Dear sales partner

Recently the first edition of the "Application Letter", the new forum of the Market Segment Team, was published.

Our aim with the Application Letter is to inform you about important, developing applications and market potential and to provide you with more background information.

We are now pleased to present the second Application Letter treating the field of transgenic application. Transgenic animals are often used as model systems for basic research investigations like gene regulation and gene expression. But also in biotechnology and pharmacology these organisms are produced in a growing number to study pathways of diseases and the effects of drugs and medicaments.

We hope that you will enjoy this second edition of the Application Letters. Please do not hesitate to send us your contributions and any suggestions for improvement. The easiest way is to use the questionnaire at the end of this Letter.

Best regards
The Market Segment Team

In general the technique of microinjection is used to introduce a dissolved substance into a living cell. The equipment that the investigator needs is an inverted microscope with microinjection devices e.g. Leica mechanical micromanipulator, electrical or hydraulic micromanipulators.

There are two different kinds of microinjection:

1) Microinjection into adherent cells (attached to the bottom of a coverslip within a petri dish)
2) Microinjection into non-adherent cells like oocytes (see below). These oocytes have to be fixed with a holding capillary for injection

The second method is described in the chapter on Transgenics and ICSI.

Before that, some more general information:
Microinjection itself is carried out with a fine pulled-out glass-capillary (inner diameter of the tip: between 0.2 µm and 1 µm). The capillaries are sold by e.g. Eppendorf or the user produces them by himself with a pipette puller (e.g. Sutter). The pipette is filled with the substance of interest and around 10% of the cell volume is transferred to the pierced cell.

Typical specimens for microinjection are DNA, RNA, proteins, antibodies, dyes, fluorescent markers or oligonucleotides.

A problem with microinjection is the potential blockage of the small diameter of the capillary by non-dissolved particles or drying out of the liquid inside the tip. To avoid that, the substance should be centrifuged before filling the capillary and the pipette should always remain in the culture medium of the cells.

Outlook: "Tissue engineering and somatic gene therapy" - biotechnology
A new and fast growing market for cell culture applications and micromanipulation of cells is the application of tissue production. This means in vitro production of specialized cells such as skin in cell culture. The skin cells grown in a petri dish can be used as a substitute for damaged human skin of victims of burning, for example.

In future, it will be possible to manipulate cells prior to their transplantation into diseased humans. Introduction of genetic information into diseased cells can compensate a deficiency, for instance the deficiency of insulin production in pancreatic cells (diabetes). Only a few weeks ago scientists were able to stimulate stem cells to differentiate into pancreatic cells which produce insulin.

Fig. 1: Microinjection into the huge nucleus of the unicellular green alga Acetabularia acetabulum
What is a transgenic animal?
In the last decade the development of DNA technology has enabled scientists to identify, isolate and find single genes that carry the key to specific cell characteristics. This isolated genetic material is now readily analyzed, modified and replicated.

Modified and replicated genes can be introduced into the reproductive cell genomes, so that the changed or new genetic material is transmitted to offspring. Animals that carry these introduced genes that now express the new trait are known as "transgenic animals."

Transgenic animals are among the most useful and exciting research tools in biological science. The first successful production of transgenic mice using microinjection was reported in 1980. Since then, transgenic animals have become a research tool in many disciplines.

How is a transgenic animal produced?
Basically, two different techniques are used:

1) Microinjection of linear DNA into the pronucleus

2) Transfer of embryonic stem cells into blastocysts

Pronuclear microinjection
Fertilized mouse eggs (one-celled embryos in the pronuclear stage) are used as the start material for this technique. Linear DNA sequences are transferred into one of the two pronuclei using microinjection. This genetic material is incorporated into one of the embryonic chromosomes.

It is very important that the DNA sequence is built into the chromosome prior to the doubling of the genetic material that precedes the first cleavage. This allows foreign DNA to be copied into each of the cells of the animal born from this fertilized egg.

