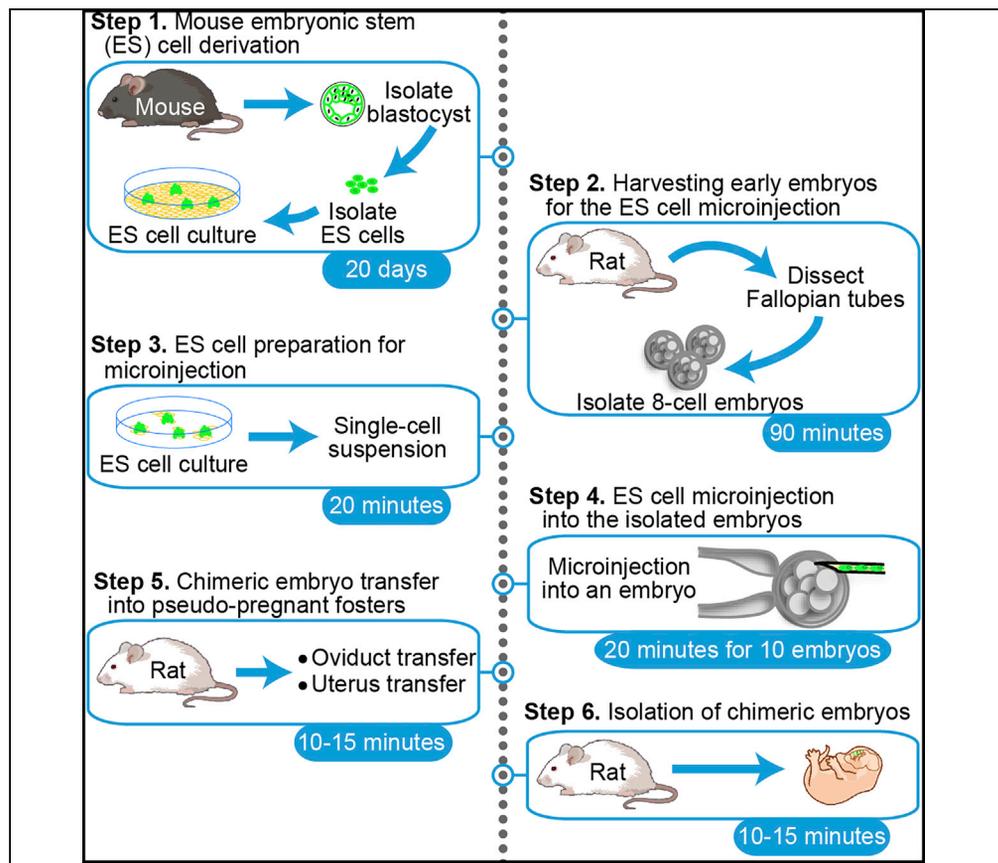


Protocol

Generation of interspecies mouse-rat chimeric embryos by embryonic stem (ES) cell microinjection



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Highlights

Isolation of ES cells from mouse blastocysts and culture of rodent ES cells

Microinjection of mouse ES cells into early rat embryos and vice versa

Transfer of chimeric embryos into pseudo-pregnant foster mothers

Generation of mouse-rat chimeric brains

Interspecies chimerism is a useful tool to study interactions between cells of different genetic makeup in order to elucidate the mechanisms underlying non-cell-autonomous processes, including evolutionary events. However, generating interspecies chimeras with high efficiency and chimerism level remains challenging. Here, we describe a protocol for generating chimeras between mouse and rat. Donor embryonic stem cells of one species are microinjected into early embryos of the other species (recipient), which are implanted into host foster mothers of the recipient species.

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Protocol

Generation of interspecies mouse-rat chimeric embryos by embryonic stem (ES) cell microinjection

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SUMMARY

Interspecies chimerism is a useful tool to study interactions between cells of different genetic makeup in order to elucidate the mechanisms underlying non-cell-autonomous processes, including evolutionary events. However, generating interspecies chimeras with high efficiency and chimerism level remains challenging. Here, we describe a protocol for generating chimeras between mouse and rat. Donor embryonic stem cells of one species are microinjected into early embryos of the other species (recipient), which are implanted into host foster mothers of the recipient species.

For complete details on the use and execution of this protocol, please refer to Stepien et al. (2020).

BEFORE YOU BEGIN

Mouse embryonic stem (ES) cell derivation

⌚ Timing: about 20 days

Note: The present protocol for ES cell derivation is a modification of a standard protocol (Evans and Kaufman, 1981). This step is only necessary if no established or commercial ES line can be used. To allow robust analysis of interspecies chimeras, it is advisable to use ES cells engineered to constitutively express fluorescent proteins for microinjection into non-fluorescent embryos of the other species or into embryos expressing a different fluorescent protein. As an example, we used mouse Tg(ACTB-EGFP)D4Nagy/J-derived GFP-labelled ES cells (Hadjantonakis et al., 1998; Stepien et al., 2020) for microinjection into non-fluorescent Wistar rat wild type embryos. A reverse approach can also be used, e.g., we microinjected non-fluorescent rat DAc8 ES cells (Li et al., 2008) into mouse Tg(ACTB-EGFP) D4Nagy/J line (Hadjantonakis et al., 1998) embryos. We did not derive the rat ES cells ourselves – for a protocol how to do that see (Li et al., 2008) – but used the existing DAc8 ES cell line (Li et al., 2008; Tong et al., 2010).

1. Animal breeding for mouse blastocyst harvesting:
 - a. Set up the mating of the desired mouse ES cell donor strain in the late afternoon or evening (4 days before the planned blastocyst harvesting) by placing 1–2 female mice in a cage with a single male mouse (the preferred age of the animals for optimal embryo yield is between 8–12 weeks for females and 12–18 weeks for males).



Note: As estrous in mice occurs every 3–4 days, plan a sufficiently large mating group to obtain the required number of plugged females, or repeat mating for 3–4 consecutive days if necessary. This point applies also to [before you begin](#) steps 6a and 7a below.

- b. On the morning after mating (designated as embryonic day (E) 0.5, as most mice mate close to the middle of the dark phase of the standard 12/12 light-dark cycle), detect the plug-positive females and move them to fresh cages (wherever possible, keep at least two females in one cage; see [troubleshooting](#)).

Note: In mice, copulation plugs are typically readily visible as a white/yellowish jelly either covering the vulva or located slightly deeper at the entrance of the vaginal canal, and can be observed either directly or upon opening the vagina with the help of round-tipped forceps.

- c. Keep the cages with plugged females in a quiet area to minimize stress until E3.5 – the time point when most embryos reach an early blastocyst stage.

2. Mouse blastocyst harvesting:

- a. At E3.5, sacrifice the plugged females by cervical dislocation.
- b. Immediately dissect out the uteri and place them in a Petri dish (Sarstedt, Tissue Culture Dish, 83.3900.500) containing M2 medium ([Nagy et al., 2003](#)) at 20°C–22°C (for recipe see: [materials and equipment](#)).
- c. Use a 3 mL syringe (Braun Omnifix, Luer Lock) with a 27G × ³/₄" needle to flush M2 medium into the uterine horns to collect the blastocysts from the uteri. Use a light microscope (Zeiss SV6 Stemi ZOOM 0.8×–5×) to make sure you collect all blastocysts.
- d. Add 200 µL of 2i medium ([Silva et al., 2008](#); iSTEM-TaKaRa, Y40010) to each well of a 96-well plate (Thermo Fisher Scientific, 167008).
- e. By mouth pipetting (using a custom-made pipetting device described below), transfer the blastocysts to the 96-well plate (one blastocyst per well). Please see [Methods video S1](#) for how to use the mouth-pipetting device.

Note: The mouth-pipetting device can be bought commercially (BioMedical Instruments) or can be made as follows: (1) take a 3-mm diameter silicon tube, about 60 cm in length (Advanced Medical Systems Ltd., Silicone Tubing 3 mm ID (inner diameter) × 5 mm OD (outer diameter), SKU: T-30-50-10), and cut it into half; (2) to one half, attach a metal pipette holder (BioMedical Instruments) to one end; (3) insert a 0.22-µm replaceable filter (Merck, Millex GV 0.22 µm 33 mm sterile, SLGV033RS) to the other end of this tube; (3) attach the second tube to the inserted filter and; (4) attach a mouth piece (BioMedical Instruments) to the other end of the second tube ([Figure 1A](#)). A handling capillary (BioMedical Instruments, ID: 115–124 µm, TL: 4 cm, pipette length: 12 cm) is then inserted into the pipette holder to transfer the blastocysts from the Petri dish to a 96-well plate.

Note: The same mouth-pipetting device can also be used to (1) transfer 8-cell stage embryos during the washing and microinjection steps, and (2) transfer the embryos into the oviduct or uterus of a pseudo-pregnant female. For transfer of 8-cell stage embryos during the washing and microinjection steps, insert a handling capillary into the pipette holder (see: [harvesting early embryos for the ES cell microinjection](#) and [ES cell microinjection into the isolated embryos](#)). For transfer of embryos into the oviduct or uterus of a pseudo-pregnant female, remove the handling capillary and insert a transfer capillary into the pipette holder (retransfer capillary, BioMedical Instruments, 114–118 µm ID) (see: [chimeric embryo transfer into pseudo-pregnant fosters](#)).

Note: 2i medium (iSTEM-TaKaRa, Y40010) should be prepared as per manufacturer's instructions, and we recommend storing the prepared 2i medium as small aliquots at –20°C.

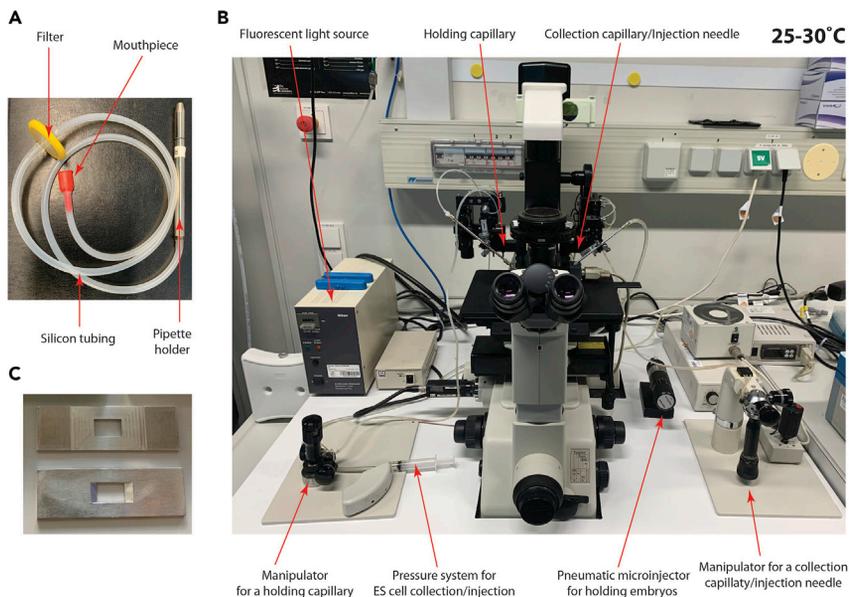


Figure 1. Setup and equipment for handling and microinjection of the embryos

(A) A mouth-pipetting device consisting of the metal pipette holder, the silicon tubing, a filter and a mouthpiece. A handling or transfer capillary should be inserted into the pipette holder to allow embryo handling.

