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Handmade Somatic Cell Cloning in Cattle

Gábor Vajta, Ian M. Lewis, and R. Tayfur Tecirlioglu

Summary

Apart from the biological and ethical problems, technical difficulties also hamper the improvement and widespread application of somatic cell nuclear transfer (NT). Recently introduced zona-free procedures may offer a solution for the latter problem. The most radical approach of these techniques is the so-called handmade cloning (HMC). It does not require micromanipulators because the manipulations required for both enucleation and nucleus transfer are performed by hand. The HMC technique includes manual bisection of zona-free oocytes, selection of cytoplasts by staining, and the simultaneous fusion of the somatic cell with two cytoplasts to produce a cloned embryo. HMC is a rapid and efficient technique that suits large-scale NT programs. It requires less expertise and time than traditional NT methods and the cost of equipment is significantly less. Production efficiency is high and embryo quality, in terms of pregnancy rates and live births, is not compromised. Although HMC has been developed particularly for bovine NT, the technique is applicable to other species. The method may become a useful tool for both experimental and commercial somatic cell cloning because it allows for standardization of procedures and provides the possibility of automation.

Key Words: Nuclear transfer; cloning; bovine; handmade; somatic cell; zona-free.

1. Introduction

The basic technical aspects of commonly used nuclear transfer (NT) methods for mammals have not changed since the original description of the first successful embryonic cell cloning ***(1)***. Most embryologists regard the zona pellucida as important for appropriate embryonic development until the expanded blastocyst stage. However, preservation of an almost-intact zona during NT requires the use of micromanipulators, highly skilled labor, and expensive equipment. In addition, these factors make standardization of the procedure difficult.

AU: nuclear transfer abbreviated throughout as NT for book-wide consistency.

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The need for an intact zona pellucida for appropriate embryo development was questioned several years ago ***(2, 3)*** and a micromanipulator-free procedure was introduced for embryonic cell cloning ***(2, 4, 5)***. However, the direct application of this technology for somatic cell nuclear transfer (SCNT) was unsuccessful initially ***(6)***. Later, a technical modification in pairing, fusion, and the application of more appropriate culture conditions made zona-free somatic cell cloning possible in cattle and pigs ***(7–10)***. Recently, other forms of the technique were described ***(11–13)***. Although some of these zona-free procedures still required the use of micromanipulation for enucleation before or after zona removal ***(9–11),*** this step can be performed by hand as well ***(7, 8, 12–21)***. For clear distinction, the micromanipulation-free, SCNT method has been referred to recently as handmade cloning (HMC; *see* **Fig. 1** and **ref. *12***). A recent modification of the fusion technique has improved the efficiency and reliability of this step considerably ***(14, 21)***.

Fig. 1

All publications dealing with zona-free NT techniques agree that these methods are easier to learn and apply, do not require specially skilled workforce, the productivity is high and the final embryo quality is comparable to that with traditional cloning. Pregnancy and calving data are still limited ***(11, 12, 14–21)***; however, initial observations do not reflect any compromised developmental ability. Viability of HMC embryos also was illustrated by the fact that pregnancies and offspring were obtained after vitrification (G. Vajta, unpublished ***[14, 15, 20, 21]***).

Additionally, in the HMC procedure, no micromanipulators or related equipment are required, making the method accessible to laboratories with limited budgets. The simple and repeatable steps of the HMC method make the outcome less dependant on individual skills, meaning that a higher level of standardization of SCNT is possible. Eventually, partial or full automation of the procedure is a realistic possibility.

2. Materials

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

1. Tissue culture medium (TCM)-199 medium (M-0650): 2.5 m*M* HEPES (H-7006), 5 mM $NaHCO_3$ (S-4019), 0.2 m*M* sodium pyruvate (P-3662), 50 µg/mL gentamycine sulfate (G-1264) adjusted to pH 7.4, 280 mosm and supplemented with either 0, 2, 10, or 20% cattle serum (T0, T2, T10, and T20, respectively).
2. Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS0; Gibco-BRL, Gaithersburg, MD; cat. no. 14190–144).
3. Fusion medium: 0.3 *M* D-mannitol (M-9546), 0.1 m*M* $MgSO_4$ (M-2393), 0.05 m*M* $CaCl_2$ (C-7902), and 1 mg/mL polyvinylalcohol (P-8136). Store in 10-mL aliquots at –20°C.
4. Mineral oil (M-8410), cell culture tested.

