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

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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.

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 system includes a number of 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, 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. As a consequence, compared to its predecessor the TransferMan NK 2 or other ICSI systems, the total time needed for ICSI is lower when using the Eppendorf TransferMan 4m system. This is of great value as the time period in which oocytes might be exposed to unfavorable external variances is shortened.

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

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® Air microinjector for holding the oocyte
- CellTram vario 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)
- VacuTip holding capillaries (Eppendorf) or others, e.g. Gynemed, Nr. 001-100-30
- TransferTip® (ICSI) injection capillaries (Eppendorf) or others. e.g. Origio® (MIC-50-30)
- 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 a Petri 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.
First, the micropipettes are integrated into the universal capillary holder, which is connected to the microinjector via a tube. When working with oil-filled systems (e.g., CellTram vario), it is essential to ensure that absolutely no air bubbles are in the system. The capillaries are gently pushed past the sealing rings inside the tool holder. 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).

To align the capillary in the vertical position, the universal 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 pipette 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 pipette needs to tilt slightly downwards so that the tail of the spermatozoon can be broken properly.

It is also necessary to prime micropipettes 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 other 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).

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 (2 T and 2 H).

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).

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 TransferTip (ICSI) capillary to position 1 T and under 200x to 400x magnification a spermatozoon is selected and immobilized either by »rolling« the TransferTip (ICSI) capillary over the tail or by gently pressing the tail against the bottom of the dish.

The spermatozoon is aspirated, tail-first, into the TransferTip (ICSI) capillary as gently as possible by rotating the knob of the CellTram vario. 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 Petri 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 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 TransferTip (ICSI) capillary which is lowered again to position 1 T and slightly varying the negative pressure of the CellTram 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 vario, 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 pipette 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 Petri 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.

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**Fig. 7:** ICSI procedure. A) Position the injection needle with spermatozoon at the 3 o’clock position, B) push the needle through the zona pellucida, C) aspirate cytoplasm (arrow), D) and push out the cytoplasm together with the spermatozoon into middle of the oocyte (arrow). (Pictures from the Centre of Reproductive Medicine and Andrology, IVF laboratory, Münster, Germany).
Literature


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<tr>
<th>Description</th>
<th>Order no. international</th>
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<tbody>
<tr>
<td>TransferMan® 4m1, Proportional micromanipulator for suspension cells</td>
<td>5193 000.012</td>
<td>5193 000.020</td>
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<tr>
<td>CellTram® Air1, Manual pressure device for the reliable holding of suspended cells</td>
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<td>CellTram® vario1, Manual hydraulic microinjector, with gears 1:1 and 1:10</td>
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<td>VacuTip1,2, 25 glass capillaries for holding large cells (e.g. oocytes) sterilized, tip angle 35°</td>
<td>5175 108.000</td>
<td>930001015</td>
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<tr>
<td>TransferTip® RPI(ICSII)1,2, Injecting microcapillary for injecting sperm into oocytes (ICSI), rigid, parallel flange, sterile, pack of 25</td>
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<td>930001074</td>
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1 This product is registered in Europe as a medical device (according to Medical Device Directive MDD 93/42/EEC).

2 Proven non-cytotoxicity by the mouse embryo development test.