight' of the ES cells and the use of a 'heavier' capillary with a large diameter, whether this is also true for SCNT or ICSI needs to be determined.

In summary we conclude that the use of the Eppendorf PiezoXpert increases overall efficiency in our workflow and is thus saving not only precious time and therefore money, but it also greatly reduces the number of mice necessary to generate germ line transmission of a construct.

Literature

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Ordering information

Product	Description	Order no. International	Order no. North America
Eppendorf PiezoXpert®[1]	Basic device incl. Actuator, Foot control and Spacer plate*	5194 000.016	5194000024
TransferMan® NK 2[1,2]	Micromanipulator with proportional movement control for suspension cells	5188 000.012	920000011
PatchMan™ NP 2	Micromanipulator with dynamic movement control	5183 000.014	920000037
CellTram® Air[1,2]	Manual pressure device for the reliable holding of suspension cells	5176 000.017	920002021
CellTram® Oil[1,2]	Manual pressure device for the reliable holding of suspension cells	5176 000.025	920002030
CellTram® vario[1,2]	Manual hydraulic microinjector, with gears for coarse and fine control	5176 000.033	920002111
VacuTip™ [1,2,3]	25 glass capillaries for holding large cells (e.g. eggs), sterilized, tip angle 35°	5175 108.000	930001015
Piezo Drill Tip™ ES[1]	25 glass capillaries for the piezo-assisted transfer of embryonic stem cells, angle 25°	5175 250.001	930001104
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)[1]	"Personal" sized CO_2 incubator offering 14L of CO_2 incubation with an LED display	CO14S-230-0000	
Galaxy® 14 S (230 V)[1]	Incubator with active 1-19 % O_2 control	CO14S-2300200	

[1] For research use only. Not for use in diagnostic procedures. This product is not registered in the U.S. as a medical device and does not have a 510(k) registration.
[2] This product is registered in Europe as a medical device (according to Medical Device Directive MDD 93/42/EEC).
[3] Proven non-cytotoxicity by the mouse embryo development test.

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