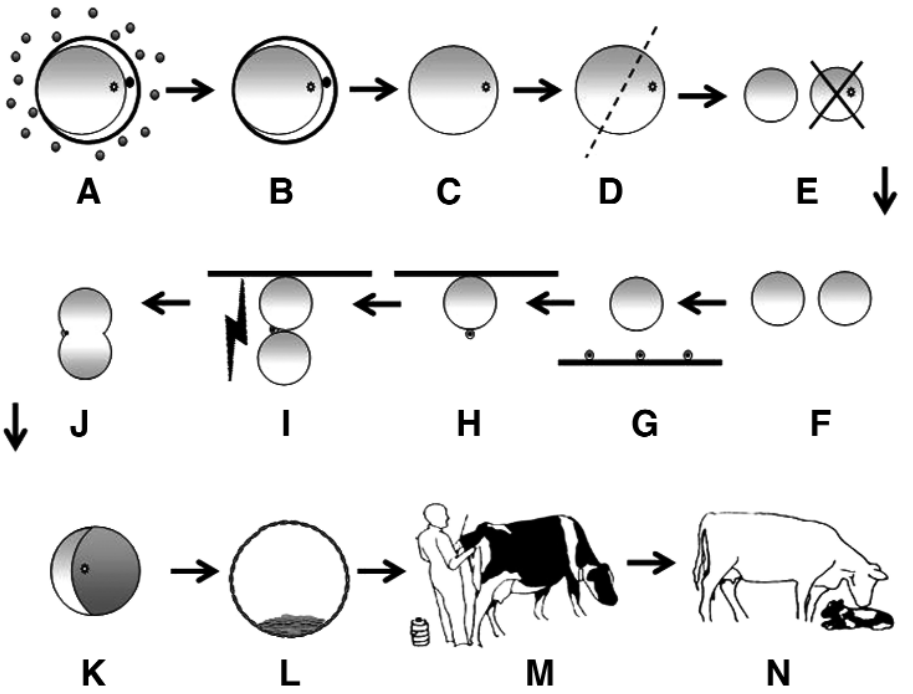

Fig. 1. Schematic illustration of handmade cloning: **(A)** Cumulus intact oocytes 18–22 h after maturation; **(B)** cumulus removal by vortexing; **(C)** zonae removal; **(D)** bisection of oocytes; **(E)** Hoechst 33342 staining; **(F)** selection of cytoplasts; **(G)** attachment of somatic cell to the cytoplast; **(I)** simultaneous fusion of two cytoplasts with a somatic cell; **(J)** round-up following fusion; **(K)** activation of embryos 3 h after reconstruction; **(L)** blastocyst 7 d after reconstruction; **(M)** embryo transfer with fresh and vitrified embryos; **(N)** offspring.

5. Phytohemagglutinin (PHA; L-8754). Prepare 5 mg/mL stock solution in T0, store in 50-µL aliquots at –20°C.
6. Protease (Pronase E; P-8811). Prepare 2 mg/mL solution in T10, store in 500 µL at –20°C. Centrifuge before use.
7. Hoechst 33342 (B-2261). Prepare 1 mg/mL stock solution in ultra-pure H_2O. Store in 20-µL aliquots at –20°C in dark (light sensitive).
8. Trypsin– EDTA (Invitrogen, Carlsbad, CA; cat. no. 25300–054). Store in 100-µL aliquots at –20°C.
9. Hyaluronidase (H-4272). Prepare 0.5 mg/mL solution in T2. Store in 500-µL aliquots in 2-mL Eppendorf tubes at –20°C.
10. Calcium ionophore II (C-7522). Prepare 1 mg/mL stock in dimethyl sulfoxide (D-2650). Keep at room temperature in a small dark bottle with tightly closed cap.

