APPLICATION NOTE No. 285

The Eppendorf TransferMan® 4r, one manipulator for all genetic engineering techniques

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Abstract

The study of genetically modified animals provides important insights into gene function. It can also be used for modelling human diseases, to either understand disease mechanisms or aid drug development.
The main techniques to generate those animal models require micromanipulation devices, but usually in slightly varying set-ups and with different settings for the respective techniques. Therefore many laboratories generating mouse or rat models prefer to have two separate workstations for the two main techniques, nucleic acid injection into fertilized oocytes and embryonic stem (ES) cell transfer into early embryos.
With the multifunctional TransferMan 4r, one workstation is adequate for all applications because of its easy angle adjustment, vibration-free movements and unique Eppendorf DualSpeed™ joystick.

Introduction

Micromanipulation of oocytes or embryos is the method for modifying the genome of laboratory animals. The most common method is microinjection of nucleic acid constructs like simple transgenes, larger bacterial artificial chromosomes (BAC), or site-specific nucleases like zinc finger (ZFN) and transcription activator-like effector nucleases (TALEN). In recent times, the microinjection of clustered regularly interspaced short palindromic repeats (CRISPR) and RNA-guided Cas9 nucleases emerged as a powerful tool for genome engineering.
The different types of constructs are either injected into one of the pronuclei of early zygotes (1-2) or into the cytoplasm (3-4). These techniques are called pronuclear and cytoplasmic injection, respectively. Another technique, though not applicable to all laboratory animals due to the lack of suitable material and/or limited accessibility of material, is the so-called ES cell transfer, i.e. the injection of genetically modified embryonic stem cells into embryos in blastocyst or 8-cell stage. In addition, the Intracytoplasmic Sperm Injection (ICSI) of either genetically modified sperms or sperms coated with nucleic acid (sperm-mediated gene transfer) into oocytes can be used to generate genetically altered offspring (5).

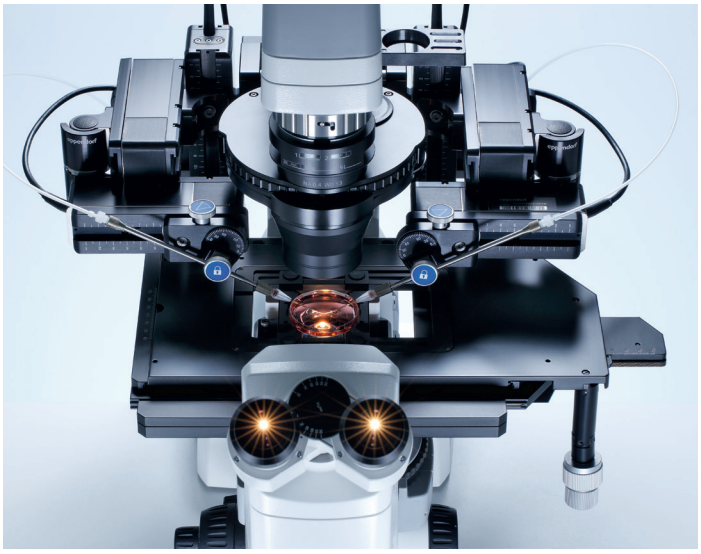

Figure 1: Eppendorf TransferMan 4r micromanipulators.

In practice, all these injection techniques demand certain precautions and considerations for optimal results. They do not only differ in the optimal injection angles, but (in our opinion) also vary in their demand of the micromanipulators used as well as in the speed of injection movements. Therefore most laboratories are using independently pre-fixed and finely adjusted workstations for each kind of injection technology.

With the TransferMan 4r (see Figure 1) Eppendorf introduces a multifunctional manipulator suitable for all these applications.

