eppendorf

APPLICATION NOTE No. 394

Precise and Oil-Free Transfer of ES Cells into Early Embryos with CellTram® 4r Air

Ronald Naumann[1], Sandra Stobrawa[2]
[1]Transgenic Core Facility, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
[2]Eppendorf AG, Hamburg, Germany

Abstract

The generation of knock-out and knock-in mice via embryonic stem (ES) cell injection is still a powerful tool to understand *in vivo* functions of a gene of interest. One critical step of this method is the uptake of selected ES cells into the injection capillary and their careful release into mouse blastula or early morula. These steps require direct and precise pressure control in the injection capillary by a manual microinjector and well trained skills of the user.

For this Application Note the pneumatic microinjector Eppendorf CellTram 4r Air was extensively tested for its suitability of ES cell collection and injection into early mouse embryos. The oil-free CellTram 4r Air allows a precise pressure control during the ES cell collection and injection and its intuitive operation facilitates even beginners to quickly succeed in this technique.

Introduction

Targeted gene knock-in/-out mouse models are powerful tools to understand the biological and molecular functions of genes *in vivo*. For example, these mouse models may be used to examine the molecular mechanisms underlying human diseases and could aid in the creation of new therapies. In the last three decades, these gene targeted mouse models were generated by creating chimeric mice via injection of homologous recombined embryonic stem (ES) cells. Recently, an efficient one-step targeting method was established by injection of CRISPR/Cas9-related components into the zygote. However, this one-step approach via zygotic CRISPR injection is still critical when larger or more complex targeting events (e.g. large humanizations, conditional Knock-outs) are required. Its lower targeting efficiency is ethically problematic when screening of gene modification is done not before the stage of living offspring. Thus, the technique via ES cell transfection, their selection via genotyping and injection is still a common method in mouse model production.

Furthermore, a cooperation of world-wide institutions, the so-called International Knock-out Mouse Consortium (IKMC) maintains and expands a cell bank of targeted ES cells for more than 20.000 mouse genes. IKMC offers researchers the free access to the ES cells for generating the mouse models in their facilities.

The injection of the targeted ES cells into 3.5 days old mouse blastocysts or into 2.5 days old morula stages requires a setup with precise and direct pressure control and manual skills. This is especially true for taking up a few selected ES cells and lining them up close to the tip within in the injection capillary for a controlled release into the early embryo. How quickly these techniques are learned by an operator depends on previous experience, routine work, natural abilities, and how intuitive and finely-adjustable the microinjection devices are.

For ES cell transfer transgenic labs are using microinjection systems with different modes of operation. Most use a hydraulic microinjector (e.g. CellTram Oil/vario) which provides a very precise and direct pressure control in the injection capillary. However, the system is sensitive to smallest air bubbles within the oil-phase of the injector and thus requires some trained skills. In our lab, we use a 10 mL syringe to manage the ES cell collection and injection via pneumatic pressure. This simple injection tool needs an extensive operator training to acquire the ability of controlled ES cell uptake and release.

The Eppendorf CellTram 4r Air is an exclusively pneumatic microinjector with precise pressure control by a separated coarse and fine control drive. We extensively tested its performance for mouse ES cell injections which are routinely performed in our transgenic facility. In this Application Note, we describe optimization of our microinjection workflow by using the CellTram 4r Air microinjector.

Materials and Methods

List of Equipment

> Inverted microscope equipped with Differential Interference Contrast (DIC), and 10x, 20 x and 40 x objectives
> Infrared Laser for laser-assisted ES cell transfer, e.g. from OCTAX® (Germany) or Hamilton Thorne (USA)
> Two TransferMan® 4r micromanipulators, Eppendorf (Germany)
> CellTram 4r Air microinjector for holding early mouse embryos, Eppendorf (Germany)
> CellTram 4r Air microinjector for collection and injection of ES cells, Eppendorf (Germany)
> CO_2 incubator, e.g. from Labotect (Germany) or Eppendorf (Germany)

List of Materials

> Holding capillaries, e.g. VacuTip I, Eppendorf (Germany)
> ES cell transfer capillaries, e.g. TransferTip® ES, Eppendorf (Germany)
> Homemade metallic frame as injection chamber
> Cover glass slide to insert into the injection chamber, 24 x 40 mm, Roth (Germany)
> ES cell-culture medium with HEPES for injection
> KSOM medium for incubation of embryos - covered by Paraffin-Oil, Sigma-Aldrich (U.S.A.)
> Cell culture dish for incubation of embryos, 35 mm, Eppendorf (Germany)

Preparation of cells, embryos and injection samples

The preparatory steps are crucial but their detailed description would go beyond the scope of this Application Note (see 1, 2). For the ES cell injection, we used the ES cells from mice with the following genetic backgrounds: VGB6 (background: C57BL/6NTac), JM8A3.N1 (background: C57BL/6N-A<tm1Brd>), R1/E (background: 129X1/SvJ x 129S1/Sv).

