Chapter 16

Tetraploid Complementation Assay

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Abstract

The generation of mouse chimeras by combining host embryos with genetically modified embryonic stem (ES) cells is a necessary step towards establishment of genetically modified mouse strains. This protocol describes the procedures necessary for the production of mouse chimeras by tetraploid complementation assay. This technique can be used to generate ES cell-derived embryos or animals, and to rescue extraembryonic defects. It provides a powerful tool for direct analysis of phenotype and for studies of cell fate during mouse development. This method can speed up the production of genetically modified strains directly from hybrid ES cells. The Transgenic facility at the Samuel Lunenfeld Research Institute of Mount Sinai Hospital and more recently at the Toronto Centre for Phenogenomics has been successfully using this protocol since early 1990s.

Abbreviations

AC	Alternating electric current
BSA	Bovine serum albumin
DC	Direct electric current
E1.5 etc	Embryonic stage 1.5 and further
EGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
hCG	Human chorionic gonadotropin
lacZ	A reporter gene encoding beta galactosidase
MEF	Mouse embryonic fibroblasts
PMSG	Pregnant mare's serum gonadotropin

Selected Vendors:
Millipore: www.millipore.com
Sigma: www.sigmaaldrich.com
Invitrogen: www.invitrogen.com
Harlan: www.harlan.com
Charles River Labs: www.criver.com

S. Pease and T.L. Saunders (eds.), *Advanced Protocols for Animal Transgenesis*, Springer Protocols,
DOI 10.1007/978-3-642-20792-1_16,

Taconic: www.taconic.com
BLS Ltd: www.bls-ltd.com
Becton Dickinson: www.BD.com
Fine Science Tools: www.finescience.com
VWR: www.vwr.com
Drummond: www.drummondsci.com

16.1 Introduction

The protocol below is based on the procedures described by Nagy et al. [1]. Several different methods can be used to induce tetraploidy (reviewed in [2]). The electrofusion of blastomeres of 2-cell stage embryos for the generation of tetraploid embryos described here was first developed by Kubiak and Tarkowski [3]. Most tetraploid embryos die shortly after implantation, but when complemented with diploid embryos, their contribution is primarily restricted to the extraembryonic tissues: the primitive endoderm of the yolk sac and the trophoblast layer of the placenta, and excluded from the primitive ectoderm lineage [4]. Aggregation of tetraploid and diploid embryos is used to segregate embryonic and extraembryonic phenotypes and rescue or bypass extraembryonic defects of the mutations [5, 6]

ES cells have limited ability to contribute to the trophoblast lineage [7]. When ES cells are complemented by tetraploid embryos they colonize the embryo proper, the amnion, the allantois as well as the mesoderm layer of the yolk sac, while tetraploid cells are excluded from these lineages and restricted to the extraembryonic tissues [8, 9] resulting in nearly completely ES cell-derived embryos. The aggregation of ES cells with tetraploid embryos provides a rapid test for the developmental potential of ES cells and allows the generation of mutant embryos directly from ES cells for analysis of their phenotype [10]. Moreover, it is possible to derive viable and fertile animals carrying mutations directly from ES cells and speed up traditional breeding using characterized F1-hybrid ES cells introduced into tetraploid embryos by blastocyst microinjection or aggregation methods [11, 12].

Occasionally, a small contribution of tetraploid cells can be observed in the embryo proper; therefore, reporters such as EGFP or lacZ are often used for the tetraploid host embryos in order to make them easily recognizable and to confirm ES cell origin of mutant embryos when they are dissected at mid-gestation [13]. For more details on the applications of the tetraploid complementation assay and chimera analysis in general, *see* [14–16].

16.2 Materials

16.2.1. Equipment, Tools, and Plasticware

- Stereomicroscope(s) with transmitted light for embryo manipulations and incident light or fiber optics with a goose-neck for embryo transfer surgery. The use of two microscopes is convenient for the fusion of 2-cell stage embryos and for embryo transfers into pseudopregnant females; a single microscope is also sufficient, however. We find that frosted instead of transparent glass in the base of the microscope with transmitted light often provides better visualization of the zonae pellucidae, which is helpful for zona removal.
- Inverted microscope with phase contrast for ES cell culture observation.
- Biosafety Cabinet for routine cell culture.
- Humidified incubator(s) at 37°C, 5% CO_2 for embryo and ES cell culture.
- Cell-fusion instrument [e.g., CF-150B pulse generator with 250 μm electrode chamber (BLS Ltd, Hungary, www.bls-ltd.com)].
- Aggregation needle DN-09 (BLS Ltd, Hungary, www.bls-ltd.com).
- Sterile Petri and tissue culture dishes (35, 60, 100 mm), organ culture dishes (Falcon 35–3037). 35 mm Easy Grip Falcon 35–3001 tissue culture plates are well suited for making depressions in the plastic for aggregates.
- Sterile Pasteur pipettes and plastic pipettes for tissue culture (1 ml, 5 ml, 10 ml).
- Sterile 1 cc syringes, 26 G 1/2 and 30 G 1/2 needles. To make a flushing needle, the sharp tip of 30 G 1/2 needle is cut off and/or polished on a sharpening stone or sand paper. The flushing needle is flushed with 70% ethanol before and after use.
- Bunsen or alcohol burner for pulling embryo-manipulating pipettes.
- Embryo-manipulating pipettes: Pasteur pipettes or glass micro-capillaries (e.g., Drummond 1-000-0400 or 1-000-0500) are drawn by hand over the flame and broken flat with an inner diameter slightly larger than an embryo (>100 μm). It is very important to flame polish the tip to prevent damaging zona-free embryos. Embryo-manipulating pipettes are connected through elastic rubber tubing (e.g., VWR 62996–350) to an aspirator mouthpiece (Drummond 2-000-000 or Sigma A5177 Aspirator Tube Assembly). Pasteur pipettes fit into standard 1,000 μl pipettor tips and microcapillaries are

inserted into silicone tips (Drummond 1-000-9003 or Sigma A5177).

