Microinjection of fertilized oocytes to produce transgenic animals

Annette Hammes
Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13092 Berlin-Buch, Germany.

Andreas Schedl
Institute of Human Genetics, International Centre for Life, Central Parkway, Newcastle upon Tyne, NE1 3BZ, U.K.

Equipment and reagents

Injection set up:

- vibration-damped table (e.g. Series 63; Spindler & Hoyer GmbH)
- Microscopes with inverted optics (e.g. Zeiss Axiovert or Leica DM IRB)

  The microscopes should be equipped with 10x eyepieces, a 32x or 40x objective and a 5x or 10x objective.
- Micromanipulators:

  mechanical micromanipulators (e.g. from Leitz, Zeiss-Jena or Narashige)

  electronically controlled micromanipulator (e.g. Eppendorf PatchMan/Micromanipulator)
- De Fonbrune microforge (Micro Instruments)
- micro-needle puller:

  vertical micro-needle puller (e.g. needle puller model 750, Kopf TM Instruments) or horizontal micro-needle puller (e.g. Sutter P-97, Sutter instruments)
- holding pipettes:

  borosilicate glass tubing without an internal filament: length=10 cm , outer diameter=1.0 mm, inner diameter=0.75 mm, s=0.125 mm (e.g from W-P Instruments TW-150)
- injection needles:

  thin walled borosilicate glass tubing, with a thin internal filament: length 12 cm, outer diameter 1.0 mm, inner diamter 0.58 mm, filament diameter=0.133 mm (e.g from W-P Instruments TW-150)
- injector:

  50 ml Syringe or Eppendorf transjector
- Microloader tips (e.g. from Eppendorf)
- Embryo-tested paraffin oil (Sigma)
- Depression slides (e.g. from Roth)
- M2 medium (e.g. from Sigma)[a]
- M16 medium (e.g. from Sigma)
- CO_2 incubator

- Embryos from Superovulation and isolation of fertilized oocytes for microinjection to produce transgenic mice
- DNA for injection from Isolation of linearized plasmid DNA constructs for microinjection to produce transgenic animals, Isolation of YAC DNA for microinjection to produce transgenic animals or Isolation of linearized BAC DNA for microinjection to produce transgenic animals.

Method

1. Prepare the holding pipette: A good holding pipette is essential for efficient injections. Holding pipettes can be pulled either by hand or using a micro-needle puller (e.g., Sutter P-97). Use a 10-15 cm long borosilicate glass tubing without an internal filament (e.g W-P Instruments TW-150, length = 10 cm , outer diameter = 1.0 mm, inner diameter = 0.75 mm, s = 0.125 mm). Hold the centre of the tubing above a small gas flame until it is soft. Remove the tube from the flame and at the same time pull strongly at both ends. Ideally, the outer diameter of the holding pipette is not much bigger than the oocyte itself (80-100 µm), and certainly not bigger than 150 µm. The glass capillary is marked with a diamond pen about 1.5 cm below the neck and broken by flicking the glass tube. Control the end under a stereomicroscope. Discard all needles with a ragged end, as only a blunt and clean break will lead to a good holding pipette. Clamp the needle into a De Fonbrune microforge and position the needle just above the heating filament. Turn on the heating until the filament is bright red. Move the holding needle closer to the heat until you see the end melting. Keep it in this position until the inner diameter of the opening is about 1/3 of an oocyte (20 µm). For controlling the oocyte through the holding pipette several methods have been employed. The commercially available AirTram system (Eppendorf) provides excellent control over the oocytes.