Preparation of the ES cell transfer workstation

The workstation is assembled of two TransferMan 4r micromanipulators controlling the movements of the holding and transfer capillaries. As injector, a CellTram 4r Air in combination with the ES cell transfer capillary is used for the transfer of the ES cells. A second CellTram 4r Air in combination with the holding capillary is used for holding of blastocysts or 8-cell-stage embryos. Both, transfer and holding capillaries are fitted into the capillary holders by insertion into the grip head 4 system. Their angled tip ends are carefully aligned parallel to the bottom of the injection chamber to ensure optimal holding support when puncturing the embryos from its opposite site.

The CellTram 4r Air injector allows to adjust its pressure responsiveness in the capillary individually and in requirement to the specific application (e.g. gentle but tight holding of embryo vs. precise uptake and release of ES cells). This pressure responsiveness is adjusted by positioning the internal piston displayed on the injector by a numerical scale. Softer and slower pressure control is achieved by working in a piston position range of 7 – 9, whereas a faster and stronger responsiveness is achieved by piston positions at of 2 – 4 (see Figure 1).

The injection chamber made of a metallic frame with a silicon grease glued glass slide is prepared.

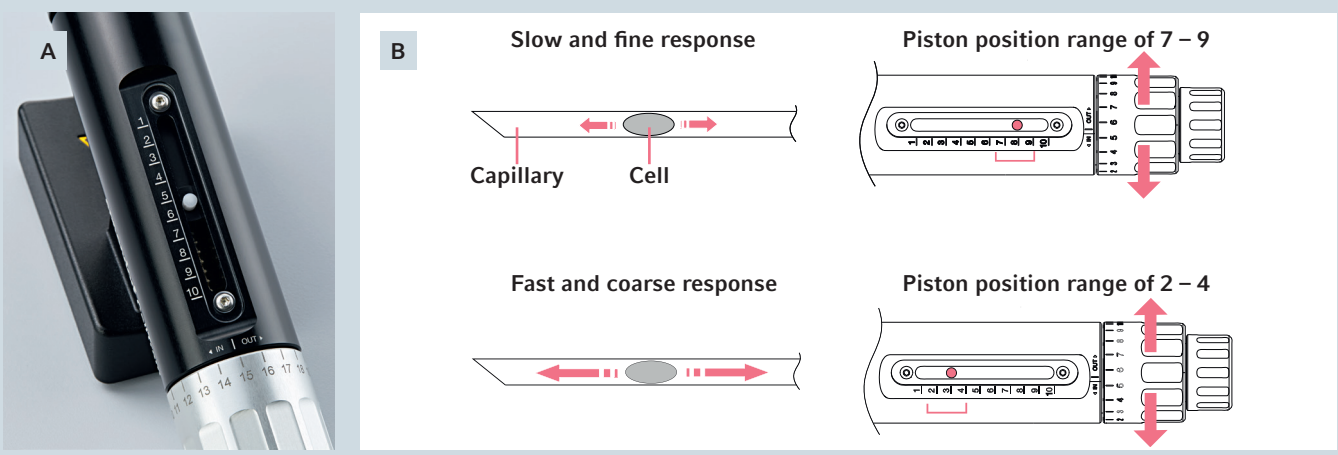

Figure 1: Adjustment of the responsiveness in pressure control of the CellTram 4r Air.
A: The numerical scale indicates the position of the internal piston of the CellTram 4r Air.
B: The pressure responsiveness can be adjusted by the piston position. Softer and slower pressure control is achieved by working in a piston position range of 7 – 9, whereas a faster and stronger responsiveness is achieved by piston positions around 2 – 4.

ES cell transfer into blastocysts or early morulae

A drop of ~ 600 µL HEPES-buffered ES cell-culture medium is placed in the injection chamber and up to 20 early embryos are transferred into one area of this drop. A few hundred ES cells in medium are inserted into the droplet of the injection chamber as well in another region. Under high magnification (200 x), individual ES cells are selected according to their quality in size and shape.

For blastocyst or 8-cell-stage injections, approx. 15 or 8 ES cells, respectively, are taken up with minimal medium into the transfer capillary and lined up close to the tip end. For the uptake and release of ES cells different piston position sets were tested at the CellTram 4r Air to identify the optimal pressure responsiveness of the injector to our individual handling.