- Surgical instruments (e.g., Fine Scientific Tools – FST): sharp fine-pointed scissors, fine forceps (e.g., Dumont #5), straight or curved blunt forceps with serrated tips, serrefine (e.g., FST18050-28).
- AUTOCLIP Wound Clip Applier (Becton Dickinson 427630) and AUTOCLIP Wound Clips, 9 mm (Becton Dickinson 427631).

16.2.2. Mouse Stock

Outbred stocks such as ICR/CD-1 (e.g., Harlan Sprague Dawley, Charles River Laboratories or Taconic) are commonly used as donors of host embryos as well as recipients of manipulated embryos. For analysis of mutant embryos at mid-gestation, outbred females are generally mated with homozygous transgenic males expressing ubiquitously a reporter such as EGFP [17] to generate tetraploid host embryos. The presence of a reporter assists in identification of cells derived from tetraploid embryos. If fluorescent reporters are not available and where albino animals have been used for the generation of tetraploid embryos, then ES cell contribution of 129B6F1 hybrid or other pigmented strains can be determined by fetal eye color, starting at E11.5. The details of mouse colony management and procedures involved in the production of superovulated embryo donors and pseudopregnant recipients are described in [18] as well as elsewhere in this book (Chaps. 6 and 25).

16.2.3. ES Cells

Before attempting to generate completely ES cell-derived embryos or animals from genetically modified ES cell clones it is very important to establish that a nonmanipulated parental ES cell line in existing culture conditions has such developmental potential. We use aggregation with tetraploid ICR embryos for R1 and G4 ES cells derived in A. Nagy's laboratory.

R1 ES cells [9] were derived from a hybrid of two 129 substrains: 129X1 female crossed with 129S1 male. 129X1 is white-bellied, pink-eyed, light chinchilla (A^w/A^w $Oca2^p$ $Tyr^{c\text{-}ch}/Oca2^p$ $Tyr^{c\text{-}ch}$) and 129S1 is white (or light)-bellied agouti (A^w/A^w). Thus, R1 ES cells are homozygous for white-bellied agouti allele at the agouti locus and heterozygous for chinchilla ($Tyr^{c\text{-}ch}$) and pink-eyed dilution ($Oca2^p$): A^WA^W *Cc-ch Pp*; they have light-bellied agouti coat color and black eyes. For more details on 129 substrains see http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml

G4 ES cells were established from the cross of 129S6/SvEvTac female and C57BL/6NTac male (129S6B6F1). They are heterozygous for white-bellied agouti allele at the agouti locus and homozygous for tyrosinase (Tyr^+) and pink-eyed

dilution ($Oca2^{+}$) loci: $A^{W}a$ *CC PP*. Nonmanipulated G4 ES cells aggregated with tetraploid ICR embryos produced completely ES cell-derived pups at a rate of ~30% per number of aggregates transferred up to passage 14. After one or two electroporations, 75% of G4 subclones produced newborn pups at ~25% rate [12, 19].

16.2.4. Reagents

16.2.4.1. Embryo Culture

During the aggregation experiments, embryos are cultured in vitro for 2 days and at least 24 h without zona pellucida. Zona removal dramatically increases the embryos' sensitivity, which makes optimal culture conditions absolutely necessary for success. Embryo culture medium can be purchased commercially (e.g., Millipore) or prepared as described in [18]; aliquots are stored at 4°C and should not be stored as such for more than 2 weeks. The quality of water is very critical for media preparation. Water should be obtained from a regularly maintained Milli-Q (Millipore) filtration system preferably pretreated by deionization or purchased commercially (e.g., Invitrogen, Sigma). Disposable plasticware is highly recommended; if glassware is used, it should never be exposed to detergents or organic solvents. All chemicals should be of highest grade, embryo tested if available and once purchased, used only for media preparation.

Embryos are cultured in organ culture dishes or in microdrops covered with embryo-tested light mineral oil (e.g., Millipore ES-005-C or Sigma M8410). As embryos are intolerant of pH and temperature fluctuations, the time between euthanizing the embryo donors and placing the embryos in the culture dish should be minimized. To test in vitro culture conditions, zygotes are cultured for 96 h. More than 80% should reach blastocyst stage. For additional details on preimplantation embryo in vitro culture, refer to [18]. The list of necessary reagents is below.

- M2 (e.g., Millipore MR-015-D) is a HEPES-buffered medium used during embryo collection and other manipulations while in room atmosphere such as electrofusion and zona removal. Aliquots are stored at 4°C and brought to room temperature prior to use. If M2 is brought to 37°C it should be done on a warm plate or in a 37°C oven. If you must use a 5% CO_2 incubator to warm M2 media, make sure the cap is tightly closed to avoid exposure of the media to CO_2 as it will alter the pH. Embryos should not be kept in M2 for a prolonged period of time and need to be rinsed through several drops of CO_2-equilibrated embryo culture medium before being placed in the incubator. Historically, we use M2 medium for all embryo manipulations outside the incubator; however, M2 can be substituted with FHM (HEPES-buffered KSOM medium) or any other HEPES-buffered media

corresponding to the embryo culture medium used in subsequent procedures.