This system includes a number of special features including application-specific masks like »Cell transfer«, »DNA injection« or »ICSI« which facilitate and simplify the individual workflow process. The unique DualSpeed joystick allows precise and intuitive movement during injection in all spatial dimensions as well as dynamic movements while e.g. collecting ES cells. Thanks to its mounting concept the workstation is sturdy and safe against external vibrations. Furthermore, all elements of the manipulator are easy and fast to install and adjust. The construction of the motor modules allows for an extremely flexible set-up and can be adapted to all major microscopes used. Position changes on the motor modules are extremely flexible and therefore comfortable for all microscopes. The angle adjustment is easily done. It is possible to set shallow angles of theoretically 0°, preferred for pronuclear and cytoplasmic injections when using straight capillaries, as well as angles up to 45° (with a maximum of 90°).

We tested the TransferMan 4r performance for manipulation techniques which are routinely performed in our lab over an extended period of time. Where possible, this was done in parallel to our standard set-up.

Materials and Methods

Devices

> Inverted microscope equipped with Differential Interference Contrast (DIC), and 10 x, 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 (one for moving the holding capillary and the second for positioning the transfer/injection capillary), Eppendorf (Germany)
> Adapter for inverted microscope, Eppendorf (Germany)
> CellTram 4r Air microinjector for holding the oocyte, Eppendorf (Germany)
> CellTram 4r Oil microinjector for transferring the ES cells, Eppendorf (Germany)
> FemtoJet® microinjector for pronuclear and cytoplasmic injections, Eppendorf (Germany)
> Holding capillaries, e.g. VacuTip I, Eppendorf (Germany)
> Injection capillaries, e.g. BioMedical Instruments (Germany)
> ES cell transfer capillaries, e.g. TransferTip® ES, Piezo Drill Tip ES, Eppendorf (Germany)
> Eppendorf Microloader™ for back-filling the injection capillaries, Eppendorf (Germany)

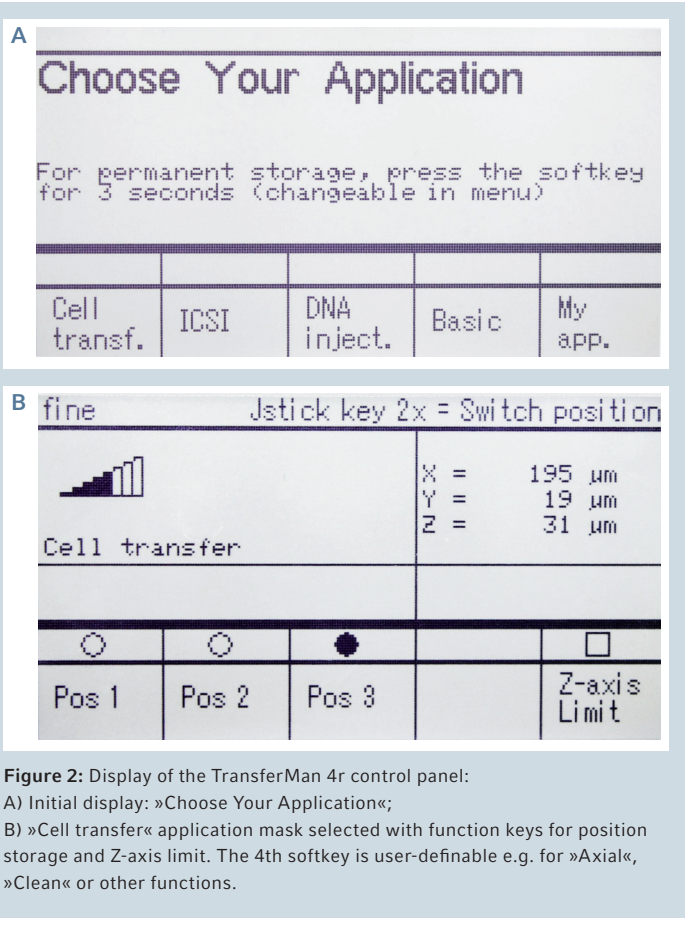

Figure 2: Display of the TransferMan 4r control panel:
A) Initial display: »Choose Your Application«;
B) »Cell transfer« application mask selected with function keys for position storage and Z-axis limit. The 4th softkey is user-definable e.g. for »Axial«, »Clean« or other functions.