(B) ES cell microinjection setup. Main system components are indicated using arrows. Use as per the instructions from the manufacturer.

(C) The custom-made aluminium plates for the microinjection chamber. A glass coverslip is inserted between these two plates.

3. Mouse blastocyst culture and ES cell isolation:

- a. Culture the blastocysts in the 2i medium for approximately 8 days (in a cell culture incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C), until the inner cell mass (ICM) forms a distinct bulk in the blastocyst cavity.
 - i. Replace half of the medium (100 μL) daily with 100 μL of fresh 2i medium.
- b. Trypsinize the blastocysts to isolate the ES cells:
 - i. Gently aspirate the 2i medium.
 - ii. Cover each blastocyst with 10 μL of chicken serum (with 0.1% trypsin) and incubate for 15 min in an incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
 - iii. Add 200 μL of ES cell culture medium (for recipe see: [materials and equipment](#)) and dissociate the ES cells by gentle pipetting.
 - iv. Transfer the suspension containing the dissociated ES cells to a new well of a fresh 96-well plate (about 1500–2000 cells per well), pre-plated with a confluent layer of inactivated mouse embryonic fibroblasts (MEFs) (see the note below for instructions on how to prepare 10-cm dishes with a confluent MEF layer).

△ CRITICAL: Always pre-warm the ES cell culture medium to 37°C before use.

Note: To prepare 10-cm dishes with a confluent MEF layer, thaw a vial (about 600,000–800,000 cells) of inactivated MEFs in a water bath maintained at 37°C. The MEF cells can either be generated in-house ([Nagy et al., 2006a](#); [Nagy et al., 2006b](#)) or bought commercially (Gibco, A34180). Immediately transfer the thawed cells to a 15-mL Falcon tube (Corning, 430791) and resuspend the cells in 10 mL pre-warmed ES cell culture medium (for recipe see: [materials and equipment](#)). Centrifuge the suspension at 123 × g (Heraeus, Biofuge Primo R) for 5 min at 20°C–22°C. Aspirate the supernatant and resuspend the cell pellet in

8 mL of fresh ES cell culture medium. To prepare one 10-cm dish (Corning, Falcon 100 mm TC-treated Cell Culture Dish, 353003) with a confluent MEF layer, add 10 mL of fresh ES cell culture medium to the dish and then add 2 mL of the MEF suspension (about 45,000–50,000 cells per 10-cm cell culture dish).

Note: The prepared dishes with inactivated MEF layer require fresh medium change every 24 h and should be used within maximum 2 weeks.

Note: The amount of cell culture medium needed and the size of the cell culture dish should be adjusted depending on the growth characteristics of the cells under study.

- c. Incubate the suspension containing the dissociated ES cells in an incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
- d. Change the medium at least once every 24 h by aspirating the old medium and adding new ES cell culture medium (200 μL per well of a 96-well plate).
- e. Split the ES cells once they reach a sub-confluent stage:
 - i. Check the growing ES cell culture under a microscope (on alternate days; 1–2 times a day), until the cells reach a sub-confluent stage.
 - ii. Aspirate the old medium and add 200 μL of pre-warmed PBS for washing.
 - iii. Aspirate the PBS and add 100 μL of chicken serum (with 0.1% trypsin) per well (for recipe see: [materials and equipment](#)).
 - iv. Incubate for 5 min in a cell culture incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
 - v. Resuspend the trypsinized cells by adding 100 μL of fresh ES cell culture medium per well (for recipe see: [materials and equipment](#)). Alternatively, at this stage, DMEM-FCS only medium (for recipe see: [materials and equipment](#)) can also be used.
 - vi. Centrifuge the suspension for 5 min at 123 × g (Heraeus, Biofuge Primo R).
 - vii. Aspirate the supernatant and add 200 μL of fresh ES cell culture medium per well to re-suspend the cells.
 - viii. Split the resuspended cells 1:4 by adding 1 volume of the resuspended cells to 3 volumes of fresh ES cell culture medium. For example, in a new 96-well plate, pre-plated with a confluent MEF layer, add 150 μL of fresh ES cell culture medium per well and then add 50 μL of the resuspended cells to each well.

△ CRITICAL: ES cells grow very fast. A delay in either changing the ES cell culture medium or splitting them on time, results in differentiation and loss of pluripotency. Please see [Figure 2](#) for representative images showing both mouse and rat, healthy appearing, ES cells after various days of culture.

- f. After 2–3 passages in the 96-well plate, split the ES cells 1:4 and plate them onto 10-cm dishes (Corning, Falcon 100 mm TC-treated Cell Culture Dish, 353003).
- g. As soon as passaging of the ES cells on the 10-cm dishes has yielded three subsequent sub-confluent dishes, cryopreserve the ES cells in 8–12 cryovials (CryoPure Tube, 1.6 mL, Sarstedt, 72.380.007) and store them in liquid nitrogen until further use:
 - i. Pre-cool the empty cryovials on dry ice.
 - ii. Aspirate the ES cell culture medium from the 10-cm dish and add 1 mL of pre-warmed PBS for washing.
 - iii. Aspirate the PBS and add 1 mL of chicken serum (with 0.1% trypsin) (for recipe see: [materials and equipment](#)).
 - iv. Incubate for 5 min in a cell culture incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
 - v. Resuspend the trypsinized cells in 1 mL of fresh ES cell culture medium (for recipe see: [materials and equipment](#)).

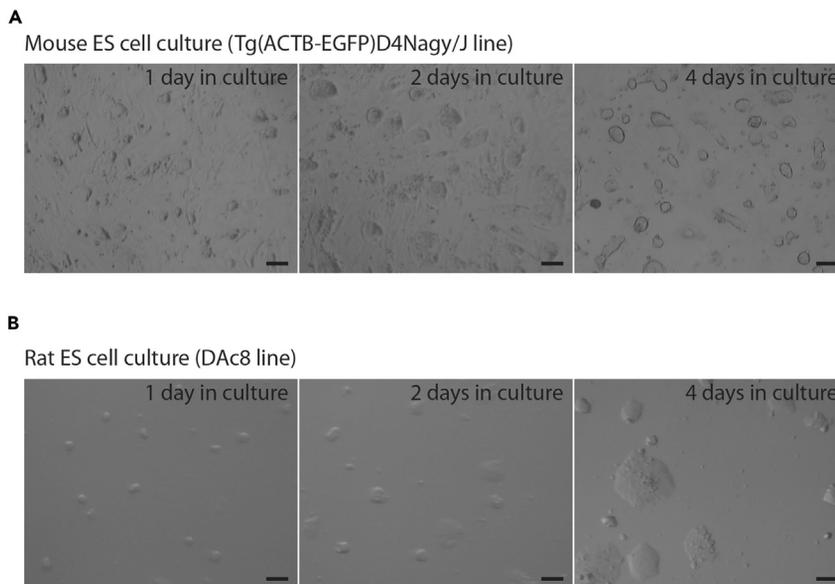


Figure 2. Images showing ES cells after different days of culture

(A) Bright-field microscopic image of mouse ES cells.

(B) Bright-field microscopic image of rat ES cells. Scale bars, 250 μ m.

- vi. Transfer the resuspended cells to a 15-mL Falcon tube (Corning, 430791) and centrifuge the suspension for 5 min at $123 \times g$ (Heraeus, Biofuge Primo R).
- vii. Aspirate the supernatant and resuspend the cell pellet in 8–10 mL of ES cell freezing medium (10% (vol/vol) DMSO / 90% (vol/vol) ES cell culture medium; for recipe see: [materials and equipment](#)).
- viii. Save a small sample of the ES cells for mycoplasma testing by PCR (a few microliters are typically sufficient).
- ix. Fill each of the pre-cooled cryovials with 1 mL of the ES cell suspension.
- x. Close the tubes and incubate on dry ice for 1 h.
- xi. Transfer the tubes to liquid nitrogen for long-term storage.

△ CRITICAL: Always prepare fresh ES cell freezing medium for freezing the ES cells.

ES cell culture for embryo microinjections

⌚ **Timing:** 6–7 days

4. Mouse ES cell culture for rat embryo microinjections:
 - a. Thaw a vial of mouse ES cells (either of the desired, established or commercial, mouse ES cell line, or of ES cells obtained by derivation as described in [before you begin](#)):
 - i. Pre-warm the ES cell culture medium (for recipe see: [materials and equipment](#)) in a water bath maintained at 37°C.
 - ii. Thaw a vial of ES cells (100,000 cells) in a 37°C water bath, transfer the thawed cells to a 15 mL Falcon tube (Corning, 430791), and immediately mix the ES cells with 10 mL of the pre-warmed ES cell culture medium.
 - iii. Centrifuge the suspension for 5 min at $123 \times g$ (Heraeus, Biofuge Primo R).
 - iv. Aspirate the supernatant and resuspend the cell pellet in 12 mL of fresh ES cell culture medium.
 - v. Plate the cells onto a 10-cm dish (Corning, Falcon 100 mm TC-treated Cell Culture Dish, 353003), pre-plated with a MEF feeder cell layer.

△ **CRITICAL:** Brief washing of the thawed ES cells with fresh ES cell culture medium is important to remove the DMSO.

- b. Culture the plated ES cells in a cell culture incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
- c. Change the medium at least once every 24 h by aspirating the old medium and adding the same volume of fresh ES cell culture medium.
- d. Split the ES cells (typically 1:4) 2–3 times (as described in [before you begin](#) step 3e) to establish a robust culture with a sufficient number of ES cells for the microinjection procedure.