- KSOM-AA (e.g., Millipore MR-121-D) is a bicarbonate-buffered medium with nonessential amino acids used for embryo culture [20–22]. Ideally, freshly prepared medium as well as the air space in a tube should be gassed with 5% CO_2 gas mixture and re-gassed after opening because medium rapidly becomes alkaline outside the incubator. Embryo culture medium is gas-equilibrated by placing the open tube or prepared microdrop dishes in the incubator at least a few hours or better 16–20 h before use.
- Embryo-tested light mineral oil (e.g., Millipore ES-005-C or Sigma M8410) is used to overlay microdrops of embryo culture medium. Unopened containers with oil can be stored at room temperature (below 30°C) away from normal light. We prefer to aliquot oil using proper aseptic procedures, store in the fridge, and incubate with loose cap overnight in CO_2 incubator.
- Acid Tyrode's solution (e.g., Sigma T1788) is used for zona removal. Aliquots are stored at –20°C. One thawed aliquot should be kept at 4°C for no longer than 2 weeks and brought to room temperature prior to use.
- 0.3 M mannitol (Sigma M4125) is used for 2-cell stage embryo electrofusion. It is prepared in ultrapure embryo-tested water (e.g., Sigma W1503) containing 0.3% BSA (e.g., Sigma A3311), filtered and stored at –20°C. Aliquots are thawed prior to use and not re-frozen.

16.2.4.2. ES Cell Culture

ES cells are typically grown on mitotically inactivated mouse embryo fibroblasts in an ES cell medium containing FBS. Before purchasing a new batch, we routinely test FBS lots in vitro as described in several publications [18, 23] and whenever possible by tetraploid complementation assay. FBS is kept frozen at −80°C up to 2 years and must be used within 4 months after thawing. If the whole bottle is not going to be used during that time, it is best to prepare aliquots and re-freeze them. The ES cell medium is made fresh as necessary, kept at 4°C and used within 3–4 weeks.

- ES cell medium
  - Dulbecco's modified Eagle's medium (DMEM) (Invitrogen 11960)
  - 15% FBS (ES cell qualified)
  - 2 mM GlutaMAX™ (Invitrogen 35050) or L-Glutamine (Invitrogen 25030)
  - 0.1 mM 2-mercaptoethanol (Invitrogen 21985–023)

  - 0.1 mM MEM nonessential amino acids (Invitrogen 11140)
  - 1 mM Sodium pyruvate (Invitrogen 11360)
  - 1,000 U/ml LIF (Millipore ESG1107)
  - 50 U/ml Penicillin and 50 μg Streptomycin (Invitrogen 15140) – optional
- 0.05% Trypsin/EDTA (Invitrogen 25300) or 0.25% Trypsin/EDTA (Invitrogen 25200)
- 0.1% gelatin in sterile water (Millipore ES-006B)
- Ca/Mg free D-PBS (Millipore ES-1006-B or Invitrogen 14190)

16.3 Protocol

16.3.1. Collection of 2-Cell Stage Embryos

Table 16.1 describes the co-ordination of mouse and ES cell protocols necessary for the generation of embryo donors and recipients as well as the preparation of ES cells for the aggregation experiment.

Two-cell stage embryos collected at E1.5 are used for generation of tetraploid embryos by electrofusion. Fused embryos are cultured overnight and aggregated with ES cells (or diploid embryos) the following day when they reach the "4-cell stage."

1. One day before embryo collection: prepare culture plates using KSOM-AA medium (e.g., one organ culture dish

Table 16.1
Timeline and co-ordination of mouse and ES cells preparation

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Mouse embryo donors	PMSG injection		hCG injection & mating	S/O plug checking	E1.5 S/O embryo donors		
Mouse embryo recipients				Mating with VAS males	VAS plug checking		E2.5 pseudo pregnant recipients
ES cells	Thawing	Media change	Passage or thawing	Media change	Passage on gelatin	Trypsinization for aggregation	
Embryo manipulations				Prep' of embryo culture dishes	E1.5 embryo collection & electrofusion Prep' aggregation plates	Zona removal & aggregation with ES cells	Uterine embryo transfer

S/O superovulation, *VAS* vasectomized males

and/or one dish with microdrops overlayed with embryo-tested mineral oil). Draw the line(s) on the bottom of the microdrop dish to distinguish two groups of embryos: those, subjected to the electric pulse but not fused yet, and those fused after the pulse. Collected embryos can be kept in an organ culture dish or in a separate drop of the same or different dish before the pulse (Fig. 16.1a, b).

2. Alternatively, place the tubes containing KSOM-AA medium and embryo-tested mineral oil with the caps loose into the incubator to equilibrate overnight or at least for a few hours before embryo collection.
3. On the day of the embryo collection and electrofusion: bring M2 medium to room temperature and prepare culture dishes using equilibrated KSOM-AA medium and oil if not prepared the day before. Pull embryo-manipulating pipettes.