Operation of the micromanipulator TransferMan 4r
Operating the TransferMan 4r micromanipulator with its functional softkeys and its single joystick for all movement dimensions and speed settings is extremely simple (see Figure 2): The selection of the application mask »Cell transfer« allows for the saving and recall of three capillary positions, plus the setting of a vertical limit and thus the avoidance of capillary breakage. The application mask »DNA injection« offers not only capillary position storage but also functions to temporarily deactivate the Y motor preventing accidental sideways tearing of the zygote during the sensitive injection procedure. This feature can be of crucial importance especially when the user is not very experienced with this injection technique. The single joystick provides easy, intuitive movement control of the microcapillary in any direction (X, Y, Z), and each TransferMan 4r can store up to five independent positions (depending on the chosen application mask). By simply pressing the joystick key twice, the capillary can be moved to one of the next preprogrammed positions.

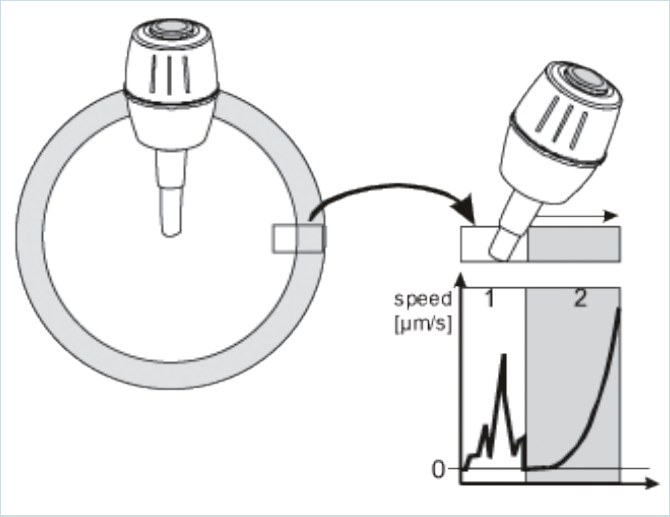

Figure 3: Principle of the DualSpeed joystick with direct (1) and dynamic deflection (2).

Furthermore, the DualSpeed joystick can be operated with two different movement modes. The standard mode is a direct, intuitive movement, a direct transfer of the hand movement to the microcapillary. When the maximum deflection of the actual path radius has been reached it is possible to press the joystick gently against its outer margin to activate the dynamic mode so that the needle proceeds straight in the desired direction with an accelerating speed as long as the joystick is deflected (see Figure 3). Using this DualSpeed joystick system, the needle can be moved carefully in the fine or extra fine (x-fine) speed mode for gentle manipulation of cells and embryos whilst still being capable of a considerable range of quick motion once the dynamic, outer zone of the joystick range is entered. By using this feature even cells which are located in the periphery of your working focus can be quickly reached and collected. The combination of both features, the user-definable application mask and the DualSpeed joystick, speeds up the entire work flow and therefore shortens the time of samples being held under the light beam of the microscope.

Preparation of cells, embryos and injection samples
The preparatory steps are of crucial importance but their detailed description would go beyond the scope of this Application Note. They will therefore not be described here, please proceed as described elsewhere (1, 6).

Many different organisms may be used for pronuclear and cytoplasmic injection, and there are also more and more laboratory models for the transfer of ES cells. Although the general procedure of both microinjection techniques is very similiar for the different species, this Application Note focuses on the generation of mouse models.

Preparation of the workstation for pronuclear (and cytoplasmic) injection

One TransferMan 4r micromanipulator is used for moving the holding capillary and the other is used for moving the injection capillary (Figure 4A).

A CellTram 4r Air microinjector in combination with a holding capillary, e.g. the VacuTip I, is used to hold the embryo. The programmable microinjector FemtoJet is used together with finely tapered microinjection capillaries to inject the nucleic acids.

The injection angle should be as flat as possible since the microcapillaries for pronuclear injection are usually not bent but straight. With the TransferMan 4r a very shallow injection angle can be chosen. Depending on the individual set-up (injection chamber, microscope stage) lower than 10° are possible. Furthermore the use of the Capillary Holder 4 Slim Shape (Order No. 5196 062.000) with its very narrow designed capillary bushing gives more space for a very flat angle adjustment (Figure 4B).