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11. 6-Dimethylaminopurine (DMAP; D-2629). Prepare 100 m*M* stock solution in PBS0. Use heat-durable container, heat in a hot water bath until completely dissolved. Store in 8-µL aliquots at –20°C.
12. Cytochalasin B (C-6762). Prepare 5 mg/mL stock dissolved in dimethyl sulfoxide.
13. Store in 2-µL aliquots at –20°C.
14. Two stereo microscopes with sharp image contrast, such as Olympus SZ60 (Olympus, Tokyo, Japan) or Nikon SMZ 2T (Nikon, Tokyo, Japan). One microscope was equipped with heated stage for manipulation, and the other microscope was maintained at room temperature for fusion.
15. Inverted fluorescent microscope, preferably with heated stage.
16. Fusion machine (Genaust; Bacchus Marsh, Victoria, Australia).
17. Fusion chamber. Microslide with 0.5-mm gap (BTX, San Diego, CA; cat. no., Model 450, 01–000209–01).
18. BLS aggregation needles (DN-09; BLS, Budapest, Hungary).
19. Microblade (Ultra Sharp Splitting Blades; AB Technology, Pullman, WA).
20. Four-well dishes (4WDs; Nunc, Roskilde, Denmark).

3. Methods

Methods for oocyte collection, maturation, and somatic cell preparation are described in other chapters of this book as well as in our earlier publications ***(8, 12)***. Briefly, 25 oocyte-cumulus cell complexes were matured in each well of a 4WD filled with 400 µL of bicarbonate buffered TCM 199 medium with additives, hormones, and 15% cattle serum, covered with 400 µL of oil. Fibroblasts were cultured in wells of a 4WD containing 400 µL of Dulbecco's modified Eagle's medium supplemented with additives and 10% cattle serum (*see* **Note 1**) and covered with 400 µL of oil for 3 to 5 d to reach almost total confluence. For one routine HMC program, 150–200 oocytes and one well of somatic cell culture were used.

This chapter deals in detail only with procedures that are specially performed in bovine HMC, that is, (1) cytoplast preparation, (2) fusion, (3) activation, and (4) embryo culture. Except where otherwise indicated, all manipulations were performed on 39°C heated stages and plates using minimum background light.

3.1. Preparation of Cytoplasts and Somatic Cell Suspension

3.1.1. Removal of the Cumulus Cells and the Zona Pellucida

1. Remove 150–200 oocytes from the maturation dish 21 h after the start of in vitro maturation (hpm) and transfer to an Eppendorf tube containing 500 µL of hyaluronidase solution.
2. Mix suspension carefully with a 1000 µL of automatic pipet until all visible cumulus clouds disappear.
3. Vortex the tube at maximum speed (8000 rpm) for 3 min.

4. Collect oocytes in a 36-mm diameter Petri dish under a stereo microscope and wash twice in T2 to remove all cumulus cells from the solution (*see* **Note 2**).
5. Fill wells of a 4WD with the following solutions:

   Well 1: Supernatant of the centrifuged pronase solution
   Well 2: 800 µL of T20
   Well 3: 800 µL of T2
   Well 4: 800 µL of T20
6. Using the smallest possible volume, transfer oocytes to well 1 and place the dish on the heated plate of a horizontal shaker and rotate at (120 rpm) for 10–15 min, then with (85 rpm) for 1 min (*see* **Note 3**).
7. Transfer zona-free oocytes collected in the middle by the slow rotation into well 2 and incubate for an additional 3 min (*see* **Note 4**).

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3.1.2. Preparation of Somatic Cell Suspension

1. Remove all medium and oil from one well of the 4WD containing the fibroblast monolayer wash three times with PBS0 during pronase digestion of oocytes.
2. Incubate cells with 100 µL of trypsin–EDTA for 4 min to lift cells and then add 1 mL of of T20 neutralize trypsin–EDTA. Mix contents rigorously, transfer to an Eppendorf tube, and store at room temperature to minimize aggregation of cells.

3.1.3. Random Bisection of Oocytes

1. Prepare a bisection dish by adding 4 mL of T20 and a cytochalasin B stock tube solution into a 36-mm diameter Petri dish.
2. Transfer approx 40–50 zona-free oocytes into the bisection dish and line them up on the bottom of the dish. Facilitate the final linear arrangement with careful tapping on the side of the dish.
3. Clean the microblade with 70% ethanol and then anchor the tip of the blade to the bottom of plastic dish close to the oocytes. Lower the cutting edge of the blade close to the middle part of the oocyte to bisect each oocyte individually. After cutting three to seven oocytes, change the position of the blade tip and repeat the procedure.
4. Tap the dish carefully again to detach oocyte halves from the dish and swirl solution to collect halves in the middle.
5. Transfer all halves to the fourth well of the 4WD and repeat bisection procedure until oocytes are bisected (*see* **Note 5**).