An embryo firmly attached at the holding capillary is brought into the injection position in focal plane. After optimal positioning the embryo (e.g. inner cell mass of blastocyst at 6 o' clock, see Figure 2A) the tip of the transfer capillary is aligned in the same focal plane as the equator of the blastocyst. With a single, continuous movement the loaded injection capillary is pushed into the blastocyst cavity at a junction between two trophoblast cells. This will minimize the damage to the embryo. The cells are slowly expelled inside the cavity (Figure 2B). For higher survival rate of the embryos it is crucial that no lysed cells are inserted into the blastocyst. After injection, the capillary is slowly pulled out of the embryo.

A more efficient penetration of the early mouse embryos is achieved by laser- or piezo-assisted micromanipulation allowing the pre-perforation of the thick zona pellucida (3, 4).

The successful injected early embryos are transferred into a cell culture dish with 100 µL KSOM medium, covered by paraffin-oil and quickly placed back into the CO_2 incubator where the embryo can recover under optimal growth conditions until their retransfer into a pseudo pregnant foster.

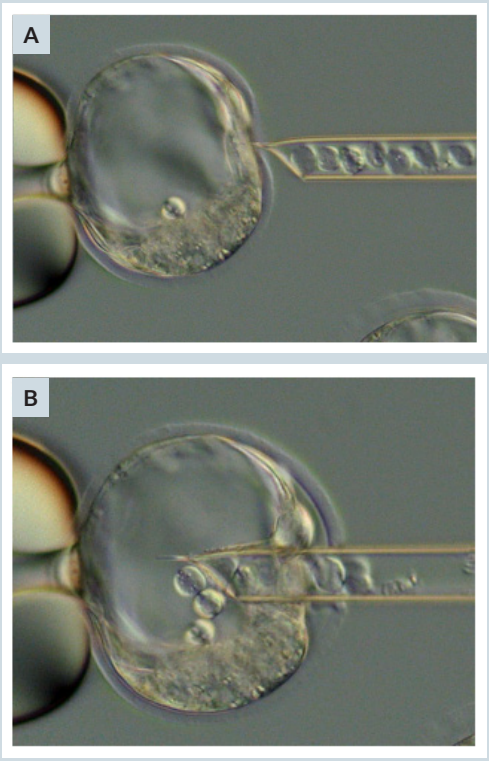

Figure 2: Blastocyst injection.
A: The targeted ES cells are collected and lined up in the transfer capillary close to the tip end. The blastocyst is firmly attached to the holding capillary with the inner cell mass to 6 o'clock. The spike of the beveled tip end is positioned between two trophectoderm cells. Then, with a single, swift and continuous movement the capillary is pushed into the blastocoel.
B: The targeted ES cells are slowly released by carefully applying positive pressure to the capillary via the CellTram 4r Air.

Results and Discussion

The pneumatic working manual injector CellTram 4r Air offers two options for precise and direct pressure control:

- **a)** by operating with the coarse or fine rotary knob (transmission ratio 1:10)
- **b)** by individual pre-setting the pressure responsiveness via its piston position

We tested the performance of the CellTram 4r Air for the ES cell transfer using different settings of the piston position of this device. Table 1 shows typical results from highly experienced operators and a trainee.

The CellTram 4r Air allows a very gentle and controlled uptake of the ES cells. The drift-free position of the cells inside the capillary and the direct response to pressure changes facilitate a good lining the cells up at the tip end prior to ejection. We also experienced a precise controlled release of the ES cells into the blastocoel or the subzonal space of early embryos operating the fine rotary knob.

Testing different pre-set piston positions, we recognized different responsiveness in pressure control within the capillary. In our hands the optimal conditions for handling the ES cells is at piston position 6. More experienced operators also like to speed up the ES cell handling using lower piston positions.

Using the CellTram 4r Air for the holding side, the injector

Table 1: Observed responsiveness of pressure control in the ES cell transfer capillary using different settings

Settings				
Pre-fill level of media in capillary	low (2 mm filling level in the capillary)		medium (5-6 mm filling level in the capillary)	
Pre-set piston position	2	4	6	8
Results				
ES cell uptake	Very direct and fast control, suitable only for experienced operators	Good control with coarse knob	Perfect control with coarse & fine rotary knob	Perfect control with coarse knob, very precise control with fine knob
Cell control inside the capillary	Drift-free position of cells; fast and very sensitive response	Drift-free position of cells; direct response to pressure changes	Drift-free position of cells; direct response to pressure changes	Drift-free position of cells; good response
Release into blastula	Good control with fine knob	Good control with fine knob	Good control with fine & coarse knob	Good control with fine & coarse knob
Release into 8-cell-stage embryo	Good control with fine knob	Good control with fine knob	Optimal control with fine knob	Good control with fine knob

works also very reliable. During the injection, the blastocysts as well as 8-cell-stage embryos stay well attached at the holding capillary tested at lower or higher piston positions. We could also confirm this when doing pronuclear injection into zygotes. For holding of embryos and zygotes, we mainly operate with the coarse rotary knob.

