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APPLICATION NOTE No. 009

Intracytoplasmic Sperm Injection (ICSI) with the Eppendorf TransferMan® 4m and CellTram® 4m

Verena Nordhoff, Centre of Reproductive Medicine and Andrology, University Hospital of Münster, Germany

Abstract

Assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are used worldwide to overcome infertility problems. In particular ICSI has become the tool of choice for the »treatment« of severe male infertility. For the implementation of this technique, advanced micromanipulation equipment attached to inverted microscopes is essential. This Application Note describes the microinjection procedure using the Eppendorf micromanipulation system fitted with the TransferMan 4m and microinjectors CellTram 4m.

Introduction

Since their first use in 1988, micromanipulation techniques to assist fertilization (1) have steadily evolved, so that today, the technique known as intracytoplasmic sperm injection (ICSI) (2) is the most valuable tool for treating the infertile couple, particularly those with male factor patients. Circumventing many of the limitations of »traditional« *in vitro* fertilization (IVF), ICSI has raised the hopes that these couples may have a child of their own and as a consequence has become the preferred method of treatment in assisted reproduction. As with IVF, the initial steps of ICSI consist of the retrieval of oocytes via follicle aspiration and the removal of the cumulus and the corona radiata cells by hyaluronidase treatment. In parallel, spermatozoa are prepared using techniques such as swim-up or density gradients, the procedures recommended by the World Health Organization™ (WHO) in their manual for the examination and processing of human semen (3). Subsequently, a single spermatozoon is selected and injected into an oocyte through a thin glass capillary (injection pipette). If fertilization occurs, the resulting embryo is transferred into the uterus 2 to 5 days after microinjection.

Fig. 1: Intracytoplasmic sperm injection (ICSI) (Picture from Centre of Reproductive Medicine and Andrology, IVF laboratory, Münster, Germany).

With the delicate manipulations needed for ICSI and the handling of highly valuable cells in mind, Eppendorf developed the TransferMan 4m micromanipulator (see Figure 2). This application note describes the ICSI technique using the Eppendorf micromanipulator and -injector setup, which enabled us to simply and speed up our workflow in the routine.

Fig. 2: Workstation for ICSI with Eppendorf micromanipulator TransferMan 4m, CellTram 4m Air and CellTram 4m Oil.

Materials and Methods

Devices

> Inverted microscope equipped with Modulation Contrast or Differential Interference Contrast (DIC), equipped with 10 x, 20 x and 40 x objectives
> Two TransferMan 4m micromanipulators (one for moving the holding capillary and another for collecting and transferring the spermatozoa)
> Adapter for inverted microscope
> CellTram 4m Air microinjector for holding the oocyte
> CellTram 4m Oil microinjector for transferring the sperm

Consumables and media

> Light mineral oil, embryo tested (e.g., M-8410 (Sigma-Aldrich® or others)
> Shallow cell culture dishes, tissue-culture-grade (e.g., no. 353655 Dish 50mm IVF Low Wall (BD Falcon®) or others)
> Holding capillary, for holding of oocytes (recommended: 15 µm inner diameter, 100 µm outer diameter, 35° tip angle)
> ICSI transfer capillary, for collecting and injecting sperms (recommended: 4 µm inner diameter, 35° tip angle)
> Culture media (HEPES-buffered, supplemented with antibiotics, protein and pyruvate)
> PVP (polyvinylpyrrolidone) or equivalent formulations

Microinjection dish preparation

It is essential to heat all media and the oil to 37 °C prior to use. For ICSI, several droplets of medium (5 µL to 25 µL) are placed in the center of the cell culture dish. Droplets intended for retrieved spermatozoa are supplemented with PVP before the addition of the sample. Additional droplets containing PVP only might be necessary for storage of selected spermatozoa before injection. All droplets are completely covered with light mineral oil to maintain their stability as well as temperature and pH. Once prepared, the microinjection dish can be placed into the incubator until use.

Materials and Methods

Set up of the micromanipulator workstation
With the TranferMan 4m micromanipulator it is possible to permanently select the application mask >>ICSI<< with optimized function keys for the application injection of spermatozoa (see Figure 3).
The ICSI mask offers 2 position storages (Pos 1 and Pos 2), the 'Y off' function, facilitating precise injection movement only in X-direction, and the 'Z-limit' function preventing that the capillary can be moved too deep breaking at the bottom of the dish.
The TransferMan 4m manipulators of the holding and injection side are both equipped with capillary holders of the injectors CellTram 4m.