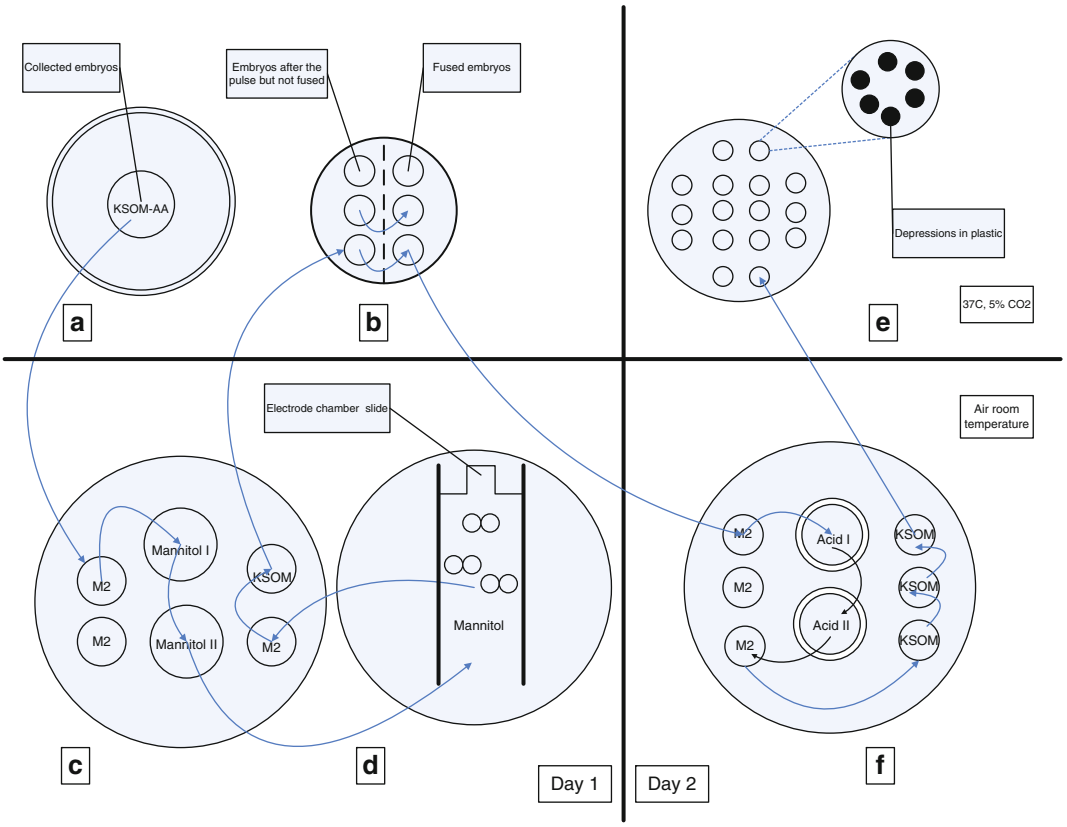

Fig. 16.1 Electrofusion and zona removal scheme. (**a**) Embryo culture dish; (**b**) Microdrop dish divided into the zones with not fused and fused embryos after the pulse; (**c**) Mannitol and media washes plate; (**d**) Electrode chamber slide with 2-cell stage embryos oriented by AC field; (**e**) Aggregation plate with depression wells made in the plastic; (**f**) Zona Pellucida removal by acid Tyrode's.

4. Dissect the oviducts and place them in a drop of M2 medium. The time between euthanizing embryo donors by cervical dislocation and placing the embryos in culture dishes should be kept to a minimum (ideally no more than 30 min). Do not dissect more donor females than can be handled in 30 min.
5. Transfer one oviduct into a small drop of M2 medium under stereomicroscope; insert the flushing needle attached to a 1 or 5 ml syringe filled with M2 into the infundibulum, and gently press the tip of the flushing needle against the bottom of the dish to hold it in place. The use of fine forceps helps to hold the needle in the right position. Flush M2 medium through the oviduct; observe its swelling. Proceed with the remaining oviducts, keeping the time of manipulations to a minimum.
6. Collect the embryos and wash them through several M2 drops to get rid of all debris and several drops of equilibrated KSOM-AA medium. Transfer the embryos into a prepared KSOM-AA embryo culture dish and place it back in the incubator. We find that leaving E1.5 embryos in the incubator for at least 15 min before applying the electric pulse improves the fusion rate.

16.3.2. Generation of Tetraploid Embryos

1. The fusion of blastomeres of 2-cell stage embryos occurs when DC electric pulse is applied perpendicular to the plane of the blastomeres' contact area. The suggested parameters for different types of electrode chambers of CF-150B BLS fusion instrument are listed in Table 16.2. The actual parameters vary depending on the instrument and the mouse strain – they need to be determined in a pilot experiment. The voltage for the square wave DC pulse applicable for electrofusion of mouse embryos is recommended to be 1–1.5 kV/cm [24]. The goal is to reach a 90% fusion rate in 30–45 min without embryo lysis. The adjustable 1 MHz AC field set up at 0.5–1 V orients the 2-cell stage embryos in the electrode chamber using nonelectrolyte solution, so that the blastomeres contact area is parallel to the electrodes and

Table 16.2
Suggested parameters for different electrode chambers of CF-150B BLS fusion instrument

Electrode chamber	DC voltage	Duration	# of pulses	AC voltage
250 μm	30 V	40 μs	1–2	1 V
500 μm	50 V	35 μs	2	2 V
1,000 μm	160 V	36 μs	2	2.2 V

enables the simultaneous fusion of a group of embryos instead of individual embryo fusion performed in electrolyte solution.