If this is not done correctly, only a few cells will incorporate the genetic material (gene).

This is the reason why the genetic material is introduced in the fertilized egg at the earliest stage. The number of foreign gene copies integrated is not controllable.

The best moment for genetic material transfer is the so-called pronuclear stage. For several hours after fertilization the male and female pronuclei are individually visible with traditional light microscopy.

After this period the pronuclei are fused and the cell forms the so-called zygote which incorporates the genetic material from both the male and the female pronucleus. During the pronuclear stage the foreign...
genetic material can be injected into one of the pronuclei. Usually, the male pronucleus is injected with the foreign genetic material as the male pronucleus is larger than the female one and it is also closer to the oocyte surface. The injection is carried out with a straight injection capillary at an angle of around 10°.

After successful transfer of the genetic material, the oocytes are transferred (surgically) into the uterus of a pseudo-pregnant recipient animal. After birth the offspring is screened (e.g. with PCR, Southern Blots or other techniques) for control of successful integration of the foreign genetic material (gene).

Embryonic stem cell transfer

Embryonic stem cells are used as start material for this technique. Embryonic stem cells (ES) are collected from the inner cell mass of blastocysts (early stage (mouse) embryos). These embryonic stem cells are non differentiated (pluripotent) cells which can still develop (differentiate) into any type of tissue.

Therefore, isolated embryonic stem cells can be placed back into another blastocyst (ES transfer). After the transfer, they will develop, divide and become part of the embryo.

Before the transfer foreign genetic material is introduced into the embryonic stem cell.

This can be done by:

1) Transfection is a technology where the foreign DNA is transferred from the surrounding substrate of the cell into the cell nucleus. The foreign DNA is incorporated in the chromosomes of the cell.

2) Infection is transfer of foreign DNA by viruses into a cell where it is incorporated in the chromosomes of the cell.

3) Electroporation is a method where the cell is opened for a short time with the use of a strong electrical pulse. The DNA from the substrate will enter the cell through pores (small openings) in the cellular membrane. This transferred material is incorporated in the chromosomes of the cell.

Transferred DNA material often also encodes for resistance against certain antibiotics. The genetically modified cells are cultured in a medium containing this antibiotic.

Only the cells that survive will have the foreign DNA built-in at the right position for expression. Other selection methods are based on molecular biology techniques. These selected embryonic stem cells are cultured further to generate enough material for the later embryonic stem cell transfer.

During stem cell transfer the embryonic stem cells are sucked into the injection pipette. A number of the embryonic stem cells are transferred into a blastocyst of a 3.5 day-old embryo. The injected cell will afterwards become part of the embryo and differentiate into various types of tissue. The injected blastocysts are transferred (surgically)
into the uterus of a pseudopregnant recipient animal. After birth the offspring is screened (e.g. with PCR, Southern Blots or other techniques) for control of successful integration of the foreign genetic material (gene).

The result is a mouse with only certain organs and functions genetically modified. Ideally the germ cells of the mouse are also genetically modified so that “normal” reproduction can be used.

Transfection

Different methods for introduction of foreign DNA into eukaryotic cells (transfection).

Fig. 6: Blastocyst stage embryo during injection

Fig. 7: Embryonic stem cell transfer. ES are injected into blastocyst.

Fig. 8: Flow chart of transfer of embryonic stem cells into blastocysts. Transfected ES are microinjected into the cavity of blastocysts. They become incorporated into the inner cell mass of host blastocysts. Several such blastocysts are introduced into pseudopregnant mouse. Screening of newborn mice for desired properties.
The Leica Solution

Leica mechanical micromanipulators (Fig. 9) are well known, widely used and very popular in the field of microinjection. Users who work with the "Leitz manipulators" say that these manipulators handle well and enable swift and precise operation.