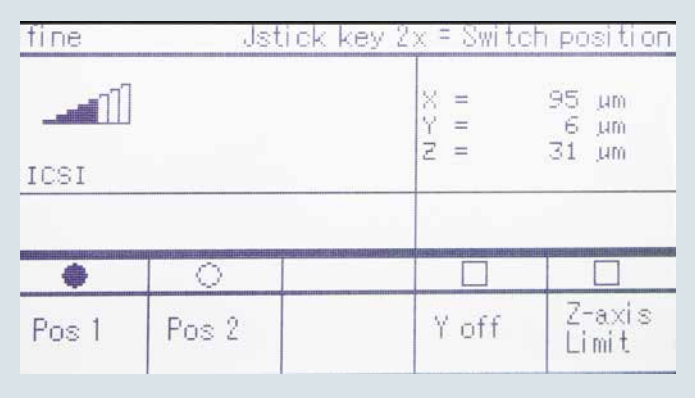

Fig. 3: Display of the TransferMan 4m control panel: ICSI application mask is selected. The central softkey is freely definable e.g, with Pos 3, Clean or other functions.

Preparation of the microinjection capillaries
Both microcapillaries need to be positioned into the holding system of the CellTram 4m microinjectors, then aligned and equilibrated prior to the ICSI procedure being started. As the holding capillary has a greater diameter than the injection pipette, it can be used as a guide for the positioning and equilibration steps.

First, the microcapillaries are integrated into the capillary holder, which is connected to the microinjector via a tube. For holding of oocytes an air-based microinjector is often preferred (e.g., CellTram 4m Air). For the injection side, many users prefer an oil-based microinjector, as it allows highly precise control of the sample to be injected.

When working with oil-filled systems (e.g., CellTram 4m Oil), it is essential to ensure that absolutely no air bubbles are in the system. The CellTram 4m Oil with its new oil filling system allows for simple oil re-filling without air bubble formation or spill-over of oil. The capillaries are gently pushed past the sealing rings inside the tool holder up to the noticable capillary stop allowing a reproducible insertion of the capillary. Then the universal capillary holder itself is added to the angle head of the TransferMan 4m and the alignment checked. The individual injection angle can be adjusted independently via the knurled screw and the angle mark on the angle head (Figure 4).

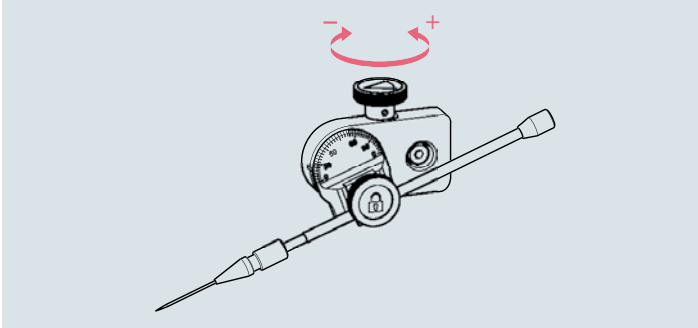

Fig. 4: Adjustment of the injection angle according to the angle of the capillary tip.

To align the capillary in the vertical position, the capillary holder can be rotated, even when the pipette is tightly gripped in place. Both pipettes must be aligned straight in the field of view. Alignment in the horizontal plane has to be done with great care, in particular the following points must be taken into account: the holding capillary must be aligned without tilt, as it needs to lie flat on the bottom of the dish in order for the aspiration of the oocyte to be conducted in a controlled manner. In contrast, the injection capillary needs to tilt slightly downwards so that the tail of the spermatozoon can be broken properly.

It is also necessary to prime microcapillaries with medium before use so that the manipulated gametes never come into contact with air or oil. Usually, this equilibration is achieved using ICSI media.

Storing of positions

Using the ICSI application mask it is possible to store up to three positions of which two softkeys are already reserved (Pos 1 and Pos 2) and the third is individually programmable (Pos 3). The capillary can be moved easily in any direction (X-/Y-/Z-axis) by means of the joystick. By pressing the joystick key twice (double-click) the capillary can be returned to preset positions needed during the ICSI procedure, namely »parking« and »working« (Figure 5).

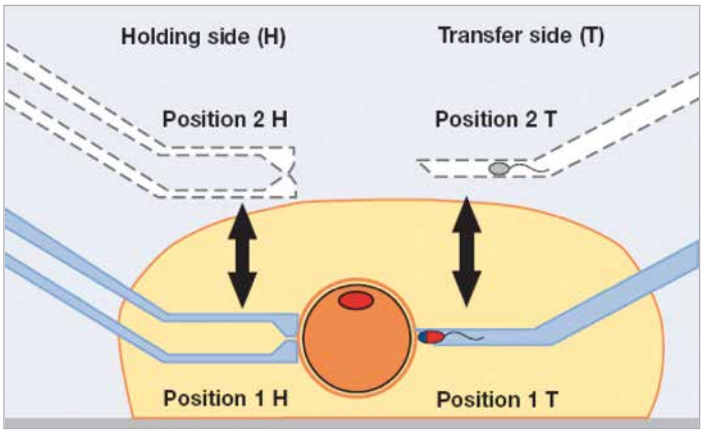

Fig. 5: Storing of positions within and above the injection droplet (schematic representation).