3.1.4. Selection of Cytoplasts

1. Add 8 µL of Hoechst stock solution to well 3 of the 4WD, transfer all oocyte halves into the well, and incubate in dark for 5 min.
2. Meanwhile, prepare cytoplast sorting dish. Pipet 200-µL drops of T2 at the 12-o'clock position into the lid of a 36-mm diameter Petri dish. Place the lid on a room temperature stage to avoid excessive evaporation. Make 130–140 small droplets (approx 1–2 µL) under the stereo microscope using a thin capillary pipet and cover both the large and small droplets with mineral oil.

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3. Transfer all stained halves into the large drop first and then into the small droplets in groups of three as quickly as possible to minimize light exposure. Register positions of half-oocytes without chromatin staining on a tape recorder using an inverted microscope and ultraviolet light,
4. Collect cytoplasts under a stereo microscope and transfer into well 4 of the 4WD for temporary storage.

3.2. Pairing and Fusion

1. Prepare a 4WD for cell–cytoplast pairing and fusion with following solutions:

   Well 1: 800 µL of T20
   Well 2: Supernatant of the PHA tube (mix 450 µL of T2 solution with PHA stock solution and centrifuge to remove precipitation)
   Well 3: 800 µL of T20
   Well 4: 800 µL of fusion medium.
2. Fill the middle part of the 4WD with 4 mL of T2 and keep the dish on the heated stage of stereo microscope. Transfer all cytoplasts into the well 1 of the 4WD for cell-cytoplast pairing and fusion.
3. Place the fusion chamber on the second microscope at room-temperature stage and secure it with sticky tape. Attach live (red) wire to Northern and ground (black) to the Southern platinum wire. Cover fusion chamber with 2 mL of fusion medium.
4. Turn the fusion machine on and set following parameters: alternating current (AC) = 14 V direct current (DC) = 168 V (3.36 kV/cm), pulse duration = 4 µs, number of pulses = 1, and post-AC pulse = 0 inducing a potential difference of 3 V on the somatic cell surface (*see* **Notes 6–8**).
5. Mix somatic cell suspension thoroughly with a 1000-µL automatic pipet and add 3 to 5 µL of the cell suspension into the middle part of the 4WD in one group avoiding extensive dispersion. Focus microscope in high magnification and adjust mirror to visualise individual cells clearly under the stereo microscope.
6. While cells are settling, remove 20–30 cytoplasts (start with 2 cytoplasts if you are inexperienced) from well 1 and transferred into well 4 containing fusion medium for equilibration. The cytoplasts were then transferred into the Southern (away from the platinum wires) part of the fusion chamber.
7. Subsequently, remove another 10–20 cytoplasts from well 1 and disperse into well 2, close to top of the PHA solution without allowing cytoplasts to touch each other or any surface of the well. Transfer all PHA coated cytoplasts within 3–4 s to the middle of the dish in a clear area away from the somatic cells.
8. Pick one cytoplast and allow cytoplast to sink over a single fibroblast that had clear/round edges. When the two cells are attached to each other, transfer cytoplast–fibroblast pair into well 4 containing fusion medium for equilibration and then to the Northern part of the fusion chamber (away from the platinum wires; *see* **Note 9**). Repeat pairing procedure with the remaining cytoplasts stored in the middle of the 4WD.

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9. Turn the AC alignment on, pick the cytoplast–fibroblast pair and expel them above the platinum wires close to the Northern wire (*see* **Note 10**). Gently steer the pair with the pipet until fibroblast facing the opposite wire. While cytoplast–fibroblast pair sink in the fusion medium, the pair move toward and then attach to the Northern wire.
10. After attaching cytoplast–fibroblast pair to the wire, pick another cytoplast and expel it close to the cytoplast–fibroblast pair. This cytoplast will float toward the pair touching the fibroblast first and then cytoplast having somatic cell is sandwiched in the middle (*see* **Note 11** and **Fig. 2**).

Fig. 2

11. Apply DC pulse, gently remove triplets and transfer into well 3 containing T20 for rounding-up. Repeat fusion steps until all cytoplasts are fused with somatic cells (*see* **Note 12**).
12. Following visible signs of fusion and round-up, transfer all reconstructed oocytes into wells of a 4WD filled with 400 µL of α-aaci medium supplemented with 5% cattle serum ***(22)*** and covered with 400 µL of oil in a well of a 4WD. Incubate the dish at 39.0°C in 5% CO_2 in air and maximum humidity for 3 h.