△ **CRITICAL:** ES cells grow very fast. Please see [Figure 2](#) for representative images of mouse and rat ES cells after different days of culture. A delay in either changing the ES cell culture medium or splitting them on time, results in differentiation and loss of pluripotency. When plating the ES cells, always plate them over a fresh confluent MEF feeder layer (prepared as in [before you begin](#) step 3b) and change the medium daily (as described in [before you begin](#) step 3d).

Note: When expanding the ES cell culture and splitting the cells, always plate them on a fresh confluent layer of MEF feeder cells. However, on the day before embryo microinjections, split the ES cells and plate them onto a dish with no feeder cells. This reduces the number of fibroblasts in the ES cell suspension, thus making it easier to pick single ES cells for microinjection.

5. Rat ES cell culture for mouse embryo microinjections:

- a. Thaw a vial of the desired rat ES cell line (in our case: DAc8 ES cell line ([Tong et al., 2010](#))):
 - i. Pre-warm the 2i medium (iSTEM-TaKaRa, Y40010) in a water bath maintained at 37°C.
 - ii. Prepare gelatin-coated plates: (1) Prepare a 0.2% gelatin solution in water (Bovine gelatin powder, Sigma-Aldrich, G9391) and sterilize the solution by autoclaving. (2) Under sterile conditions, add 500 µL of the gelatin solution to each well of a 12-well cell culture plate (ThermoFisher Scientific, Nunclon Delta Surface, 150628) and leave the plates at 20°C–22°C for 1 h. (3) Remove the gelatin solution and let the plates dry for 10–15 min under a laminar flow hood.

Note: The prepared gelatin-coated plates can be sterilized (if needed) under UV light for a minimum of 30 min.

Note: The prepared gelatin-coated plates can be sealed with Parafilm and stored at 4°C for up to a week.

- iii. Thaw a vial of ES cells (100,000 cells) in a 37°C water bath, transfer the thawed cells to a 15 mL Falcon tube (Corning, 430791), and immediately mix the ES cells with 10 mL of the pre-warmed ES cell culture medium.
- iv. Centrifuge the suspension for 5 min at 123 × g (Heraeus, Biofuge Primo R).
- v. Aspirate the supernatant and resuspend the cell pellet in 1.5 mL of fresh 2i medium (iSTEM-TaKaRa, Y40010).
- vi. Plate the cells onto a gelatin-coated plate prepared as above (about 250,000 cells per well of 12-well plate).
- b. Culture the plated ES cells in 2i medium (iSTEM-TaKaRa, Y40010) in a cell culture incubator (Heraeus, Heracell incubator), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
- c. Change the medium at least once in every 24 h by aspirating the old medium and adding the same volume of fresh 2i medium.
- d. Split the ES cells (see below) 2–3 times (as described in [before you begin](#) step 3e) to establish a robust culture with a sufficient number of ES cells for the microinjection procedure.

Note: Rat ES cells in 2i medium typically grow faster than mouse ES cells in ES cell culture medium (see [Figure 2](#)), and therefore, may require more frequent splitting, or splitting at a 1:8 ratio instead of the standard 1:4 ratio.

Note: On the day before microinjection, split the rat ES cells and plate them onto a fresh 6-well plate (ThermoFisher Scientific, Nunclon Delta Surface, 140675) instead of a 12-well plate (about 100,000 – 150,000 cells per well of 6-well plate).

△ **CRITICAL:** Make sure that the ES cells never overgrow – check the plates twice a day, and split the cells when the culture reaches around 50% confluency (see Figure 2).

Embryo donor and foster animal breeding

⌚ **Timing:** 3 days for mouse and 4 days for rat

6. Donor animal breeding for the collection of embryos for microinjection:

Note: In-house animal breeding to collect early-stage embryos is not always necessary, as plugged donor females can often be obtained commercially. In our study, we acquired pregnant Wistar strain (CrI:WI) rat donor females directly from Charles River Laboratories, while the Tg(ACTB-EGFP)D4Nagy/J mouse line was bred in-house. As in-house animal breeding for a limited number of experiments is not always economical and may lead to surplus animals, it must always be evaluated as per the 3Rs of the animal welfare policy.

Note: The overall quality of embryos, which is very important for ES cell microinjection, is higher from natural matings, and therefore we do not recommend using methods of super-ovulation to obtain pregnant females.

- a. Set up mating (in the late afternoon or evening) of mice or rats of the desired strain by placing 1–2 females in a cage with a single male.

Note: The mating should be set up 3 days before the planned 8-cell stage embryo harvesting from mice, and 4 days before the planned 8-cell stage embryo harvesting from rats.

Note: The preferred age of the animals for optimal embryo yield is between 8–12 weeks for mouse females, 10–16 weeks for rat females, and 10–18 weeks for males of either species. Using too young or too old animals compromises the number of available, good-quality embryos.

- b. Detect the plug-positive females on the morning after mating (designated as embryonic day (E) 0.5) and move the plugged females to a fresh cage.

Note: In mice, copulation plugs are typically readily visible as a white/yellowish jelly either covering the vulva or located slightly deeper at the entrance of the vaginal canal, and can be observed either directly or upon opening the vagina with the help of round-tipped forceps. In rats, however, the copulation plugs are typically located deeper in the vaginal canal and can often be observed only with the help of a speculum, which is used to gently open the entrance to the vagina to expose the plug. Therefore, only experienced animal caretakers should do the plug detection in rats.

- c. Keep the cages with plugged females until E2.5 for mice and E3.5 for rats – the respective optimal time points for harvesting 8-cell stage embryos from these species.

Note: To minimize stress, keep the plugged females in a quiet area and avoid daily handling.

7. Animal breeding for pseudo-pregnant foster animals:

- a. Set up the mating (in the late afternoon or evening) of the females of the desired foster strain by placing 1–2 females in a cage with a single vasectomized male.

△ **CRITICAL:** For oviduct transfer, pseudo-pregnant foster animals are needed at an equivalent stage of E0.5, and therefore the mating should be set up one day before the planned embryo transfer. For uterus transfer, however, pseudo-pregnant foster animals are

needed at an equivalent stage of E2.5, and therefore the mating should be set up three days before the planned embryo transfer.

Note: The preferred age of the females for successful surgery and high implantation rates is between 9–12 weeks for mice and 10–14 weeks for rats. The age of vasectomized males is of less importance provided they are healthy and can continue to mate readily.

Note: Vasectomized mouse or rat males can be generated in-house or obtained commercially. Choose strains that are healthy, non-aggressive and that show robust mating behavior. In our hands, vasectomized males from mouse outbred strains (e.g., RjOrl:SWISS or Crl:CD1(ICR); in-house colony) and rat Sprague Dawley strain (Crl:CD(SD); Charles River Laboratories) worked well.

Note: Three weeks prior to the start of the experiment, the vasectomized males of either species were continuously mated with training females of the respective species to ensure a robust mating behavior.

Note: Choose healthy strains for foster females, which are relaxed during handling and have good behavior of maternal care. In our hands, mouse outbred strains (e.g., RjOrl:SWISS or Crl:CD1(ICR); in-house colony) and rat Sprague Dawley strain (Crl:CD(SD); Charles River Laboratories) worked well.

- b. Check for the plug-positive females (as described in step 6b above) on the morning after mating (designated as E0.5) and move the plugged females to a fresh cage (whenever possible, keep at least two females in one cage; see [troubleshooting](#)).
- c. To prevent any stress, keep the plugged females in a quiet environment until surgery.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken polyclonal anti-GFP	Aves Labs	Cat#GFP-1020; RRID:AB_10000240
Goat anti-chicken Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11039; RRID: AB_2534096
Chemicals, peptides, and recombinant proteins		
Sodium pyruvate	Sigma-Aldrich	Cat#P4562
Calcium chloride dihydrate	Merck	Cat#1023820500
Sodium chloride	Merck	Cat#106401000
Potassium chloride	Merck	Cat# 1049361000
Potassium dihydrogen phosphate	Merck	Cat# 1048731000
Magnesium sulfate heptahydrate	Merck	Cat#1058861000
Sodium lactate	Sigma-Aldrich	Cat#L7900
Glucose	Sigma-Aldrich	Cat#G6152
Penicillin	Sigma-Aldrich	Cat#P3032
Streptomycin	Sigma-Aldrich	Cat#S9137
Sodium bicarbonate	Merck	Cat#1063291000
Phenol red	Sigma-Aldrich	Cat#P3532
HEPES	Roth	Cat#91053
2-Mercaptoethanol	Sigma-Aldrich	Cat#M6250
DMSO	Sigma-Aldrich	Cat#276855
EDTA disodium salt	Merck	Cat#1084181000
Bovine gelatin powder	Sigma-Aldrich	Cat#G9391
KnockOut DMEM	Gibco	Cat#10829018
2i	iSTEM-Takara	Cat#Y40010

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant human LIF	In-house	N/A
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A3912
Fetal calf serum	Sigma-Aldrich	Cat#F7524
MEM	Gibco	Cat#11140035
L-Glutamine	Gibco	Cat#25030024
Penicillin-streptomycin 10,000 U/mL	Gibco	Cat#15140122
Trypsin	Thermo Fisher Scientific	Cat#15090046
Chicken serum	Thermo Fisher Scientific	Cat#16110082
Ketamine 100 mg/mL	WDT/Medistar	Ketamidor
Xylazine 2%	WDT/Medistar	N/A
Acepromazine	CP-Pharma	Tranquisol 1%
<i>Experimental models: cell lines</i>		
Mouse: Tg(ACTB-EGFP)D4Nagy/ J ES cells	(Hadjantonakis et al., 1998)	N/A
Rat: DAC8 ES cells	(Li et al., 2008; Tong et al., 2010)	RRID:CVCL_E220
<i>Experimental models: organisms/strains</i>		
Mouse: Crl:CD1(ICR)	In-house colony	N/A
Mouse: RjOrl:SWISS	In-house colony	N/A
Rat: Wistar	Charles River Laboratories Inc.	Crl:WI(Han); RRID:RGD_2308816
Rat: Sprague Dawley	Charles River Laboratories Inc.	Crl:CD(SD); RRID:RGD_734476
<i>Software and algorithms</i>		
Fiji ImageJ 2.0.0-rc-61/1.51h	(Schindelin et al., 2012)	https://fiji.sc/ RRID:SCR_002285
<i>Other</i>		
Zeiss sv6 stemi	Zeiss	N/A
Axiovert 200 M microscope	Zeiss	N/A
96-Well plate	Thermo Fisher Scientific	Cat#167008
Mouth-pipetting device	BioMedical Instruments	N/A
Silicone tube	Advanced Medical Systems Ltd	Cat#SKU: T-30-50-10
Metal pipette holder	BioMedical Instruments	N/A
0.22- μ m Replaceable filter	Merck	SLGV033RS
Mouth piece	BioMedical Instruments	N/A
Handling capillary	BioMedical Instruments	VRE-ID-TL
Cryovials	CryoPure	Cat#72.380.007
Vacuum filtration system	Corning Filter System	Cat#431153
Manipulators	Narishige	MMN-1 and MMO-4
Celltram 4r Air pneumatic microinjector	Eppendorf	Cat#5196000013
Fluorescent light source	Nikon	HB-10104
Laser source	Hamilton Thorne Biosciences	XYClone laser system
Holding capillary	Harvard Apparatus	GC100T-15
Micropuller	Sutter Instruments	P-97
Microforge	Narishige	MF-900
Collection capillaries/injection needles	BioMedical Instruments	VRE-ID-TL
Heating pad	Labortechnik Göttingen	Hot Plate 062, 13854
Gas anesthesia apparatus	Harvard Apparatus	Cat#34-1041SV
Activated charcoal filters	Eickemeyer Or VetEquip	Cat#21348801 Cat#931401
Surgical sutures	Ethicon	Cat#V493H
Autoclip and Autoclip Applier	Agthos	Cat#59023 And Cat#59024
Acupuncture needle	SEIRIN	J-type, no.8
Plastic Pasteur pipette	Fisherbrand	Cat#13439108
Isoflurane	WDT	Cat#21311