A further advantage we experienced with the new CellTram 4r Air is the new grip head system (Figure 3). This new grip head allows an easier insertion of the capillary into it when mounting the capillary onto the injector. Within this system, two o-rings with a small sleeve in between ensure that the capillary is perfectly fixed and stabilized during penetration.

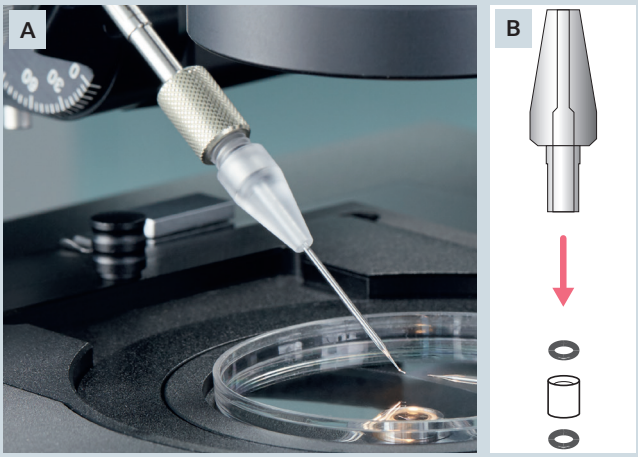

Figure 3: Grip head 4 system.
A: The new grip head mounted onto the capillary holder of the CellTram 4r Air is holding the capillary stable and tightly.
B: The grip head is stacked with two o-rings and a small metallic sleeve in the depicted order.

Conclusion

The Eppendorf CellTram 4r Air is an exclusively pneumatic injector – even without the use of oil in its pressure control system, this manual microinjector is an excellent tool for precise collection and injection of the ES cells. By simply operating the fine and coarse rotary knobs very accurate pressure changes can be generated in the attached capillary facilitating the controlled uptake and release of the ES cells. Furthermore, the responsiveness of this pressure control can be even individually adjusted via the piston position, which allowed us to handle the ES cells according to our personal training skills and preferences. Especially for trainees without a lot of ES-cell injection experiences, it is a great value using the Eppendorf CellTram 4r Air microinjector. Furthermore, the device is all the time ready to use and you don't need any preparation time for this tool as required for an oil-based microinjector. As we also used the device successfully for embryo holding and in embryonic cell biopsy experiments, the CellTram 4r Air proved itself as a multipurpose microinjector in a transgenic facility lab.

Literature

[1] Nagy A, Gerstenstein M, Vintersten K, Behringer B. Manipulating the Mouse Embryo, a Laboratory Manual. Third edition 2003. Cold Spring Harbor Laboratory Press
[2] Pease S, Saunders TL (editors). Advanced Protocols for Animal Transgenesis. An ISTT Manual. 2011. Springer Protocols
[3] DeChiara TM, Poueymirou WT, Auerbach W, Frendewey D, Yancopoulos GD, Valenzuela DM. VelociMouse: Fully ES Cell-Derived F0-Generation Mice Obtained from the Injection of ES Cells into Eight-Cell-Stage Embryos. In: Wurst W, Kühn R (editors). Gene Knockout Protocols. Methods in Molecular Biology 2009, 530:311-324. Humana Press
[4] Kawase Y, Iwata T, Watanabe M, Kamada N, Ueda O, Suzuki H. Application of the piezo-micromanipulator for injection of embryonic stem cells into mouse blastocysts. Contemp Top Lab Anim Sci 2001, 40:31-34.

Ordering information

Description	Order no. International	Order no. North America
CellTram® 4r Air, manual pneumatic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000.013	5196000013
CellTram® 4r Oil, manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000.030	5196000030
TransferMan® 4r, micromanipulator with DualSpeed™ joystick for direct and dynamic movement control	5193 000.012	5193000020
Microscope Adapter, for mounting onto different inverse microscopes, compatible with major microscope brands	Available on request	Available on request
Eppendorf PiezoXpert®, for piezo-assisted micromanipulation, incl. actuator and foot control	5194 000.016	5194000024
TransferTip® ES, transfer capillary for ES cells, 20° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.079	5195000079
Piezo Drill Tip ES, capillary for piezo-assisted mouse ES cell transfer, 25° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.095	5195000095
VacuTip I, holding capillary, 35° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.036	5195000036
Galaxy® 48 R, CO_2 incubator, 48 L, 230 V/50/60 Hz, High-Temp Disinfection, 0.1–19 % O_2 Control	CO48310042	CO48210045

Your local distributor: www.eppendorf.com/contact
Eppendorf AG · Barkhausenweg 1 · 22339 Hamburg · Germany
eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com