3.3. Activation

1. Activate oocytes 28 h after the start of maturation (approx 3–4 h after the fusion). Incubate reconstructed embryos first in 1 mL of T20 containing 2 µ*M* calcium ionophore (2 µL of stock solution) for 5 min at room temperature and then wash twice in T20.
2. Further incubate reconstructed embryos individually in 5-µL droplets of culture medium containing 2 m*M* DMAP (content of one stock tube dissolved in 400 µL of culture medium) and covered with oil in 5% CO_2 in air at 39°C for 6 h (*see* **Notes 13** and **14**).

3.4. Embryo Culture

1. Add 400 µL of culture medium to each well and cover with 400 µL of oil.
2. Support the bottom of the wells with a thick glass microscope filter and score 20–50 well of the wells (WOWs) in each well by applying a strong, steady vertical pressure with aggregation needle. Flush wells individually with culture medium and incubate dishes at 39°C in 5% CO_2, 5% O_2, and 90% N_2 and maximum humidity. WOWs were prepared according to the modification ***(9)*** of the original description ***(3)***.
3. Wash reconstructed embryos three times in culture medium (*see* **Notes 15** and **16**) approx 34 hpm and culture individually in WOWs at 39°C in 5% CO_2, 5% O_2, and 90% N_2. To maintain constant conditions, do not change medium or assess the cleavage rate during the culture period (*see* **Note 17**). The number of blastocysts per reconstructed embryo is determined under a stereo microscope 7 d after reconstruction.

4. Notes

1. The use of cattle serum instead of fetal calf serum was suggested in our earlier publication ***(12)***. The high protein content of cattle serum efficiently decreases

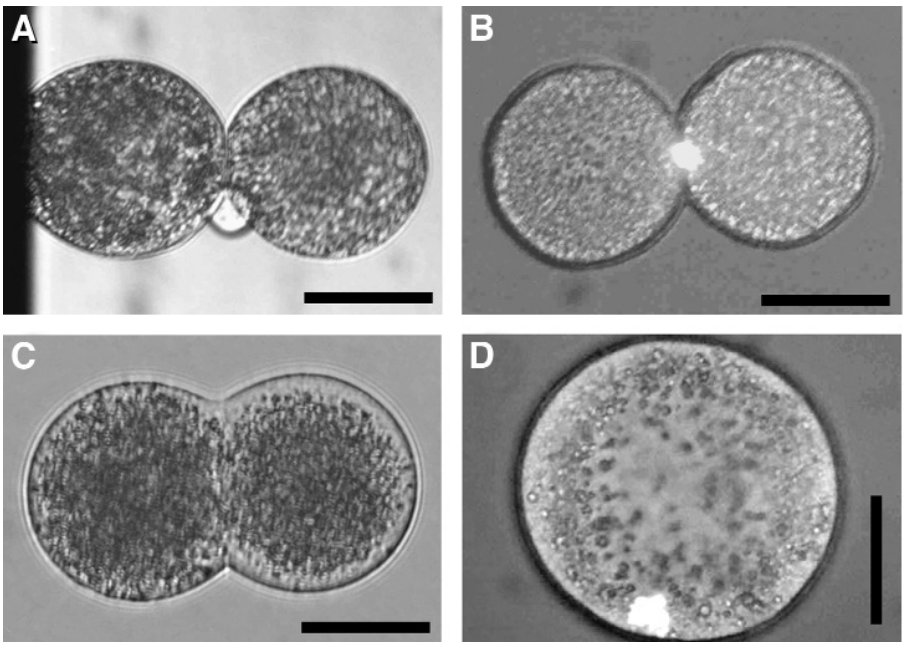

Fig. 2. One-step fusion in handmade cloning. **(A)** Simultaneous fusion of two-cytoplasts and a somatic cell before the direct charge (DC) pulse; **(B)** 1 min; **(C)** 30 min; and **(D)** 6 h after the DC pulse. **(A)** and **(C)** visible light, **(B)** and **(D)** ultraviolet epifluorescence after Hoechst 33342 staining. Scale bar, 50 μm

attachment of bisected demi-oocytes to the bisection blade, improves recovery after fusion, and also may help to avoid blastomere spreading and embryo disintegration before compaction. High protein content in the medium was also suggested by Peura et al. ***(4)***. Moreover, the growth factor content of cattle serum is at least twice as high as that of fetal calf serum ***(12)***, which also may contribute in the high developmental rates of HMC embryos in culture.