MATERIALS AND EQUIPMENT

M2 medium	Final concentration	Amount
Stock A (see below)	N/A	10 mL
Stock B (see below)	N/A	1.6 mL
Stock C (32.7 mM sodium pyruvate)	0.327 mM	1 mL
Stock D (171.4 mM calcium chloride dihydrate)	1.714 mM	1 mL
Stock E (see below)	N/A	8.4 mL
BSA	4 mg/mL	400 mg
ddH ₂ O	N/A	78 mL
Total	N/A	100 mL

Note: The M2 medium has to be filtered before use. Use a vacuum filtration system with a 0.22 μm filter (Corning Filter System, 431153). Filtered M2 medium can be stored at –20 °C for 6 months.

Stock A for M2 medium	Final concentration	Amount
Sodium chloride	9.5 M	5.534 g
Potassium chloride	47.75 mM	0.356 g
Potassium dihydrogen phosphate	12 mM	0.162 g
Magnesium sulfate heptahydrate	12 mM	0.293 g
Sodium lactate	0.2329 M	2.61 g
Glucose	55.5 mM	1 g
Penicillin (1650 U/mg)	462 U/mL	0.028 g
Streptomycin (765 U/mg)	803.25 U/mL	0.105 g
ddH ₂ O	N/A	to 100 mL
Total	N/A	100 mL

Stock B for M2 medium	Final concentration	Amount
Sodium bicarbonate	250 mM	2.101 g
Phenol red	0.28 mM	0.01 g
ddH ₂ O	N/A	to 100 mL
Total	N/A	100 mL

Stock E for M2 medium	Final concentration	Amount
HEPES	250 mM	5.958 g
Phenol red	0.28 mM	0.01 g
ddH ₂ O	N/A	to 100 mL
Total	N/A	100 mL

Note: Adjust stock E for M2 medium with NaOH to pH 7.4. All stock solutions can be stored at –20 °C for 6 months.

ES cell culture medium	Final concentration	Amount
Knockout DMEM medium	0.77 ×	385 mL
Fetal calf serum (FCS), ES cell-tested and heat-inactivated	20%	100 mL
2-Mercaptoethanol	0.1 mM	3.5 μL
Non-essential amino acids	1 ×	5 mL
L-Glutamine (200 mM)	2 mM	5 mL
Recombinant human LIF 2,500,000 U/mL (made in-house)	1000 U/mL	200 μL
Penicillin-Streptomycin 10,000 U/mL	100 U/mL	5 mL
Total	N/A	500 mL

Note: ES cell culture medium can be stored at 4 °C for 7 days. Of the various components of the ES cell culture medium, 2-mercaptoethanol should be stored at 20 °C–22 °C, non-essential amino acids at 4 °C, and all others at –20 °C, until the expiry date.

Alternatives: In principle any ES cell-tested serum and other ES cell culture grade components can be used; e.g., L-glutamine can be replaced by GlutaMax Supplement (ThermoFisher Scientific, 35050061), and human LIF can be replaced by mouse recombinant LIF (ThermoFisher Scientific, A35933).

△ **CRITICAL:** Test beforehand how the ES cells to be used perform in the ES cell culture medium and the cell culture system, and avoid changing ingredients (including using different batches) over the course of the experiment. This is particularly important for the FCS.

△ **CRITICAL:** Inhalation, ingestion or skin contact with 2-mercaptoethanol is harmful. It can cause nasal/respiratory tract irritation, skin irritation, vomiting and stomach pain. Store in a chemical safety cabinet. Wear personal protection and use a fume hood while handling. Consult the safety data sheet before use.

ES cell freezing medium	Final concentration	Amount
ES cell culture medium (see above)	90%	9 mL
DMSO	10%	1 mL
Total	100%	10 mL

Note: The freezing medium should be prepared fresh every time, immediately before use.

△ **CRITICAL:** Inhalation, ingestion or skin contact with DMSO is harmful. Use personal protective equipment and handle with caution. Consult the safety data sheet before use.

KSOM medium	Final concentration	Amount
Stock A (see below)	N/A	10 mL
Stock B (see below)	N/A	10 mL
Stock C (20 mM sodium pyruvate)	0.2 mM	1 mL
Stock D (170 mM calcium chloride dihydrate)	1.7 mM	1 mL
Stock F (0.1 M EDTA disodium salt)	10 μM	10 μL
Stock G (200 mM L-glutamine)	1 mM	0.5 mL
BSA	1 mg/mL	100 mg
ddH ₂ O	N/A	to 100 mL
Total	N/A	100 mL

Note: Adjust KSOM medium with HCl to pH 7.4. The medium has to be sterile-filtered before use. Use a vacuum filtration system with a 0.22-μm filter (Corning Filter System, 431153). Filtered KSOM medium can be stored at –20 °C for 6 months.

Stock A for KSOM medium	Final concentration	Amount
Sodium chloride	1 M	5.55 g
Potassium chloride	25 mM	0.186 g
Potassium dihydrogen phosphate	3.5 mM	0.0476 g
Magnesium sulfate heptahydrate	2 mM	0.0493 g

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Stock A for KSOM medium	Final concentration	Amount
Sodium lactate (60% solution, 1.32 g/L)	0.86%	1.87 g
Glucose	2 mM	0.036 g
Penicillin (1650 IU/mg)	990 U/mL	0.06 g
Streptomycin (765 IU/mg)	382.5 U/mL	0.05 g
ddH ₂ O	N/A	to 100 mL
Total	N/A	100 mL
Stock B for KSOM medium	Final concentration	Amount
Sodium bicarbonate	250 mM	2.1 g
Phenol red	28 μM	0.001 g
ddH ₂ O	N/A	to 100 mL
Total	N/A	100 mL
Chicken serum trypsin solution	Final concentration	Amount
PBS (made in-house)	≈ 1 ×	475 mL
EDTA disodium salt	0.68 mM	0.1 g
Glucose	5.6 mM	0.5 g
Chicken serum	1%	5 mL
Trypsin (2.5%)	0.1%	20 mL
Total	N/A	500 mL

Note: The trypsin solution has to be sterile-filtered before use. Use a vacuum filtration system with a 0.22-μm filter (Corning Filter System, 431153). Aliquot into 20 mL tubes and store at –20°C for up to 6 months.

Ketamine-based anesthesia mixture	Final concentration	Amount
Ketamine (100 mg/mL)	5 mg/mL	1 mL
Xylazine (2%)	0.08 %	0.8 mL
Acepromazine (1%)	0.015%	0.3 mL
Physiological buffer or saline (e.g., 0.9% NaCl)	N/A	to 20 mL
Total	N/A	20 mL

Note: Anesthetic solution is stable for up to 3 months at 4°C–8°C.

ES cell microinjection setup

In principle any type of manipulator which works for ES cell microinjections can be used. The microinjection setup used in this study (Figure 1B) had two manipulators (Narishige MMN-1 and MMO-4), (1) the holding capillary manipulator to immobilize the embryo for microinjection, and (2) the collection capillary / injection needle manipulator to both collect and inject the ES cells into the embryo. Each manipulator had a Three Axis Hydraulic Joystick system attached to them to enable its smooth three-dimensional movement. Each manipulator also had an attached metal holder for either the holding capillary or the collection capillary / injection needle, depending on the manipulator. (Note that the term "collection capillary" and the term "injection needle" refer to the same glass capillary and are used when describing the collection and microinjection, respectively, of the ES cells.) To immobilize the embryos at the holding capillary, a pneumatic microinjector (Eppendorf, CellTram 4r Air) connected to the holding capillary was used to control the holding pressure. For both, the collection of the ES cells into the collection capillary and their microinjection from that capillary (i.e., the injection needle) into the embryo, we used a custom-made arrangement. One end of a silicone tube was attached to the end of the capillary holder of the collection capillary / injection needle, and a 5-mL syringe was attached to the other end of the tube. Alternatively, a second

pneumatic microinjector (Eppendorf, CellTram 4r Air) can be attached to the other end of the tube with the collection capillary / injection needle. The syringe was used to manually control the pressure needed to both collect and inject the ES cells. Please note that for both the holding capillary and the collection capillary / injection needle, the respective pressure control systems were placed on the opposite side to the respective manipulators. This arrangement allowed us to control the pressure with one hand and the needle position with the other.

The setup was also equipped with a fluorescent light source (Nikon HB-10104, Mercury lamp power supply) to detect the fluorescence and with a laser source (Hamilton Thorne Bioscience, XYClone laser system) for laser-assisted microinjections.

Holding capillaries and collection capillaries/injection needles

Holding capillaries, with an inner diameter of 110 μm , were pulled from glass capillaries (Harvard Apparatus, GC100T-15) using a micropuller (Sutter Instruments Puller). The tip was cut open and fire-polished with a Narishige Microforge (MF-900) to generate an open end with an inner diameter of 25 μm . Since the metal holder for the holding capillary had an oblique position (Figure 1B), the holding capillary at a position about 1 mm proximal to its open tip was bent to an angle of 30° so as to position this part parallel to the surface of the microinjection chamber.