2. Prepare the microinjection needles: Microinjection needles are prepared on a puller. The tip of the microinjection needle should be so fine that you cannot see the opening under a light microscope. It is necessary to vary different settings of the microinjection-needle puller such as heating, pull force, etc. to obtain tips with an optimal shape. Some settings will produce needles with a fused end. In this case you can choose a different setting or break the needle by touching carefully the holding pipette (under the microscope). The size of the opening will depend also on the construct that is injected. A standard plasmid construct requires a diameter of less than 1 µm. For larger constructs such as YACs or BACs we suggest to use a bigger opening. We usually obtain this by breaking one of the standard injection needles on the holding pipette. The opening should be big enough so that you can just see it under the 400x magnification. The larger opening not only reduces the risk of shearing the DNA during the injection process, but also allows an easier cleaning of the injection needle in case it is blocked up.

3. Start the microinjection as soon as the pronuclei of the oocytes are clearly visible.

4 Prepare a flat drop of about 40 μl of M2 medium (about 0.8 cm diameter) on a depression slide and overlay it with paraffin oil (embryo-tested).

5 Transfer 10–20 fertilized oocytes into the medium and mount the slide onto the microscope stage. The oocytes should be kept no longer than 30 min outside the incubator. If they start to show any sign of shrinking, transfer them back to the M16 micro-drop culture and place them into the CO_2 incubator (5% CO_2 and 37°C) for recovery. They can be injected at a later stage.

6 Using an Eppendorf microloader transfer about 1–2 μl of DNA into the back of the injection needle and insert it into the needle holder. Try to avoid introducing air bubbles into the needle. The capillary force will pull the liquid to the tip of the needle.

7 Slowly lower the injection needle and the holding pipette to the plane of the oocytes, taking care not to break them at the base of the depression slide.

8 For efficient injections we suggest that a routine should be developed for arranging the oocytes on the injection slide. Uninjected eggs can be kept at the top, injected eggs at the bottom and lysed or unfertilized eggs at the left of the depression slide. Initially, you will find it necessary to switch from high to lower magnification when moving the oocytes around. However, after some practice you will get used to the distances between the three locations of oocytes and you may want to try moving the stage under the oocyte instead of moving the holding pipette. This routine increases the speed and also reduces the risk of accidentally breaking the injection needle with your holding pipette.

9 Under low-power magnification (10× objective) pick up a fertilized oocyte by applying suction to the holding needle and return the needle to the centre of the field of vision (alternatively move the stage to position the oocytes next to the holding pipette).

10 Switch to high-power magnification. Position the embryo in such a way that the male pronucleus (usually the bigger one containing only a single nucleolus) is in the equatorial plane of the oocyte and relatively close to the injection needle. If the pronucleus is not in the equatorial plane the injections will be difficult since the oocyte will turn away when you try to enter with the injection needle.

11 Slowly insert the injection needle into the pronucleus. In the ideal situation the pronucleus will start swelling as soon as you apply pressure to the injection pipette. Try to avoid the nucleolus: it is extremely sticky and once touched you will pull out a substantial amount of DNA (visible as a string). If you find a small bubble arising at the end of the microinjection needle and clearly distinguishable from the pronucleus, you have not entered the pronucleus properly. The outer membrane of the oocyte can be extremely flexible and form a thin layer along the injection needle. To disrupt this you can either try to continue the pressure until the bubble bursts or insert the needle further into the inner part of the oocyte. Sometimes it helps to move the needle beyond the pronucleus. Once the

membrane is disrupted retract the needle to the pronucleus to carry out the injection.

12 As soon as the diameter of the pronucleus has increased by a factor of ~ 1.5–2, pull the needle out off the oocyte. We have found that the survival rate of the oocytes improves dramatically if this retraction is performed rapidly.

13 If the cell has survived, transfer it to the bottom of the injection slide. If the cell has started to lyse (granules of the cytoplasm will start leaking out of the cell) keep the cells to the left or right side of the slide.

14 Pick up the next oocyte to inject as described.

15 Transfer the injected oocytes back into the M16 micro-drop culture and place them into the CO_2 incubator.

Notes

a While M16 medium is optimized for culturing oocytes, its pH changes quickly when kept outside the CO_2 incubator. Manipulation of oocytes (e.g. washing steps and injection) should therefore always be performed in M2 medium