2. Thaw a frozen aliquot of 0.3 M mannitol, turn on the cell-fusion machine (we leave it on during the embryo collection). Make sure the switch on the back of the machine is on the "nonelectrolyte" or "normal" setting and set up other parameters. We routinely apply one or two pulses of 30 V and 40 μs for the fusion of ICR embryos in a 0.3 M mannitol solution using a CF-150B cell-fusion BLS instrument with a 250 μm electrode chamber.
3. Wipe the electrode chamber slide with 70% ethanol. The chamber must be cleaned thoroughly before use.

Note: Do not immerse the electrodes in ethanol for any length of time as it will lead to the electrodes' damage!

4. Connect the electrodes to the pulse generator and place the slide into a 100 mm Petri dish. Use the electrode clip or tape to secure it to the dish to prevent it from moving. The same dish can be used for all embryo washes before and after application of the electric pulse if only one microscope is available. Alternatively, set up an additional plate for washes on the second microscope; it helps to speed up the manipulations without the need to move the dish and adjust the focal plane. We find it more convenient.
5. Place several drops of the M2 medium (e.g., 50 μl) for "before" and "after" the pulse and 1–2 drops of the mannitol solution in 100 mm Petri dish (e.g., 100–200 μl) See Fig. 16.1c.
6. Pick up 2–3 groups of 25–30 embryos from embryo culture dish (Fig. 16.1a) and transfer them into "before the pulse" M2 drops (Fig. 16.1c). The number of embryos in the group is determined by the total time they can all be handled, so their manipulations outside the incubator do not exceed 15–20 min. No more than 2–3 groups of 10–20 embryos should be used for initial experiments.
7. Place one large mannitol drop over the chamber to cover both electrodes.

Note: The mannitol drop over the electrode chamber should not be used for longer than 15 min at a time. It must be replaced with fresh mannitol for every new batch of embryos.

8. Pick up the first group of 25–30 embryos in a minimal volume of M2 media and transfer it into the first mannitol drop. Quickly collect the embryos (they will be floating) and move them into the second mannitol drop (Fig. 16.1c). The embryos should be well equilibrated in mannitol before

placing them in the electrode chamber or they will float to the surface of the mannitol instead of resting between the electrodes. It is important not to transfer M2 medium into the electrode chamber otherwise embryo orientation by the AC field will not work efficiently. However, embryos should be kept in mannitol for a minimum amount of time.

9. Place the embryos between the electrodes, leaving space between them (Fig. 16.1d). An AC field set up in advance will orient the embryos so the blastomeres' contact area will be parallel to the electrodes, while they are dropped into the chamber. It is also possible to manually adjust the AC field after placing all the embryos into the electrode chamber, gradually increasing the voltage until all of them are fully aligned with the cleavage plane parallel to the electrodes (Fig. 16.2a).

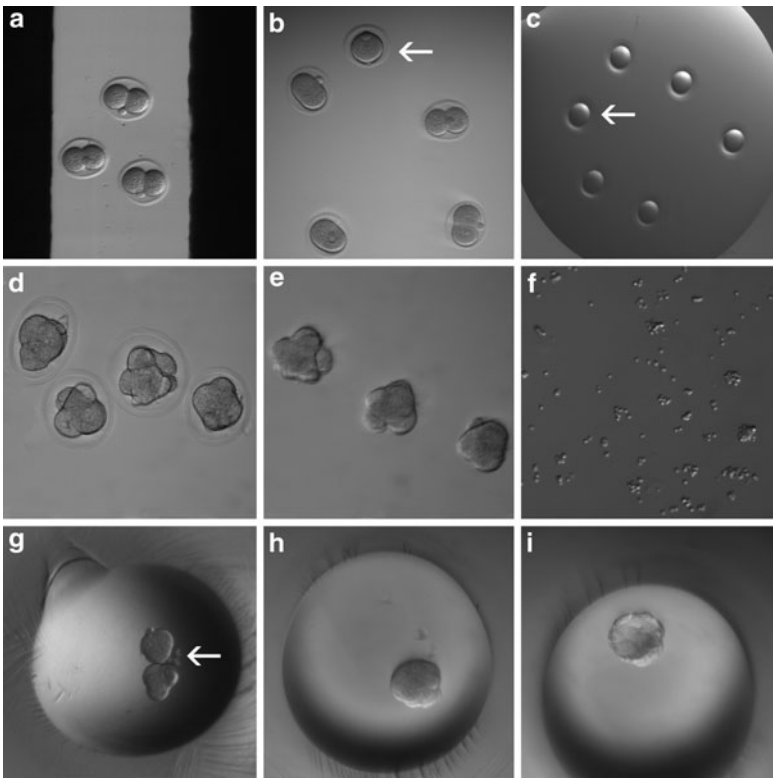

Fig. 16.2 Production of tetraploid embryos and their aggregation with ES cells. (**a**) Orientation of embryos in electrofusion chamber between electrodes under AC field; (**b**) Two-cell stage embryos undergoing fusion after application of the pulse, *arrow* indicates fused embryo; (**c**) Depression wells (one marked by the *arrow*) in embryo culture microdrop; (**d**) "Four-cell" stage tetraploid embryos after overnight culture; (**e**) Zona removal; (**f**) Lifted ES cell colonies ready for aggregation; (**g**) Aggregate of two "4-cell" stage tetraploid embryos and a clump of ES cells marked by the *arrow*; (**h**) Compacted morula after overnight culture; (**i**) Blastocyst after overnight culture.