Figure 4: A) Workstation set-up for microinjection of nucleic acids into the zygote: TransferMan 4r, CellTram 4r Air and FemtoJet 4i in combination with Leica® DMi8 microscope. B) Shallow injection angle <10° possible using the TransferMan 4r set-up with the Capillary Holder Slim Shape.

There are different injection chambers available to serve as a microenvironment for the zygote during the injection process (e.g. plastic Petri dish with glass bottom, depression slide injection chamber or metal frame/glass slide injection chamber). In our lab, we use a homemade injection chamber according to our specification. One drop of M2 medium is placed in the chamber. The zygotes to be injected (10 to 20) are placed in one area of this drop. The whole droplet is covered with oil.

The FemtoJet offers an automatic (injection time ti is preset) and a manual injection mode. Using the latter the injection is triggered by a hand control or optionally by a foot control. For pronuclear as well as cytoplasmic injections we recommend using the continuous flow option because the pronuclei can differ in size and the pronuclear swelling for each zygote can be achieved by individual injection time. Furthermore, combined cytoplasmic and pronuclear injection e.g., of CRISPR/Cas9, TALEN or ZFN components can easily be achieved in one injection step when using the continuous flow option. Depending on the inner diameter of the injection capillaries, the basic settings for the compensation pressure (pc) and the injection pressure (pi) should be determined empirically. Both, holding and injection capillaries are to be fitted into the capillary holder and carefully aligned before injection.

Before the actual injection, the capillary should be cleared from any clogging by triggering the »Clean« function using either hand control or the clean function key of the FemtoJet.

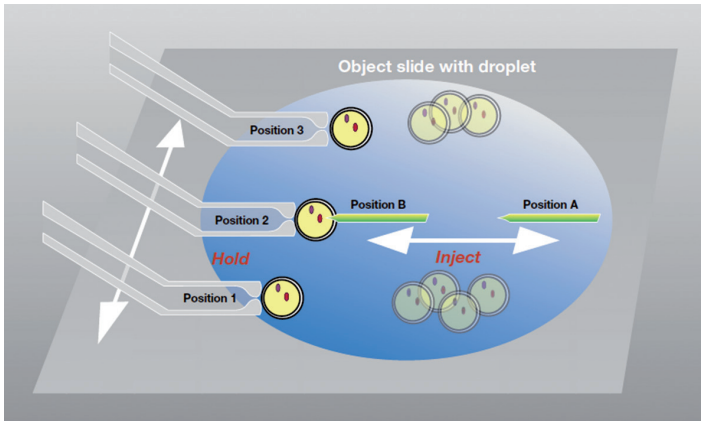

Figure 5: Storage of positions for nucleic acid microinjection into zygotes. For the holding capillary (left side) three different positions are stored for uptake, injection and collection of the zygotes. For the injection capillary two positions (injection and parking) are set.

To optimize the injection workflow under the microscope, we considered following procedure as the best (see Figure 5): On the holding side, the TransferMan 4r storage function »Position 1« is activated when the capillary has reached the area where the uninjected zygotes are placed within the M2 drop.

The CellTram 4r Air microinjector with mounted VacuTip I holding capillary can easily take up a single zygote which is then moved to a central position for injection of nucleic acids. This position is stored as »Position 2«. After injection, the zygote is transferred to another area of the M2 drop to collect the injected zygotes separately from the uninjected ones. This capillary position is set as »Position 3«.
On the injection side, »Position B« brings the injection capillary into focus close to the fixed zygote which is to be injected whereas »Position A« serves as the »parking position«.