The new "mouse" system Leica AS TP (Fig. 10) is a cooperation between Eppendorf and Leica, two main players in the field of microinjection. The result of this consolidation of experience is a system which facilitates handling during the production of transgenic animals. One control element enables the operation and supervision of microscope functions as well as manipulator functions. The motorized and automated Leica DM IRE2, the new Eppendorf micromanipulator generation Transferman NK2 and the innovative control element offer enhanced speed and convenience for complex applications.

Some of the images and textual input were provided by Kristina Vintersten, EMBL Heidelberg, Germany (5,6) and Eppendorf/Hamburg (2,3,7,8). Special thanks to Prof. M. Okabe, Osaka University, Japan for the permission to publish his famous green mice (4).

ICSI

(Intra Cytoplasmatic Sperm Injection)

The methods of In Vitro Fertilization (IVF) are considered to be successful techniques in reproductive medicine. Since infertility has increased in recent years, intracytoplasmatic sperm injection is gaining importance in assisted reproduction, overcoming the problems of tubal sterility or male subfertility to a wide extent. At least this technique contributes to increasing the hopes of infertile couples of having children of their own.

The principle

The function principle is to actively inject a sperm (after defined preparation procedures) into an oocyte which has been obtained by means of follicle aspiration. The injection is carried out with a thin glass pipette under microscope control. If fertilization is identified successfully, the embryo is transferred into the uterus where further development can take place in the natural way.

Instrumental outfit

For ICSI an inverted microscope like the Leica DM IRBE/DM IRE2 is used with 10x, 20x and 40x objectives provided with a suitable contrasting technique such as LMC (Leica Modulation Contrast) or Differential Interference Contrast.
(DIC). The microscope has to be equipped with two micromanipulators, one for a “holding” pipette that keeps the oocyte in place and one for the "injection" pipette which injects the sperm cell.

The manipulators can be mechanical, electrical or hydraulic types. Often a combination is used.

Both pipettes need an "injection" device that controls the positive or negative pressure.

The method

Sperms and ova are put onto a petri dish in several drops of an overlay medium. With 200x magnification (20x objective) the sperm cell is immobilized with the "injection" pipette by pressing the tail of the sperm against the bottom of the petri dish until it stops moving. This step needs some practice so as not to break the pipette which is very thin and fragile at the tip (the inner diameter should be around 4-5µm).

After demobilization, the sperm cell is aspirated into the "injection" pipette by adding slightly negative pressure to the injection device. The aspiration should catch the sperm from the tail end as flat as possible. The injection pipette containing the sperm is now moved to the overlay medium containing the oocyte.

Changing to 100x magnification (10x objective) the oocyte is focused and held in place with the holding pipette (inner diameter approx. 20 µm) and slight negative pressure on the injection device on the holding side.

With the aid of the "injection" pipette the oocyte has now to be brought into a position where the polar body of the cell is pointing either to the top or to the bottom of the field of view. For this step the pressure on the holding side has to be varied slightly to support the positioning of the ovum.

The oocyte and the "injection" pipette are now focused at 400x magnification (40x objective).

The tip of the injection pipette should point to the middle of the side of the oocyte. By adding a little positive pressure to the injection pipette, the sperm is brought to the tip of the capillary.

The system is now ready for the injection process.

The tip of the injection pipette is now carefully pushed through the outer layers of the oocyte, the zona pelucida and the oolemma. The perforation of these membranes has to be done very gently and with minimal damage. To support an atraumatic perforation, the tip can be pressed against the outer membranes of the oocyte and negative pressure added to the injection pipette until the membrane perforates.

When the tip has been successfully placed into the ooplasm inside the ovum, the sperm cell is injected. After the sperm has left the capillary, the pipette is gently withdrawn and the oocyte is released from the holding pipette.

References (Transgenics):


References (ICS1):


Useful web links:
http://www.embl-heidelberg.de/ ExternalInfo/transgenicService/ Beginning%20frame.html