2. In contrast to the routine procedure in traditional nuclear transfer and also to some zona-free cloning procedures ***(2, 4, 5, 11)***, selection of polar body-containing metaphase II phase oocytes is not performed in HMC, as exclusive use of polar body-containing metaphase II oocytes did not improve blastocyst rates ***(7–10, 12, 14, 20)***. For HMC, all oocytes without visible degenerative changes or signs of physical damage were processed.
3. The potential harmful effect of pronase to the oocyte membrane was minimized by the supplementation of the solution with 10% cattle serum according to the suggestion of T. Peura (personal communication). This way, as many as 300 oocytes could be digested together while the proportion of damaged embryos did not exceed 1%.

4. Proper handling and moving of oocytes and embryos with glass pipets is a key to success in HMC. Zona-free oocytes, cytoplasts, and reconstructed embryos are more fragile than zona-enclosed ones. They attach easily to surfaces and to each other and may be destroyed with even slightly increased flows of media. Moreover, HMC requires delicate movements both at enucleation, pairing, and fusion. Very finely drawn, perfectly shaped, and lightly fire-polished glass pipets should be used. Before use, pipets should be flushed with T20 to cover the walls with a thin protein layer to avoid attachment. Oil and air bubbles should be aspirated so that the handling of cells can be controlled. If manipulations cannot be controlled perfectly, a new pipet should be used or further training undertaken. For cytoplast preparation, the capillary diameter should be approx 200–250 µm (approx 1.5–2 times the size of a zona-enclosed oocyte). For fusion, the right size is approx 110–140 µm, which is about 1.5–2 times the diameter of a zona-free cytoplast (half-size of oocyte). Working with slightly polished capillaries of the appropriate size can avoid losses of 50% or more.
5. Methods for enucleation of zona-free oocytes can be divided into four groups: (1) Oriented enucleation can be performed either with Hoechst staining, ultraviolet illumination and micromanipulation ***(11)*** or positioning the polar body attached to the oocyte membrane with previous PHA incubation ***(2, 4, 5)***; (2) for random enucleation, high-speed centrifugation ***(23)***, micromanipulation ***(9, 10)***, and manual bisection ***(8, 12)*** can be used with subsequent ultraviolet selection of cytoplasts; (3) a method for chemically assisted enucleation using either micromanipulation manual bisection was established in sheep by Peura ***(13)***; (4) recently, a chemically induced enucleation was introduced in mouse by Wang and Overstrom ***(24)***. The manual bisection procedure described here is simple, rapid, efficient, and highly reliable. The fact that bisection necessitates the use of two cytoplasts for one reconstructed embryo is counterbalanced by the use of all of the oocytes, without polar body selection, and also the exceptionally high blastocyst per reconstructed embryo rates (approx 50% ***[12]***).
6. Commercially available fusion machines vary greatly in terms of pulse amplitude, pulse width, and reliability. It is recommended to test pulse parameters, preferably with a graphic pulse analyzer and an oscilloscope before initiating a cloning program. Disregard any values presented on the fusion machines and adjust pulse parameters according to readings from the analyzer.
7. Microslide fusion chambers with various gaps can be used for cell fusion, however DC amplitude (V) should be re-adjusted according to effective electrical field (E) and the gap between the wires (in centimeters: E = V/cm).
8. Effective field strength and pulse duration can be calculated for different cell types according to following formulas: $\Delta\Psi = Fg(\lambda)rE\cos\theta$ and $\tau = rC\ (\lambda_{int} + 2\ \lambda_{out}) / 2\ \lambda_{int}\ \lambda_{out}$ ***[14, 20, 25]***). Higher field strength is needed to cause disruption of the cell membrane of smaller cells compared with larger ones. However, when an intense external electrical field is applied, the extra energy that is needed to fuse smaller cells may be too strong for larger ones and can cause lysis if the pulse duration is not decreased.