Preparation of the workstation for ES cell transfer into blastocysts or morulae

For the transfer of ES cells into embryos two TransferMan 4r devices are used for controlling the holding and transfer capillaries (Figure 6).
The CellTram 4r Air used in combination with the VacuTip I holding capillary actually holds the blastocysts or morulae. The CellTram 4r Oil used in combination with the TransferTip ES capillary transfers the ES cells. If laser-assisted transfer is performed, blunt end tips (Piezo Drill Tip ES) can be used instead of spiked needles. The same applies for piezo-assisted transfer. Both, holding and transfer capillaries are fitted into the capillary holder. The transfer capillary is filled with mineral oil by turning the fine wheel of the CellTram 4r Oil prefilled with mineral oil. Both capillaries are carefully aligned at the bottom of the injection chamber. We use a homemade injection chamber according to our specification. One drop of M2 medium is placed in the chamber and up to 20 early embryos are transferred into one area of this drop.

Figure 6: Workstation set-up for ES cell transfer into blastocysts: two TransferMan 4r, CellTram 4r Air and CellTram 4r Oil in combination with Zeiss® Axio Vert.A1.

Additionally, a few hundred ES cells in medium are inserted into the injection chamber.

To optimize the workflow under the microscope we consider following procedure as the best (see Figure 7): On the holding side, »Position 1« is stored when the holding capillary has reached the area where the uninjected embryos are placed. The CellTram 4r Air microinjector with a mounted VacuTip I holding capillary can easily take up a single embryo which is then moved to a central position for injection of the ES cells. This capillary position is stored as »Position 2«. After injection the embryo is transferred to another area of the M2 drop to collect the injected embryos separately from the uninjected ones. This capillary position is set as »Position 3«.

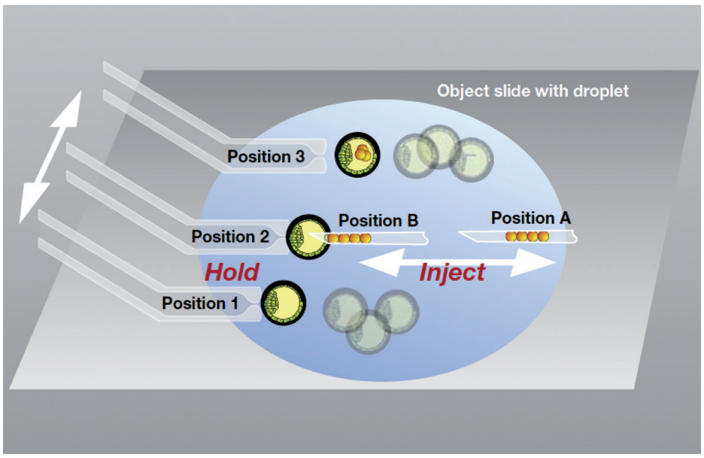

Figure 7: Storage of positions for ES cell transfer into blastocysts. For the holding capillary (left side) three different positions are stored for uptake, injection and collection of the embryos. For the injection capillary two positions (injection and cell collection) are set.

Under 20 x magnification and phase contrast the individual quality of ES cells can be judged and ES cells can be selected according to their size and shape. For blastocyst injection, 15 to 20 ES cells are taken up into the capillary together with a minimal amount of medium and then positioned near the opening of the tip. An embryo is brought into the injection position and firmly held to the holding pipette using the CellTram 4r Air. After positioning the blastocysts (the inner cell mass should be either at 6 or 12 o'clock position) the tip of the injection needle is aligned to the same focal plane as the equator of the blastocyst. By carefully touching the surface of the embryo with the tip, the right plane can be found. With a single, continuous movement the loaded injection capillary is pushed into the blastocyst cavity and the cells are slowly expelled into the cavity. It is crucial that no oil bubbles or lysed cells are inserted into the blastocyst. After injection, the capillary is slowly pulled out of the embryo.

For the injection of ES cells into 8-cell stage embryos (morulae) penetration of the microcapillary into of the embryo can be supported by perforating the zona pellucida using a laser. While the 8-cell stage embryo is being restrained by the holding capillary the laser shoots a slit into the zona pellucida with an irradiation time as short as possible in a region as far away as possible from any blastomeres.
Insert an injection capillary through the perforation in the zona pellucida and introduce approximately five to eight ES cells into the perivitelline space. Withdraw your needle carefully and release the embryos from the holding capillary.