10. Manually adjust the few embryos that are not aligned by the AC field. Push the trigger pulse and immediately transfer the embryos from the electrode chamber into an "after the pulse" M2 drop (Fig. 16.1c).
11. Proceed with the rest of the embryos until all the embryos in the first batch receive the pulse.
12. Wash the embryos through 2–3 drops of M2 medium to dispose of all traces of mannitol and then through 2–3 drops of equilibrated KSOM-AA medium. Place the embryos into the embryo culture plate in the incubator (Fig. 16.1b).
13. Wipe the electrode chamber slide with 70% Ethanol.
14. Proceed with the rest of the collected embryos as described in Subheading 16.3.2, **steps 6–12**.
15. Approximately 30 min after the pulse application, carefully assess the morphology of the fusing embryos after the second batch is placed in culture (Fig. 16.2b). Under optimal conditions, around 90% of embryos should fuse in 45–60 min. Select successfully fused embryos that look like a 1-cell stage embryo, and move them to new KSOM-AA drops that are marked as "fused" (Fig. 16.1b). Remove the embryos that cleaved before fusion, they can be used for aggregation as diploid embryos in a parallel experiment if necessary. Return the dish to the incubator.

Note: It is very important to select only completely fused embryos and transfer them into a fresh drop on the "fused" side of the dish. Since embryos are recovered at the late 2-cell stage, the second mitotic division is expected soon after the fusion. If not checked in time, fused and cleaved tetraploid embryos cannot be distinguished from nonfused diploid 2-cell stage embryos.

16. A second pulse can be applied to the embryos that did not fuse after 1–1½ h.
17. After overnight incubation, the "4-cell" stage embryos are used for aggregation in the afternoon as described in Subheading 16.3.6. The development of fused embryos to the "4-cell" stage should be at least 80% in optimal culture conditions. Embryos arrested at the "1-cell" stage are not used for aggregations. "2-cell" stage embryos are delayed and some may be aggregated later in the day after they have developed to the "4-cell" stage. Aggregation of "2-cell" stage tetraploid embryos is not efficient in our hands.

16.3.3. Preparation of the Aggregation Plates

The plates are usually prepared in the afternoon of the day before aggregation; alternatively use equilibrated KSOM-AA medium and oil if it is done on the day of aggregation.

1. Place microdrops (~3 mm in diameter or 10–15 μl) onto 35 mm Falcon 353001 Easy Grip dish using 1 cc syringe filled with KSOM-AA medium or micropipettor. We usually make two rows of four to five drops in the middle of the plate and two more rows of three drops on each side but the actual configuration of the microdrops is an individual choice (Fig. 16.1e). Immediately cover the drops with embryo-tested mineral oil.
2. Wipe the aggregation needle with 70% ethanol, if required the needle can be autoclaved. Press the needle into the plastic and make a slight circular movement. Do not press too hard or the plate will crack; however, not enough pressure will result in too shallow of a depression. The goal is to create a small cavity with a smooth and transparent surface that is deep enough to hold the aggregate safely when moving the plate to the incubator. Make six to eight depressions per microdrop, position them in the circle approximately halfway between the center and the edge of the microdrop (Fig. 16.2c). Do not make depressions too close to the edge – the embryos will be difficult to manipulate. Avoid the center, so that in the event that air bubbles are accidentally introduced to the drop, the embryos will remain visible.
3. Leave a few microdrops on the side without depressions; they will be used for the final selection of ES cell clumps as described in Subheading 16.3.6. We usually make depressions for 40–60 aggregates per plate to limit the time of embryo manipulations outside the incubator.
4. Place the aggregation plate in the incubator until it is needed.

16.3.4. Zona Removal

Acid Tyrode's solution is used to dissolve the glycoprotein membrane surrounding the embryo called the zona pellucida. If the acid is diluted with HEPES-buffered medium, it will not work efficiently; on the other hand any acid transferred into embryo culture media will damage the embryos. Hence, it is very important to transfer minimal amounts of solutions between drops and use multiple washes.

1. Place several drops of M2 media and acid Tyrode's in 100 mm Petri dish (Fig. 16.1f). The use of the tissue culture plate's lid or bacterial grade dish helps to reduce stickiness. The temperature of the acid Tyrode's should not exceed room temperature or it will act too quickly and may lead to the embryos' damage, increased stickiness, and difficulties in embryos' manipulations.
2. Transfer a few groups of embryos from the embryo culture dish and place them in M2 drops. The number of embryos manipulated at a time depends on the speed of manipulations.

With practice, it is possible to manipulate 30–50 embryos, but initially start with no more than 5–10 embryos in each group. Manipulations outside of the incubator should be limited to 20 minutes.

3. Transfer a group of embryos with a minimal volume of media into the first acid drop. Gently pipet the embryos around in acid and place them into second fresh drop of acid (Fig. 16.1f). Keep moving the embryos and observe zona dissolution that should happen within a few seconds, unless too much M2 was carried over with embryos (Fig. 16.2d, e).

Note: It is very important to fire-polish the tip of the pipette as zona-free embryos can be easily damaged by sharp edges.

4. As soon as the zona dissolves, immediately transfer the embryos with a minimal volume of acid into the drop of M2 media. Rinse them through 2–3 drops of M2 media to remove any remaining acid. Spread the embryos in the last drop of M2 and do not allow them to touch each other (Fig. 16.2e).
5. Proceed with the zona removal on the remaining embryos.
6. Wash all denuded embryos through several drops of equilibrated KSOM-AA; place them individually into the aggregation plates, directly inside or outside the depression wells (Fig. 16.3) depending on the way the aggregates will be assembled, as described in Subheading 16.3.6.
7. Keep the plates with embryos in the incubator until the ES cells are ready.