The »working« position is chosen in the focal plane of the holding side (position 1 H) as well as the injection side (position 1 T). The transfer capillary and the holding capillary are directed in the focal plane, and the positions stored as Pos 1 on both micromanipulators, respectively. As the name suggests, the »parking« position is one where both capillaries can be placed slightly above the droplet so that they do not interfere with the gametes as the dish is moved around the stage. The positions in the overlay medium are defined as Pos 2 (see Figure 5: positions 2 H and 2 T).

Selection of spermatozoa using the DualSpeed™ joystick

The DualSpeed joystick of the TransferMan 4m has the advantage that it does not need to be re-positioned by declutching if the maximum displacement of the actual path radius has been reached. Instead, it is possible to press the joystick gently against its outer margin and after a short transition phase the dynamic mode is activated and the needle proceeds in the desired direction. The speed of the dynamic movement can be adjusted in relation to the proportional movement. Using this feature, the needle can be moved carefully in the fine or extra fine (x-fine) speed mode whilst still capable of a considerable range of quick motion once the dynamic, outer zone of the joystick is entered (Figure 6).

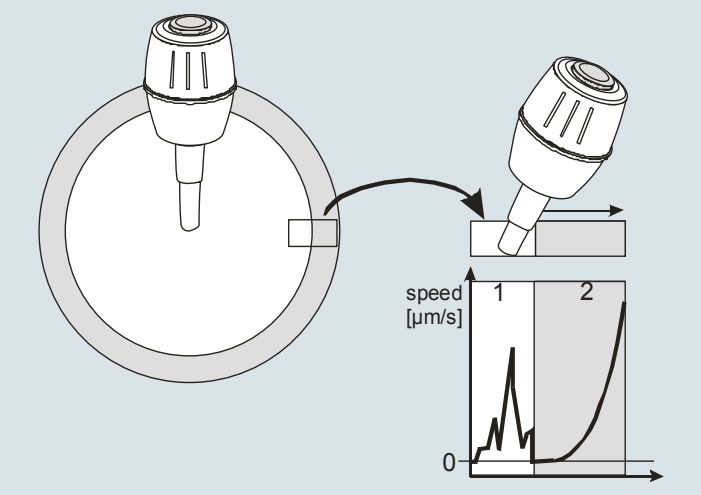

Fig. 6: DualSpeed joystick with proportional (1) and dynamic deflection (2).

Microinjection

A reasonable amount of the sperm sample is loaded into a drop pre-filled with PVP, whilst oocytes are placed into the designated medium drops. The joystick key is pressed twice to lower the ICSI transfer capillary to position 1 T and under 200x to 400x magnification a spermatozoon is selected and immobilized either by »rolling« the ICSI transfer capillary over the tail or by gently pressing the tail against the bottom of the dish.

The spermatozoon is aspirated, tail-first, into the ICSI transfer capillary as gently as possible by rotating the knob of the CellTram 4m Oil. The joystick key is then pressed twice to move the transfer capillary containing the spermatozoon up into the overlay medium (i.e. position 2 T). The cell culture dish is moved to one of the drops containing an oocyte and the cell brought into focus. The joystick key of the other TransferMan 4m is pressed to move the holding capillary from position 2 H to position 1 H. The oocyte is attached gently but firmly to the holding capillary by the negative pressure created by the CellTram 4m Air device. Injection of the oocyte is normally undertaken with the first polar body being positioned at either 6 o'clock or 12 o'clock. To achieve this orientation it may be necessary to turn the oocyte, this can be done with the aid of the ICSI transfer capillary which is lowered again to position 1 T and slightly varying the negative pressure of the CellTram 4m Air until the polar body has reached the desired position.

The injection capillary is now focused in the same plane as the maximal diameter of the oocyte becomes evident. By rotating the knob of the CellTram 4m Oil, the spermatozoon is gently moved along the capillary until it is positioned at the very tip (Figure 7A). By slight moving of the joystick the transfer capillary is then carefully pushed through the zona pellucida (Figure 7B) and subsequently through the oolemma into the ooplasm at the 3 o'clock position. The oocyte should be pricked in the middle so that the oolemma membrane is gently and atraumatically broken. To ensure that this has occurred, a small amount of ooplasm is gently aspirated into the injection capillary as a sign of membrane rupture (Figure 7C). The aspirated ooplasm and the spermatozoon are then deposited towards the center of the oocyte (Figure 7D).