Results and Discussion

Pronuclear injection
For microinjection of nucleic acids into mouse zygotes we experienced that it is very helpful to move the injection capillary with electric motor-driven systems with completely vibration-free motion. The exact injection movement ensures a minimized mortality rate. For this reason we use the Eppendorf TransferMan® NK 2 for routine injections.

When we tested the successor TransferMan 4r on several injection days and transgenic projects for pronuclear injection, we received the same injection results as when using the TransferMan NK 2 (see Table 1). Thanks to the DualSpeed joystick the movements on the holding side were much more flexible. After a short training period the dynamic movements using the outer ring of the joystick became a helpful tool for easy access to the cells in the main working area.

While using an additional 10° adapter for shallow injections with the TransferMan NK 2, with the TransferMan 4r we could easily adjust the angle for injection with a straight capillary to approximately 5° thus enabling us to perform ultra-shallow injections.

ES cell injection
The technique that has been used for the longest time in our facility is classical blastocyst injection, which results in chimeric animals with variable ES cell contribution.
When performing ES cell transfer, the injection movement needs a more dynamic and straight force. In daily routine we realize this with a hydraulic controlled system, which allows a direct transmission of movements from the hand over the joystick to the glass capillary. For ES cell injection projects with the TransferMan 4r we first tested the laser assisted 8-cell embryo injection and proceeded with blastocyst injection, manually as well as laser-assisted, in order to form an opinion on the overall performance of the manipulator.
It was a pleasant surprise when we discovered that although the TransferMan 4r is a motor driven system it works very dynamic and direct, comparable to a mechanical or hydraulic system. The new motors and joystick transmission allow short and impetus injection capillary movements. This is essential for efficient blastocyst injections. We could proceed with our routine without any constraints and could even speed up the workflow considerably using the dynamic movement mode. For logistic reasons we could not perform ES cell injection in parallel with our own system as we did for pronucleus injection, so a direct statistical comparison of the results is not possible. But when we examine the data obtained with our hydraulic system in a similar season and with similar ES cell strains the year before, we can conclude that using the TransferMan 4r all ES cell injections into 8-cell embryos as well as into blastocysts were successful and absolutely comparable to our hydraulic system.

Table 1: Results of pronuclear injection experiments performed during a time period of four weeks.

Manipulator	Zygotes injected	Lysis rate of injected zygotes	Injected zygotes transferred	Founder born
TransferMan NK 2	1024	23.4 %	784	5
TransferMan 4r	1335	18.4 %	1090	5

Summary

In summary we can conclude that the TransferMan 4r convinced us in every aspect. In transgenic routine, a working week is often divided into three pronuclear injections days and two ES cell transfer days. Using the TransferMan 4r it takes just a minimum of time to adjust the angle and switch to another application mask to change the whole set-up from a pronuclear injection to an ES cell transfer workstation. With the new motor and joystick concept, the TransferMan 4r is a fantastic option for an all-in-one solution especially for those laboratories with only one injection stage.

Literature

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Ordering Information

Description	Order no. international
TransferMan® 4r, Micromanipulator with DualSpeed™ joystick for direct and dynamic movement control	5193 000.012
FemtoJet® 4i, Programmable microinjector with internal pressure supply	5252 000.013
VacuTip I, holding capillary, 35° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.036
TransferTip® ES, Transfer capillary for ES cells, 20° tip angle, 15 µm inner diameter, sterile, set of 25	5195 000.079
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
Femtotip II, Injection capillary for aqueous solutions, 0.5 µm inner diameter, sterile, set of 20	5242 957.000
Microloader™, Pipette tip for back-filling microinjection capillaries, set of 2 x 96 pcs	5242 956.003
CellTram® 4r Air, Manual pneumatic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000.013
CellTram® 4r Oil, Manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000.030
Microscope Adapter, Adapter for micromanipulators, available for different inverse microscopes of major brands	Available upon request
Capillary holder 4 (slim shape), for mounting of flat injection angles	5196 062.000

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