Regarding the ES cell collection capillaries / injection needles, the end of these capillaries had a spike-shape, which allows the piercing through the robust zona pellucida of an 8-cell stage embryo. These collection capillaries / injection needles can be made in-house or be purchased commercially. The collection capillaries / injection needles used in this study were manufactured by BioMedical Instruments. The shapes of a holding capillary and a collection capillary / injection needle can be seen in [Methods videos S2, S3, and S4](#).

Surgical setup

The surgical setup consisted of an operating table equipped with a heating pad (Labotect, Hot Plate 062, 13854) and additionally, in case of isoflurane-based inhalatory anesthesia, a gas anesthesia apparatus (Harvard Apparatus, Isoflurane Key-Fill Vaporizer, 34-1041SV). The gas anesthesia apparatus consisted of (1) an oxygen source, (2) an isoflurane source and gas mixer (the vaporizer), (3) a tubing system connected to the vaporizer on one end and to either a plexiglass box (for initial anesthesia) or mask (for continuous gas delivery during surgery) on the other end, (4) valves for controlling the delivery of the anesthesia gas mixture, (5) activated charcoal filters to adsorb excess isoflurane gas (Eickemeyer, EickSorber Narkosegasfilter, 21348801 or VetEquip, VaporGuard, 931401), and (6) a stereo microscope (Zeiss Stereo Zoom microscope, SV6).

Alternatives: Other types of heating tables and magnifying devices (including a light source) can be used. Examples of mouse and rat surgical setups are shown in [Figure 3A](#).

A set of surgical tools and components, necessary or helpful during the procedure, is shown in [Figure 3B](#). It includes a set of sharp- and blunt-end forceps and scissors, surgical sutures (Ethicon, Vicryl, P3, USP 5-0, 45 cm, V493H), Autoclip and Autoclip Applier (Agnthos, 59023 and 59024), acupuncture needle (used specifically for rat oviduct transfers: SEIRIN, J-type No.8 (0.30) \times 30 mm) and a plastic Pasteur pipette (FisherBrand, 13439108) for applying warm PBS over the operation site. Clean and heat-sterilize the tools before use (unless disposable tools are used).

△ CRITICAL: Take care when using isoflurane-based anesthesia. Isoflurane is harmful upon repeated exposure and may cause irritation to mucosal membranes and drowsiness. Make sure the isoflurane delivery system is free of leaks, and weigh the activated charcoal filters before and after each use in order to ensure that their adsorption capacity is not exceeded. Ensure proper ventilation of the room. Consult the safety data sheet before use.

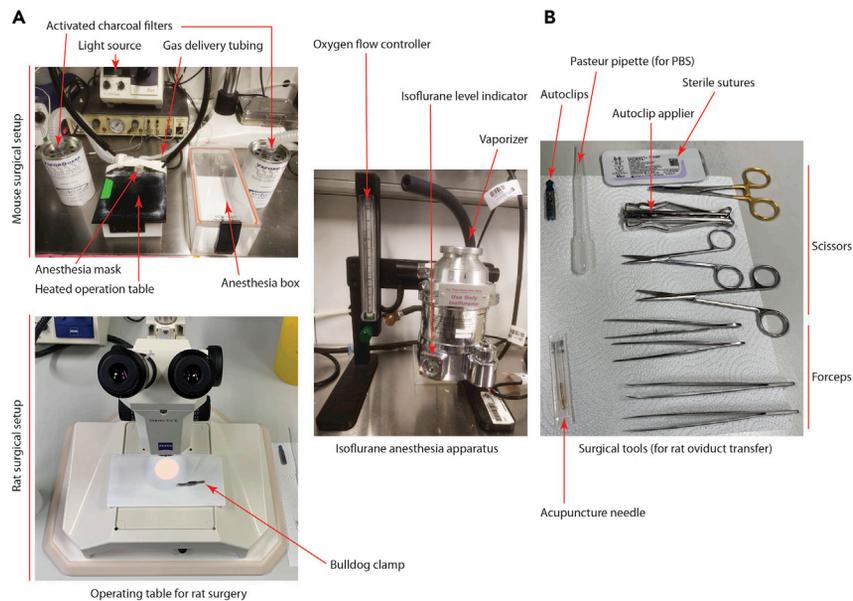


Figure 3. Surgery setups and the surgical tools for the embryo transfer procedure

(A) Examples of the surgical setup. Top left image – surgical setup for mouse surgery showing the operating table (with temperature control), a light source, anesthesia box, a gas delivery tubing, a mask attached to the operating table and two activated charcoal filters. Right image – a vaporizer with the isoflurane source and an attached oxygen flow controller. Bottom left image – operating table for rat surgery.

(B) Surgical tools. Setup components and tools are indicated using arrows.

STEP-BY-STEP METHOD DETAILS

Note: This protocol for the generation of interspecies mouse→rat or rat→mouse chimeric embryos has been based on the standard mouse chimera generation protocol (Poueymirou et al., 2007).

Harvesting early embryos for the ES cell microinjection

⌚ Timing: 90 min, followed by incubation for up to 4 h

In the following steps, 8-cell stage embryos from either mouse or rat are collected and prepared for the microinjection with the ES cells of the respective other species.

1. Embryo collection and washing:

- a. Sacrifice the plugged donor females (E2.5 mice or E3.5 rats) by cervical dislocation. In the case of rat, anesthetize the animals with 4% isoflurane before cervical dislocation.

Note: For breeding the animals, we used a light-dark cycle of 12 h, starting at 6 p.m. Under this condition, to ensure the right stage of the embryos (8-cell stage), embryo isolation should be done early in the morning (before 9 a.m.). Alternatively, blastocysts can also be used for microinjections; in that case, collect mouse blastocysts at E3.5 and rat blastocysts at E4.5.

Note: If the embryos are not collected early in the morning, some of the embryos may also enter the uterine horn, in which case flushing of the uterine horn may also be required to collect all the embryos. Depending on the light-dark cycle of breeding and the strain of the animal used, one may need to standardize the timing of embryo collection.

- b. Immediately dissect out the Fallopian tubes and place them in a Petri dish (Tissue Culture Dish, Sarstedt, 83.3900.500) containing M2 medium (for recipe see: [materials and equipment](#)).
- c. Use a needle (Hamilton, 33-gauge N-Needle, 90033) screwed on a 3-mL syringe (Braun Omnifix, 4617022V) to flush the embryos out of the oviducts (at 20°C–22°C).
 - i. When collecting from mice, flush the oviducts by injecting the M2 medium directly into the ampulla of the oviduct.
 - ii. When collecting from rats, first rupture the oviducts with fine forceps and a 27G × 3/4" needle (BD-Microlance, 302200), and then use the syringe to flush the embryos out with M2 medium.

△ **CRITICAL:** Unlike in mouse, it is not easy to flush the embryos from rat oviducts, and therefore rupturing of the oviduct, as described in step 1cii, is extremely important.

- d. The flushed embryos are then collected by mouth-pipetting (using a custom-made pipetting device as described in [before you begin](#), step 2e, equipped with a handling capillary) and transferred to a fresh Petri dish filled with M2 medium.

Note: Typically, one can expect to collect 6–8 good-quality embryos per one rat donor (Wistar Crl:WI strain) and 4–6 good-quality embryos per one mouse donor (in our case Tg(ACTB-EGFP)D4Nagy/J line in BL6 background). Embryo yield can differ, and may be lower, if inbred or sub-healthy animals are used as donors.

- e. In a separate Petri dish, wash the isolated embryos 10 times in drops of M2 medium (100 µl per drop):
 - i. Fill the handling capillary with a small amount of M2 medium and introduce an air bubble. This air bubble serves to prevent the capillary forces to cause an uncontrolled aspiration of the embryos.
 - ii. Pick all the healthy 8-cell embryos by mouth pipetting (using the custom-made pipetting device described in [before you begin](#), step 2e, equipped with a handling capillary) and wash them in the first drop of M2 medium.

Note: After all the embryos are transferred to the first M2 medium drop, either change the handling capillary or clean it with 70% ethanol before using it to move the embryos to the next drop of M2 medium.

- iii. Using the handling capillary, collect the embryos from the first drop of the M2 medium and pass the embryos to the second drop of M2 medium.

Note: When transferring the embryos, first fill the capillary with the fresh M2 medium from the next drop and do not forget to introduce the air bubble.

- iv. Repeat the above steps until all the embryos are in the last drop of the M2 medium – the embryos are considered clean after having been moved through 10 drops and thereby washed to remove debris.

2. Embryo maintenance prior to the microinjection:

- a. After the embryos have been washed, move all of them to 200 µl of KSOM medium ([Nagy et al., 2003](#)) in a Petri dish (Sarstedt, Tissue Culture Dish, 83.3900.500) and cover the KSOM medium with paraffin oil (Merck, 1.07160.1000).
- b. Incubate the isolated embryos in a cell culture incubator (Labotect, C16), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C, until the start of microinjection. They can be kept for optimally 1–2 h, but no longer than 4 h.

ES cell microinjection into the isolated embryos

⌚ **Timing:** 20 min per batch of embryos (do not exceed 4 h in total)

In the following steps, the embryos from the recipient species are microinjected with the ES cells of the donor species (e.g., rat embryo microinjected with mouse ES cells or *vice versa*) to generate chimeric embryos for transfer to the pseudo-pregnant foster females of the recipient species.

3. Prepare a single-cell ES cell suspension (from cells cultured as described in [before you begin](#), steps 4 or 5):
 - a. Aspirate the culture medium (mouse ES cell culture medium from mouse ES cells or 2i medium from rat ES cells) from the ES cell culture dishes/plates and wash the ES cells with 1 mL of pre-warmed PBS per 10-cm dish or per well of the 12-well plate.
 - b. Aspirate the PBS and add chicken serum (with 0.1% trypsin) (for recipe see: [materials and equipment](#)) – 1 mL per 10-cm dish or per well of the 12-well plate.
 - c. Incubate for 7–8 min in a cell culture incubator (Heraeus, HeraCell), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C.
 - d. Resuspend the trypsinized cells in ES cell culture medium (for both species) and centrifuge the suspension for 5 min at 123 × g (Heraeus, Biofuge Primo R).

Note: The serum in the ES cell culture medium serves to quench trypsin activity. 2i medium does not contain serum and is therefore not suitable for this purpose.

- e. For mouse ES cells, aspirate most of the supernatant, leaving about 500 μL medium for resuspending the cell pellet. For rat ES cells, aspirate the supernatant completely and add 500 μL of 2i medium to resuspend the cell pellet.
- f. Resuspend the cell pellet with gentle shaking of the medium and store the suspension on ice until the microinjection (for maximum 4 h).