In order to minimize the volume of medium and PVP introduced into the cytoplasm, the transfer capillary is gently withdrawn after the head of the sperm cell has left the capillary tip. Once this has been completed, the injected oocyte is released from the holding capillary and both capillaries are returned to position 2 by pressing the joystick key twice.

If several oocytes are to be injected, only 3 to 6 oocytes should be placed in the cell culture dish at the same time to avoid stress to the oocytes (e.g. temperature and pH changes). Once the injection procedure is completed, the oocytes are placed into an appropriate culture medium and incubated overnight.

Assessment of fertilization and embryo transfer
Approximately 15 to 18 hours after microinjection, the oocytes are assessed for fertilization. Normally fertilized oocytes should contain two pronuclei and two polar bodies. Embryo transfer into the uterus is performed 2 to 5 days after microinjection.

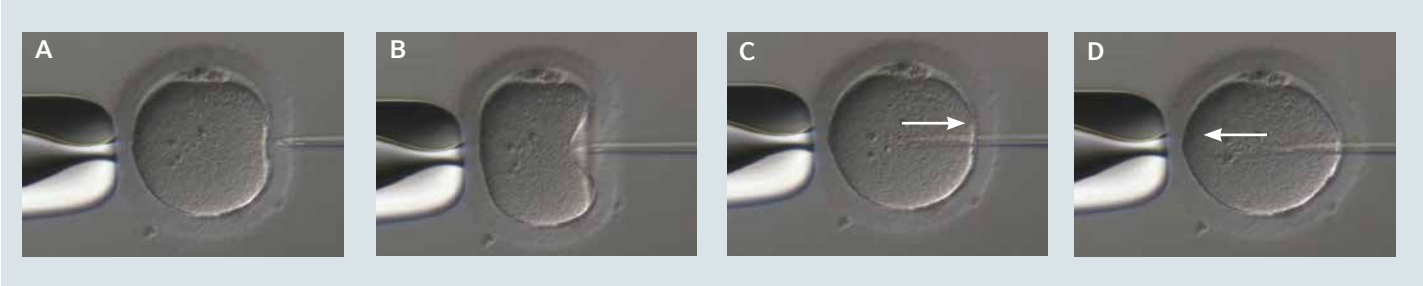

Fig. 7: ICSI procedure. A) Attach the oocyte at the holding capillary by gentle suction. Polar bodies should be either positioned at 12 o'clock (as shown) or at 6' clock. The injection capillary with spermatozoon should be at 3 o'clock position. B) Push the capillary through the zona pellucida, C) aspirate ooplasm (arrow), D) and push out the ooplasm together with the spermatozoon into the center of the oocyte (arrow).
(Pictures from the Centre of Reproductive Medicine and Andrology, IVF laboratory, Münster, Germany).

Discussion

ICSI is a stressful procedure for an oocyte and thus the setting of this technique should minimize any physical stress the oocyte is exposed to. The micromanipulator TransferMan 4m presents several special features including application specific "masks" which facilitate and ease the individual workflow process. Use of the application mask "ICSI" allows for the saving of two positions (in and above the medium droplet), plus the setting of a vertical limit and thus the avoidance of capillary breakage. The unique DualSpeed joystick enables precise and intuitive movement during injection in all three dimensions as well as dynamic movements while "catching" spermatozoa. In addition, the microinjector CellTram 4m Oil allows a very gentle and controlled uptake as well as a precise release of the sperms operating its fine and coarse rotary knobs. Consequently, compared to its predecessor TransferMan NK 2, the total time needed for ICSI is lower when using the Eppendorf system TransferMan 4m & CellTram 4m. This is of great value as the timeframe in which oocytes might be exposed to unfavorable external variances is shortened.

Literature

[1] Lanzendorf SE, Maloney MK, Veeck LL, Slusser J, Hodgen GD, Rosenwaks Z. A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil Steril* 1988 May; 49(5):835–42.
[2] Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992 Jul 4; 340(8810):17–8.
[3] WHO. (2010) Laboratory Manual for the Examination and Processing of Human Semen, 5th edn. Cambridge University Press, Cambridge, UK.

Ordering Information

Description	Order no. International
TransferMan® 4m[1], Micromanipulator with DualSpeed™ joystick for direct and dynamic movement control	5191 000 015
Microscope adapter, Adapter for micromanipulators, available for different inverse microscopes of major brands	Available upon request
CellTram® 4m Air[1], Manual pneumatic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000 021
CellTram® 4m Oil[1], Manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection	5196 000 048
Antivibration Pad, Pads in different weight ranges available, for protecting micromanipulation workstations from external vibrations	Available upon request

[1]This product is registered in Europe as a medical device (according to Medical Device Directive MDD 93/42/EEC).
For availability in your country, please contact your local sales organization.

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

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