16.3.5. Preparation of ES Cells for Aggregation

It is important to have optimal ES cell culture conditions at all times, but particularly for ES cell clones introduced into mice to preserve their developmental potential. ES cells should be kept subconfluent in order to maintain their undifferentiated state, i.e. split at densities that are neither too high nor too low. Generally, ES cells are not kept for more than 2 days without passaging (1:5–1:7) and are used for experiments when growing exponentially. The medium is changed daily and never allowed to become yellow. More details on ES cell culture are provided in [18] and elsewhere in this book (Chap. 17).

1. Thaw a vial of ES cells on a plate with mitotically inactivated mouse embryonic fibroblasts (MEF) 3 days before aggregation; the surface depends on the number of ES cells frozen in the vial, 35 mm or 60 mm plates are sufficient. If the cells were frozen properly, they should be subconfluent and ready for passage in 2 days. A vial of unknown viability should always be thawed earlier, e.g., 5 or more days before aggregation, to ensure their timely recovery and provision of the quantity of cells required for experiment. Change the medium the day after thawing.

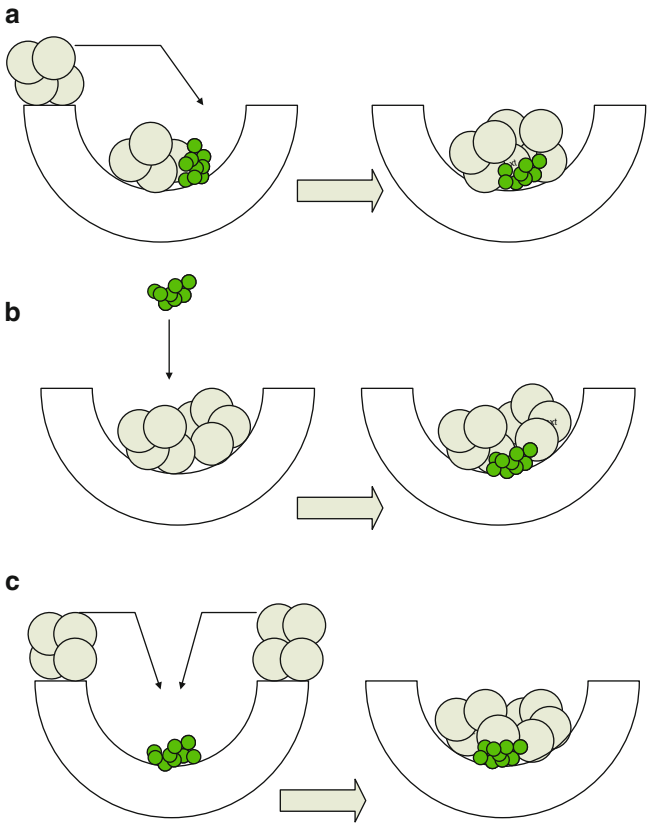

Fig. 16.3 Assembly of tetraploid embryos <–> ES cells aggregates. Right after zona removal denuded embryos are placed into microdrops of aggregation plate inside the depressions in one of the following ways: (**a**) one embryo in each depression well, (**b**) two embryos in each depression well; or (**c**) beside the depressions making sure that they do not touch each other. When the ES cells are ready the clumps are positioned next to the embryos (**a** and **b**) or distributed into the empty depressions (**c**), the embryos are then placed inside the depression next to the clumps. In all cases, special care should be taken to make sure both embryos and ES cell clumps touch each other.

2. One day prior to the aggregation, passage subconfluent ES cells on gelatinized plates as described below. For most clones 24 h is enough, while 48 h may be necessary for slower growing clones. This sparser than usual passage should produce the colonies of 8–15 cells that will be lifted by gentle trypsinization immediately before aggregation.
   (a) Add 0.1% gelatin to two to three dishes (e.g., 2 ml per 60 mm dish).
   (b) Aspirate the medium from ES cell plate and wash the cells with PBS.
   (c) Add trypsin to cover the cells (e.g., 1 ml per 60 mm dish), place it in the incubator for 5 min.

(d) Neutralize the trypsin with ES cell medium containing FBS and re-suspend the cells by gentle pipetting, ensure single cell suspension.

(e) Transfer the cell suspension to a tube and centrifuge at 200 g for 5 min.

(f) Remove the supernatant and gently re-suspend the pellet in fresh ES cell medium. The volume depends on cell density and the surface, e.g., a subconfluent 60 mm dish can be re-suspended in 5 ml of medium.

(g) Leave the tube undisturbed for 3–5 min to allow for the majority of large ES cell clumps and feeders to settle. Alternatively, place the cell suspension back into the original dish and put it back in the incubator for 10 min to allow the MEF to reattach (preplating).

(h) Aspirate the gelatin solution from the plates prepared earlier.

(i) Seed the cells from the top portion of the cell suspension in the tube or from carefully tilted original dish on new gelatinized plates using different dilutions (e.g., 1:10–1:50). For example, 0.2 ml, 0.4 ml, and 0.6 ml of 5 ml cell suspension from a subconfluent 60 mm dish can be plated on three 60 mm plates. Check the cell density and adjust the volume if necessary.