Note: The ES cell suspension should be kept on ice during the procedure to ensure cell stability and to avoid aggregation. Use an aliquot from this suspension for microinjecting the embryos.

4. Inject the embryos of the recipient species with ES cells from the other species to generate chimeric embryos:
 - a. Fill the microinjection chamber with HEPES-buffered ES cell culture medium (prepared by adding 47 mg HEPES into 10 mL ES cell culture medium).

Note: The microinjection chamber is a custom-made flat-bottom chamber assembled by inserting a glass coverslip (Roth, Deckgläser 24 × 40 mm, 1870) between two custom-made aluminium plates as shown in [Figure 1C](#). The glass slide is secured to the plates using silicon paste (Obermeier GmbH, Korasilon-Paste high viscosity, 0857.1).

Note: This custom-made flat-bottom microinjection chamber allows placement of the ES cells and the embryos on the opposite side of each other. It also allows an easy sorting of the already microinjected embryos from the un-injected embryos because the flat-bottom shape prevents any crowding in the middle.

- b. Use the mouth-pipetting device equipped with a handling capillary (described in [before you begin](#), step 2e) to place a vertical strip of the ES cells from the single-cell suspension (2000–4000 cells) into the microinjection chamber. ES cells should be placed on the side where the collection capillary / injection needle manipulator is located.
- c. Clean the handling capillary and use it to place the embryos (10–12 per batch) on the side of the chamber opposite to the ES cells.

Note: Inject the embryos in batches (10–12 embryos per batch) to avoid a prolonged incubation in the unfavorable conditions under the microinjection microscope. For subsequent batches, replace the ES cells in the microinjection chamber with fresh ES cells.

- d. From the ES cells that were placed in the microinjection chamber, collect 80–100 ES cells (enough for 10–12 embryo microinjections by an experienced experimenter) into the collection capillary / injection needle. ES cell collection into the collection capillary / injection needle is shown in [Methods video S2](#).

△ **CRITICAL:** Make sure that the ES cells collected in the collection capillary / injection needle have minimal gaps between one another. This avoids microinjecting excess medium into the embryo, which can potentially damage the embryo.

- e. Attach an 8-cell stage embryo to the holding capillary of the microinjection system, such that a region of the embryo that is devoid of blastomeres is oriented away from the holding site.
- f. Insert the collection capillary / injection needle (in the following steps simply referred to as injection needle) through the zona pellucida into the perivitelline space, in a region devoid of blastomeres, by one of the following two methods:
 - i. Manually: simply push the injection needle through the zona pellucida (see [Methods video S3](#)) (referred to as piercing).
 - ii. Using a laser-assisted system: from a XYClone laser system (Hamilton Thorne Bioscience), deliver a tangential laser pulse for 640 μ s, at 64% power, to the outer margin of the zona pellucida, in a region far away from any blastomeres. Then insert the injection needle through the laser-created puncture (see [Methods video S4](#)) (referred to as puncturing).

△ **CRITICAL:** Take extreme care not to damage any blastomeres during the piercing/puncturing of the zona pellucida.

Note: Laser-assisted microinjections are recommended because they are easier and less disruptive with regard to the zona pellucida.

- g. Slowly inject 8–10 ES cells into the embryo.
- h. Gently withdraw the injection needle after the microinjection of the ES cells.
- i. Release the microinjected embryo from the holding capillary and repeat the procedure with another embryo, until the whole batch is microinjected.

Note: Successful microinjections can be observed by light microscopy by observing the presence of the small ES cells next to the large native blastomeres ([Figure 4A](#)). If the microinjected ES cells carry a fluorescent transgene, the successful microinjections can also be confirmed by the presence of small fluorescently-labeled ES cells next to large non-fluorescent blastomeres ([Figure 4B](#); [Methods video S5](#)).

5. Using the handling capillary and the mouth-pipetting device (described in [before you begin](#), step 2e), transfer the microinjected embryos (after washing 2–3 times in KSOM medium, as described in step 1e) to a 200- μ L drop of fresh KSOM medium (for recipe see: [materials and equipment](#)) in a 3.5-mm cell culture dish. Cover the KSOM medium with paraffin oil (Merck, 1.07160.1000) and place the dish in the cell culture incubator (Labotect, C16), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C, until the embryos are transferred into pseudo-pregnant fosters, which must be carried out on the same day (try to avoid a delay of more than 3–4 h between the beginning of the embryo microinjection and transfer into pseudo-pregnant fosters).

Note: In principle, blastocyst microinjections can be performed in the same way as the 8-cell stage embryo microinjections.

Chimeric embryo transfer into pseudo-pregnant fosters

⌚ **Timing:** 5–10 min per foster (depending on the transfer type)

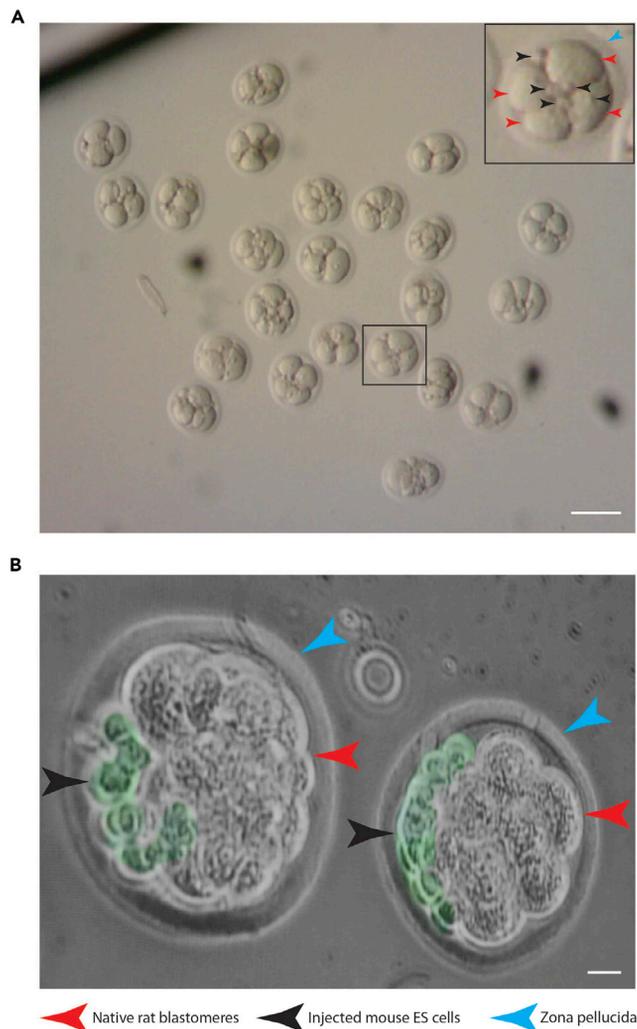


Figure 4. Images of embryos after successful ES cell microinjection

(A) Bright-field microscopic image of Wistar rat embryos microinjected with mouse ES cells. The area indicated by the black box is shown at higher magnification. Note the cell size difference between the large native rat blastomeres (red arrowheads) and the small ES cells (black arrowheads). All embryos are surrounded by an intact zona pellucida (blue arrowhead). Scale bar, 65 μ m.

(B) Higher magnification view under a fluorescent microscope of the Wistar rat embryos microinjected with Tg(ACTB-EGFP)D4Nagy/J-derived GFP-labeled ES cells. The rat blastomeres are large and non-fluorescent (red arrowheads), and the microinjected ES cells are small and easily distinguishable due to their green fluorescence (black arrowheads). Embryos are surrounded by a largely intact zona pellucida (blue arrowheads) – a small post-injection puncture can be seen in the embryo on the right. Scale bar, 14 μ m.

Note: For chimeric embryo transfer into the oviducts of pseudo-pregnant mouse foster females, the timing is 5–10 min per unilateral oviduct transfer into one foster. For chimeric embryo transfer into the oviducts of pseudo-pregnant rat foster females, the timing is 10 min per unilateral oviduct transfer into one foster. For chimeric embryo transfer into the uterus of pseudo-pregnant rat foster females, the timing is 10 min per uterine horn transfer into one foster.

In this step the chimeric embryos are transferred into the oviducts of (A) mouse or (B) rat pseudo-pregnant foster females. In an alternative version of this protocol, the chimeric embryos are transferred into the uteri of rat foster females (C).

6. Prepare the microinjected embryos for transfer into the foster females:
 - a. Wash the microinjected chimeric embryos 5–6 times in consecutive 100 μ l drops of M2 medium to remove any traces of KSOM medium (as described in [step-by-step method details](#), step 1e).
 - b. Move the washed embryos to a new sterile Petri dish containing M2 medium.
 - c. Collect the embryos by mouth-pipetting (using the device described in [before you begin](#), step 2e) into a transfer capillary (retransfer capillary, BioMedical Instruments, 114–118 μ m inner diameter):
 - i. Fill the transfer capillary with a small amount of M2 medium and introduce an air bubble. This air bubble serves to both prevent the capillary forces to cause an uncontrolled aspiration of the embryos and to monitor the successful transfers.
 - ii. Collect 6–8 chimeric embryos per unilateral oviduct transfer into a rat foster and 11–13 chimeric embryos per unilateral transfer into a mouse foster.

Note: For each animal, prepare one individual transfer capillary per unilateral transfer.

Note: The transfer capillary used for transferring embryos into a foster female is different from the handling capillary used for transferring the embryos between media drops and cell culture dishes. Make sure you are using the appropriate capillary. The embryos should be positioned very close to the tip of the capillary. Also, make sure that the capillary has only one air bubble.

7. Version A: Chimeric embryo transfer into the oviducts of pseudo-pregnant mouse foster females.
 - a. Prepare the pseudo-pregnant (E0.5) foster females for oviduct transfer surgery. Place the animal under anesthesia using one of the following two methods:
 - i. Intraperitoneal injection of a ketamine/xylazine/acepromazine mixture (65/11/2 mg/kg body weight) ([Arras et al., 2001](#)).
 - ii. Continuous inhalation of 2.5%–4% isoflurane (CP Pharma/WDT, 21311) with 0.4–0.8 L/min O₂ flow for the duration of surgery.
 - b. Place the animal with its back facing upwards and wait until there is an observable loss of the righting and palpebral reflexes.

△ CRITICAL: Protective cream (Medpex, Bepanthen, 1578681) must be applied to the animal's eyes to prevent drying.