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9. Pairing of cytoplasts and donor cells in zona-free embryonic cell NT initially was performed using the AC effect in the fusion chamber ***(2, 4, 5)***. However, the relatively small size of somatic cells does not allow this method of pairing in zona-free SCNT. The attachment facilitated by PHA was found to be an efficient way for pairing ***(7, 8, 12)***. Subsequently, successful use of the same procedure has been reported elsewhere ***(9–11, 13)***.
10. The use of AC to facilitate proper alignment at fusion is a common method in zona-free NT procedures ***(2, 4, 5, 7–10 ,12)***. Recently, a so-called automated fusion procedure was described by Oback et al. ***(11)***, where the alignment was entirely performed entirely by the AC current, using various intensities according to the requirement of the pairs. However, the reported compromised fusion rates did not fully justify this innovation.
11. The use of this sandwich type arrangement and one-step fusion ***(14, 20)*** resulted in some unexpected benefits. The two cytoplasts stabilize the cell triplets in the correct position, allowing up to 10 or 20 triplets to be fused together. This decreases the time required for one fusion considerably. The sandwich position resulted in a uniquely increased pressure on the attaching membranes between both somatic cell and cytoplasts and between the two cytoplasts. This close physical contact might contribute considerably to the very high fusion efficiency (90–95%). Additionally, the unique location of the somatic cell does not allow contact and attachment to the bottom of the culture dish after fusion, which may be a source of fusion failure in other zona-free techniques.
12. At fusion, a careful balance between different factors should be maintained. These factors include proper equilibration in the fusion medium while keeping its toxic effects to a minimum; proper alignment of cytoplasts and somatic cells while keeping the AC effect relatively moderate; strong attachment between cytoplasts and somatic cells, but weak attachment to the wire; and having the DC pulse strong enough to cause high fusion rates but moderate enough not to cause lysis or deformities in the reconstructed embryos. *See* the following points for troubleshooting:

    No AC effect (i.e., no visible movement of pairs and cytoplast to the wire). First, check connections and parameters. Another possible source of the problem is that there is albumin or serum in the medium or on the surface of the wires. Flush the area of wires and between the wires rigorously with fusion medium, and replace the entire fusion medium. If no improvement is seen, remove the fusion medium, disconnect the chamber, remove it from the microscope, flush with water, clean with paper towel, flush with 70% ethanol, clean with paper towel, dry and then repeat the whole preparation procedure. b. The AC current pulls the pairs to the wire too aggressively. This is almost always followed with the explosion of the pairs at fusion. Common reasons include inappropriate equilibration and/or ions around the pairs. Stop the AC current, remove the pair, flush the pipet and area between wires, and start the process again. c. Swirling, dancing of pairs and cytoplasts between the wires at AC: again, ions in the medium, as in **item b**.

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13. DMAP makes the membrane of zona-free embryos sticky; therefore incubation of embryos individually in small droplets is essential to avoid aggregation.
14. Although it may be difficult to insert into the conventional working schedule, extended incubations between fusion and activation and also in the DMAP (4 and 6 h, respectively) improve developmental rates ***(12)***, in accordance with previous observations made on zona-enclosed embryos ***(26–28)***.
15. Thorough and long washing, both after Ca-ionophore and DMAP incubation, are essential to prevent toxic injury during the further incubations in relatively small volumes (drops and WOWs, respectively) caused by the remnants of these chemicals.
16. At the end of DMAP incubation, the stereo microscopic view of reconstructed embryos changes significantly, including ovoid-, egg- or kidney-shaped, central dark and peripheral white areas, with well preserved, double-refracting cell membranes. Additionally, several minutes after release from DMAP, a considerable number of embryos look fragmented, some of them mimicking two- or four-cell stages. Although they appear unusual, these changes usually are correlated to efficient activation and may predict high blastocyst rates.
17. Although some zona-free NT systems still use microdrops for embryo culture ***(11)***, most researchers have found microdrops inadequate and have turned to alternative ways including the glass oviduct system of Thouas et al. ***(12, 29)***, or microwells formed either from agar ***(13)*** or on the plastic bottom of the dish. The benefits of using the WOW system for single embryo culture as well as for supporting the development of zona-free embryos has been described earlier ***(3)***. With the modification of Booth et al. ***(9)***, the preparation of WOW has become simple, and the high blastocyst per reconstructed rates achievable in the system justifies its application ***(12)***.

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