(j) Seed the rest of the cells on one or more plates, they will serve as a back-up and/or may be frozen.

(k) Incubate overnight.

3. Next day lift small colonies of 8–15 cells by gentle trypsinization immediately before the aggregation as follows.

(a) Aspirate the medium and wash the cells first with PBS, and then with trypsin (this is optional, it helps to loosen up the cells and minimize the amount of trypsin in the next step).

(b) Add a minimal amount of trypsin to just cover the cells, e.g., 0.3–0.5 ml per 60 mm plate, place in the incubator for 1–2 min or leave at room temperature. Watch under the microscope, gently swirl the plate to detach the colonies and tap at the microscope stage until all colonies are lifted. Do not over-trypsinize, as cells will become sticky and hard to manipulate.

(c) As soon as the colonies are detached, quickly add ES cell medium to the plates (e.g., 4 ml per 60 mm dish). Do not pipette. However, if ES cell clumps are larger than required 8–15 cells, a very gentle pipetting can be used being careful not to break the clumps into single cell suspension.

4. Loosely connected ES cell clumps (Fig. 16.2f) are now ready for aggregation within next 1–2 h and can be kept in the original plate. Alternatively, for ease of transportation, gently transfer the suspension of clumps into 5 ml tubes (e.g., Falcon 352063 or 352058) using a pipette; be careful not to break them into single cells. Keep the cells at room temperature, as they will start attaching to the plate if placed in the incubator.

Note: If ES cells are grown on MEF up until the day of aggregation, colonies can be lifted by a very short trypsinization at room temperature leaving the majority of feeders behind. Transfer the floating ES cell clumps into new dish with medium, gently pipette if necessary to reach the clumps of the right size.

16.3.6. Assembly of Aggregates

We routinely use two tetraploid embryos at the "4-cell stage" to aggregate with ES cells in a sandwich manner as this method has proven to be more efficient in our hands. However, if it is necessary for the experimental design to have tetraploid mutant embryos of different genotypes that should not be mixed or the number of embryos is not sufficient, single tetraploid embryos may also be used. The aggregates can be assembled in either of the ways described in Subheading 16.3.6, **step 2**; they work equally well and the choice depends upon individual preference. Special care should be taken that all three components of the aggregates (two tetraploid embryos and an ES cell clump) touch each other, so that they form one embryo with integrated ES cells after overnight culture.

1. Under the dissecting microscope, collect ES cell clumps of roughly the required size and transfer them into the microdrops that do not contain depressions for the final selection and rinse from ES cell medium.
2. Select clumps of 8–15 cells and carefully transfer them individually into the depression wells using one of these three approaches, (Fig. 16.3):
   (a) Place the clump of ES cells next to one embryo, then carefully drop the second embryo into the depression to "sandwich" the ES cell clump (Fig. 16.3a).
   (b) Place the clump of ES cells next to two denuded embryos already positioned inside the well and likely fused into one embryo by that time (Fig. 16.3b).
   (c) Distribute the ES cell clumps into all empty depressions of the plate, then drop both denuded embryos into each well on top of ES cell clump (Fig. 16.3c).
3. Assemble all the aggregates in the plate (Fig. 16.2g). Check and make sure that all the embryos touch corresponding ES cell clumps.
4. Carefully put the plate in the incubator and culture overnight.
5. Proceed with the rest of the plates.

16.3.7. Embryo Transfer

The following day the majority of aggregates should reach blastocyst stage with some remaining late morulae (Fig. 16.2h, i) and are ready to be transferred into the uteri of E2.5 pseudopregnant females as described in [18] and elsewhere in this book (Chap. 17). The implantation rate of zona-free embryos is lower than for embryos with the zona intact. Two days in culture and the addition of ES cells decreases this still further. If embryos are to be dissected at different stages to assess in vivo expression we usually transfer 12–15 embryos per recipient. For the experiments left to term 18–22 embryos per recipient are transferred. In case of recipient shortage, aggregates can be transferred into the oviducts of E0.5, the uteri of E3.5 or cultured for an additional night and transferred the following day into E2.5 uteri. All these options work but we find them less efficient that the standard uterine transfer into E2.5 recipients.

16.3.8. Troubleshooting

- Low number of embryos is obtained: test different doses of hormones, time of injection, and age of donor females.
- A lot of embryos are developing to 4-cell stage before they can be fused: adjust the time of superovulation – early hormone injections promote embryo development.
- Embryos are not fusing efficiently: test different pulse parameters changing one at a time, e.g., increasing the voltage or duration and monitoring the time of fusion in different groups of embryos.
- Embryos are lysing after pulse application: likely too high AC field was used. Adjust and test with the same or different pulse parameters.
- Embryos are not aligning in the electrode chamber: too much HEPES-buffered medium was transferred with the embryos into Mannitol drop. Make sure embryos are well equilibrated in Mannitol before moving them to electrode chamber.
- Aggregates do not look viable after overnight culture: too large a clump of ES cells was used.
- Aggregates look like two embryos attached to each other after overnight culture: two embryos and ES cell clump did not touch each other at the time of aggregation to form one embryo.
- There is no pregnancy: embryo transfer surgery technique failed or recipient did not ovulate.
- There are no pups born but there are implantation sites: ES cell quality and culture conditions do not support the survival of ES cell-derived animals. Dissect at mid-gestation to determine the embryonic stage of lethality, test parental ES cell line and other subclones. Try different FBS lot for culture of ES cells.

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