△ CRITICAL: The operating table or heating pad must be set to maintain 37°C. The animal must be kept on the operating table or heating pad for the entire duration of both surgery and anesthesia recovery to avoid hypothermia. It applies regardless of the type of anesthesia used.

Note: When using isoflurane-based anesthesia, treatment against post-operative pain should be given by subcutaneous injection of metamizole, administered 10 min before the start of surgery (200 mg/kg of body weight).

Note: No treatment against post-operative pain is required after intraperitoneal injection of ketamine/xylazine/acepromazine because this mixture already contains a painkiller.

- c. Shave the hairs around the operating region (dorsal side, parallel to the mid-line and close to the ovary position for the oviduct transfers) with a razor blade. Clean and disinfect the skin at the shaven site with 70% ethanol.
- d. Perform the surgery to transfer the chimeric embryos into the oviduct of a foster female:
 - i. Open the skin and the abdominal cavity at the shaven area by making a 0.8–1 cm long incision with 11-cm fine scissors.
 - ii. Expose the region of oviduct proximal to the ovary by gently pulling out the fat pad.
 - iii. Fix the fat pad with a bulldog clamp (Fine Science Tools, 18051-35).

- iv. Locate the infundibulum and the ampulla.

△ **CRITICAL:** For successful embryo transfer it is important to use only the females with clear signs of pseudo-pregnancy. Therefore, after exposing the oviduct, but before the embryo transfer, check for a swollen ampulla and the presence of a corpus luteum in the ovary.

- v. Insert the transfer capillary containing the embryos into the ampulla through the infundibulum.
- vi. Using gentle pressure by mouth-pipetting (using the device described in [before you begin](#), step 2e), transfer the embryos from the transfer capillary into the ampulla (a successful transfer can be recognized by the presence of the air bubble inside the ampulla).
- vii. Slowly remove the capillary.
- viii. Gently place the oviduct back into the abdominal cavity, in its original position.

△ **CRITICAL:** When placing the oviducts back into the abdominal cavity, do not apply pressure to the oviducts to avoid squeezing any of the microinjected embryos out of the transfer site.

- ix. For bi-lateral oviduct transfers, expose the oviduct on the other side and repeat the procedure.
- x. Close the peritoneum by suturing with sterile surgical sutures (Ethicon, Vicryl, P3, USP 5-0, 45 cm, V493H).
- xi. Close the skin using 9-mm metal Autoclips (Agnthos, 59023 and 59024).
- xii. Move the operated animal to a fresh cage and monitor it during the period of recovery from anesthesia.

△ **CRITICAL:** Keep the animal warm by using either infrared light or by placing a heating pad under the cage, and monitor the animal for the entire period of recovery from anesthesia. A recovered animal must not show any signs of pain and should start moving freely. Typical recovery times are up to 10–15 min for isoflurane-based anesthesia and up to 25–35 min for ketamine/xylazine/acepromazine-based anesthesia. The recovery time can increase after longer surgery times (>30 min) and for certain animal lines.

- xiii. Place the operated foster females in a calm and quiet environment and provide post-operative pain treatment (metamizole 1.33 mg/mL in drinking water for two days post-operation).

Note: Regularly monitor the foster females for signs of pain for the next few days. Avoid opening the cage to minimize disturbance of the animal.

Note: Foster mothers can be periodically weighed and examined for the signs of pregnancy after mid-gestation. However, avoid daily disturbances that could cause excessive stress, which in turn could result in termination of the pregnancy.

- xiv. After the skin has healed (typically 5 days post-operation), carefully remove the metal clips with a clip remover (Agnthos, Clip Remover, 59026) to avoid pain and discomfort to the foster female due to skin stretching in the course of pregnancy.

8. Version B: Chimeric embryo transfer into the oviducts of pseudo-pregnant rat foster females.

- a. Prepare the E0.5 pseudo-pregnant foster females for oviduct transfer surgery. Place the animal under isoflurane-based anesthesia – continuous inhalation of 2.5%–4% Isoflurane (CP Pharma/WDT, 21311) with 0.4–0.8 L/min O₂ flow for the duration of surgery (detailed in step 7a–ii above).

△ **CRITICAL:** Rats must be anesthetized with 2.5%–4% isoflurane because the duration of anesthesia upon intraperitoneal injection of the ketamine/xylazine/acepromazine mixture is not reliable in this species.

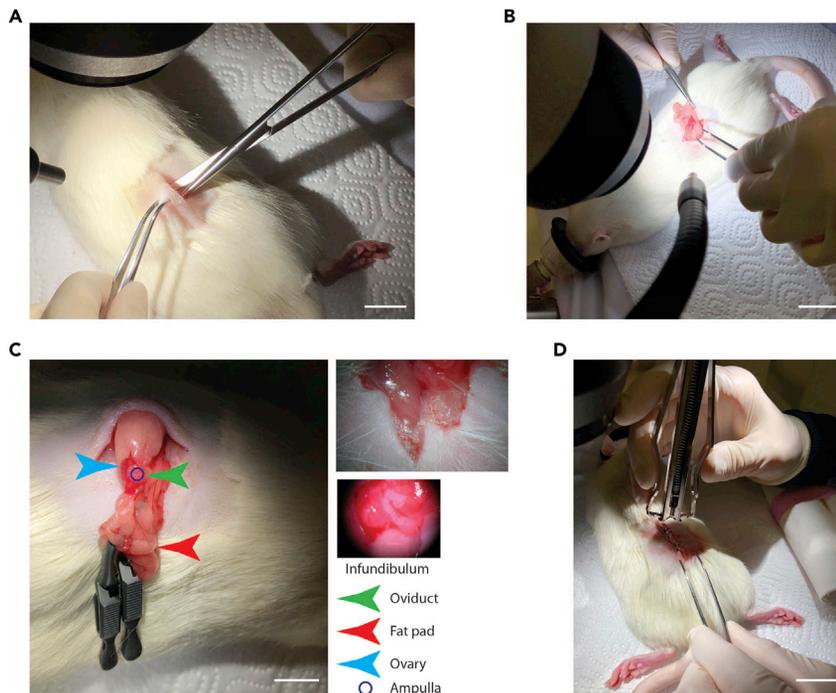


Figure 5. Critical surgical steps during the oviduct embryo transfer into a rat foster

(A) Rat foster female, in the prone position, placed under isoflurane anesthesia. The operated area (shown before the incision) at the back of the animal was cleaned with ethanol and shaved. Scale bar, 20 mm.

(B) Incision through the skin was made and the abdominal cavity was opened to expose the region containing the ovary and the oviduct. Forceps are used to pull away the fat pad to allow access to the oviduct. Note the anesthesia mask covering the nose and the mouth of the animal. Scale bar, 25 mm.

(C) Close-up of the incision site with the exposed oviduct. Colored arrowheads indicate the ovary (blue), oviduct (light green) and the fat pad (red). The dark blue circle indicates the position of the ampulla. Note how the surgical clamps are used to pull the fat tissue away from the oviduct. The infundibulum is not well visible in the rat. The insets on the right show the ampulla with the inserted transfer capillary (upper panel) and the infundibulum (lower panel). Scale bar, 15 mm.

(D) The incision of the skin is closed with metal clips. Multiple clips, placed close to one another, are used to avoid the opening of the wound. Scale bar, 25 mm.

- b. Place the animal with its back facing upwards and wait until there is an observable loss of the righting and palpebral reflexes.

△ **CRITICAL:** Protective cream (Medpex, Bepanthen, 1578681) must be applied to the animal's eyes to prevent drying.

△ **CRITICAL:** The operating table or heating pad must be set to maintain 37°C. The animal must be kept on the operating table or heating pad for the entire duration of both surgery and anesthesia recovery to avoid hypothermia.

Note: Treatment against post-operative pain should be given by subcutaneous injection of metamizole, administered 10 min before the start of surgery (200 mg/kg of body weight).

- c. Shave the hairs around the operating region (dorsal side, parallel to the mid-line and close to the ovary position for the oviduct transfers) with a razor blade. Clean and disinfect the skin at the shaven site with 70% ethanol – the operating region prepared for incision is shown in [Figure 5A](#).
- d. Perform the surgery to transfer the chimeric embryos into the oviduct of a foster female (see [Methods video S6](#)):

- i. Open the skin and the peritoneal wall at the shaven area by making a 2.5-cm long incision with 11-cm fine scissors – the incision with an exposed fat pad and oviduct is shown in [Figure 5B](#).
- ii. Expose the oviduct in the region proximal to the ovary by gently pulling out the fat pad.
- iii. Fix the fat pad with a bulldog clamp.
- iv. Locate the ampulla and the infundibulum – the fixation method for the exposure of the oviduct is shown in [Figure 5C](#).
- v. Use an acupuncture needle (SEIRIN, J-type No.8 (0.30) × 30 mm) to first open the ampulla and then insert the transfer capillary directly into the ampulla through this opening.

Note: The position of the infundibulum is very unfavorable in the rat and therefore the transfers are done directly into the ampulla, which must first be opened with an acupuncture needle to enable insertion of the transfer capillary.

- vi. Using gentle pressure by mouth-pipetting (using the device described in [before you begin](#), step 2e), transfer the embryos from the transfer capillary into the ampulla (a successful transfer can be recognized by the presence of the air bubble inside the ampulla).

△ CRITICAL: If the transfer is taking more than 10 min, a few drops of warm PBS must be applied onto the oviducts and the nearby organs to avoid drying.

- vii. Slowly remove the capillary from the ampulla.
- viii. Gently place the oviduct back into the abdominal cavity, in its original position.

△ CRITICAL: When placing the oviducts back into the abdominal cavity, do not apply pressure to the oviducts to avoid squeezing any of the microinjected embryos out of the transfer site.

- ix. For bi-lateral oviduct transfers, expose the oviduct on the other side and repeat the procedure.
- x. Close the peritoneum by suturing with sterile surgical sutures (Ethicon, Vicryl, P3, USP 5-0, 45 cm, V493H).
- xi. Close the skin using 9-mm metal Autoclips (Agntos, 59023 and 59024) – the closed skin with the metal clips is shown in [Figure 5D](#).
- xii. Move the operated animal to a fresh cage and monitor it during the period of recovery from anesthesia.

△ CRITICAL: Keep the animal warm by using either infrared light or by placing a heating pad under the cage and monitor the animal for the entire period of recovery from anesthesia. A recovered animal must not show any signs of pain and should start moving freely. Typical recovery time is up to 10–15 min. The recovery time can increase after longer surgery times (>30 min) and for certain animal lines.

- xiii. Place the foster females in a calm and quiet environment and provide post-operative pain treatment (metamizole 1.33 mg/mL in drinking water for two days post-operation).

Note: Regularly monitor the foster females for signs of pain for the next few days. Avoid opening the cage to minimize disturbance of the animal. Whenever possible avoid housing rat foster females in single-animal cages as this is very stressful to rats.

Note: Foster mothers can be periodically weighed and examined for the signs of pregnancy after mid-gestation. However, avoid daily disturbances that could cause excessive stress, which in turn could result in termination of the pregnancy.

- xiv. After the skin has healed (typically 5 days post-operation), carefully remove the metal clips with a clip remover (Agntos, Clip Remover, 59026) to avoid pain and discomfort to the foster female due to skin stretching in the course of pregnancy.

Note: Either one or both oviducts can be used for embryo transfer into a single animal. We typically use both oviducts for rat fosters and a single oviduct on the left side for mouse fosters. Using only one side may be preferable if shorter surgery times are necessary for the faster recovery of rat foster females.

9. Version C: Chimeric embryo transfer into the uterus of pseudo-pregnant rat foster females.
 - a. Prepare the E2.5 pseudo-pregnant foster females for uterine transfer surgery as in steps 8a and 8b above.
 - b. Shave the hairs around the operating region (the hairs on the medial side on the back) with a razor blade. Clean and disinfect the skin at the shaven site with 70% ethanol.
 - c. Perform the surgery to transfer the chimeric embryos into the uterine horns of foster females:
 - i. Open the skin and the peritoneal wall at the shaven area by making a 1-cm long incision with 11-cm fine scissors.
 - ii. Expose the uterine horn region about 6–8 mm distal to the end of the oviduct.
 - iii. Use fine blunt-end forceps to hold the end of the uterine horn.
 - iv. Use a 27G × $\frac{3}{4}$ " needle (Braun Omnifix, Luer Lock) to make a hole into the uterine wall.
 - v. Insert the transfer capillary containing the embryos into the uterine horn through this opening.
 - vi. Using gentle pressure by mouth-pipetting (using the device described in [before you begin](#), step 2e), transfer the embryos from the transfer capillary into the uterine horn.
 - vii. Slowly remove the transfer capillary from the uterine horn.

Note: Take extreme care while removing the capillary from the uterine horn. Withdrawing it too fast may cause a backflow of the transferred embryos out of the uterine horn.

△ **CRITICAL:** If the transfer is taking more than 10 min, a few drops of warm PBS must be applied onto the uterus and the nearby organs to avoid drying.

- viii. Gently place the uterine horn back into the abdominal cavity, in its original position.

△ **CRITICAL:** When placing the uterine horn back into the abdominal cavity, do not apply pressure to the uterus to avoid squeezing any of the microinjected embryos out of the transfer site.

- ix. For bi-lateral transfers, expose the uterine horn on the other side and repeat the procedure.
- d. Close the peritoneum by suturing with sterile surgical sutures (Ethicon, Vicryl, P3, USP 5-0, 45 cm, V493H).
- e. Close the skin using 9-mm metal Autoclips (Agnthos, 59023 and 59024).
- f. Move the operated animal to a fresh cage and monitor it during the period of recovery from anesthesia.

△ **CRITICAL:** Keep the animal warm by using either infrared light or by placing a heating pad under the cage and monitor the animal for the entire period of recovery from anesthesia. A recovered animal must not show any signs of pain and should start moving freely. Typical recovery time from isoflurane-based anesthesia is up to 10–15 min. The recovery time can increase after longer surgeries (>30 min) and for certain animal lines.

- g. Place the foster females in a calm and quiet environment and provide post-operative pain treatment (metamizole 1.33 mg/mL in drinking water for two days post-operation).

Note: Regularly monitor the foster females for signs of pain for the next few days. Avoid opening the cage to minimize disturbance of the animal. Whenever possible avoid housing rat foster females in single-animal cages as this is very stressful to rats.

Note: Foster mothers can be periodically weighed and examined for the signs of pregnancy after mid-gestation. However, avoid daily disturbances that could cause excessive stress, which in turn could result in termination of the pregnancy.

- h. After the skin has healed (typically 5 days post-operation), carefully remove the metal clips with a clip remover (Agnthos, Clip Remover, 59026) to avoid pain and discomfort to the foster female due to skin stretching in the course of pregnancy.

Isolation of chimeric embryos

⌚ **Timing:** up to 10–15 min (depending on the experimental purpose)

At this step the embryos are isolated from the uteri of pregnant foster females for further analysis.

10. Embryo isolation:

- a. Sacrifice the pregnant foster females at the desired stage of the embryos (in our experiments at E18-19.5) by cervical dislocation (in case of rats, first anesthetize the animals with 4% isoflurane)
- b. Dissect the uteri and open them using surgical scissors to collect the developing embryos – if the females are not pregnant or no live embryos are found, check: **Troubleshooting**.
- c. Proceed according to the specific aims for which the embryos are to be used.

Note: If either the progeny of the microinjected ES cells or the native cells of the recipient embryo express a fluorescent protein, the degree of chimerism can be assessed immediately under a fluorescent microscope. For example, in the mouse→rat chimeric embryos generated by microinjecting mouse Tg(ACTB-EGFP)D4Nagy/J-derived GFP-labelled ES cells (which are male) into Wistar rat recipient embryos (Stepien et al., 2020), a high degree of chimerism was observed under a standard fluorescent microscope (Nikon, HB-10104AF) as patches of GFP-expressing green regions and dark non-fluorescent regions upon dissection of the embryonic brain. However, a low degree of chimerism may not be detectable in this way and may need to be assessed using more sensitive methods (e.g., PCR-based genotyping of tissue samples or immunofluorescence; see Figure 7).

Optional: Genotyping of tissue and organ samples by PCR can be done to detect moderate degrees of chimerism. Small samples of tissues and organs can be collected during embryo dissection and either processed directly or frozen until use. DNA is then isolated using isopropanol extraction. The detailed protocol for genotyping can be found in (Stepien et al., 2020). We suggest using multiple primer pairs in PCR reactions to distinguish the cells from the two species. As an example, we used three sets of primers: rat-specific and mouse-specific primers (Steube et al., 2008), EGFP-amplifying primers (Stepien et al., 2020) and gender-specific primers (Jimenez et al., 2003) to distinguish the EGFP-positive male mouse cells from wild type rat cells.

EXPECTED OUTCOMES

We used the described protocol to generate 1) mouse→rat chimeric embryos by microinjecting mouse EGFP-expressing ES cells (derived from the Tg(ACTB-EGFP)D4Nagy/J mouse line maintained in a Bl6 background) into Wistar rat embryos, which were then implanted into Sprague Dawley rat foster mothers; and 2) rat→mouse chimeric embryos by microinjecting rat DAc8 ES cells into mouse embryos from the Tg(ACTB-EGFP)D4Nagy/J line (maintained in a Bl6 background), which were then implanted into either RjOrl:SWISS or Crl:CD1(ICR) mouse foster mothers. In the case of mouse→rat chimeric embryo transfers, we were able to obtain, in total, 17 pregnancies from 36 transfers into rat fosters (47% pregnancy rate), resulting in 16 live embryos (27% of all detected implantation events; E18.5-E19.5), 5 of which (31%) were confirmed to be chimeric using genotyping

Table 1. Live and interspecies chimeric embryos obtained from ES cell microinjection

Mouse→rat chimeras					
Transfer type	No. of fosters	No. of pregnancies	Implantations	Live embryos	Chimeras
Oviduct	23	9 (39%)	38	5 (13%)	1 (20%)
Uterus	13	8 (62%)	21	11 (52%)	4 (36%)
Total	36	17 (47%)	59	16 (27%)	5 (31%)
Rat→mouse chimeras					
Transfer type	No of fosters	No of pregnancies	Implantations	Live embryos	Chimeras
Oviduct	25	16 (64%)	82	23 (28%)	5 (22%)

(Table 1). Both the pregnancy rates (62% vs. 39%) as well as fractions of live (52% vs. 13%) and chimeric embryos (36% vs. 20%) were higher using the uterus than the oviduct transfer protocol. In the case of rat→mouse chimeric embryo transfers, we were able to obtain, in total, 16 pregnancies from 25 transfers into mouse fosters (64% pregnancy rate), resulting in 23 live embryos (28% of all detected implantation events; E19.5), 5 of which (22%) were confirmed to be chimeric using genotyping (Table 1).

The presence of chimerism was determined by genotyping of embryonic tissue and organ samples as described above. Additionally, brains were dissected from all the obtained embryos and assessed for GFP fluorescence as the readout for the chimerism. The degree of chimerism in embryonic brains was assessed by visual inspection of the brain cryosections for the presence of GFP fluorescence. The images of coronal brain sections from the mouse→rat (Figure 6A) and rat→mouse (Figure 6B) chimeric embryos, subjected to GFP immunofluorescence (stained with primary chicken polyclonal antibodies, Aves labs, GFP-1020, 1:600 dilution; and secondary goat anti-chicken Alexa Fluor 488 antibodies, ThermoFisher Scientific, A-11039, 1:1000 dilution) showed the presence of distinct segments (visible particularly well in the cortical region) derived from the cells of either species (green for mouse cells, and non-green for rat cells). Higher magnification allowed distinguishing individual mouse and rat cells by the presence and absence of the GFP signal and of nuclear speckles, respectively (Figure 6C). In most mouse→rat chimeric brains other than the one shown in Figure 6A, the degree of chimerism was low, often showing only single mouse cells surrounded by the host rat tissue. In this context, as noted by others (Eszter et al., 2021), it is critical to confirm that the progeny of the ES cells introduced into the embryo of the respective other species has acquired the identity of the specific tissue of interest. Specifically, we confirmed the identity of the GFP-positive cells in the neocortex by immunofluorescence for neuronal layer-specific markers. This showed that in the mouse→rat chimera, the GFP-positive mouse cells in the embryonic neocortex, like the neighboring GFP-negative rat cells, expressed deep-layer and upper-layer neuron markers, confirming their neuronal identity (Figure 7). We did not assess the degree of chimerism in other organs. All images were acquired with a Zeiss LSM confocal microscope and processed with Fiji (Schindelin et al., 2012).

A recently published protocol for generating rat→mouse chimeras compared three different methods: (1) 8-cell stage embryo aggregation (co-culture of ES cells and embryo); (2) ES cell microinjection into 8-cell stage embryos and; (3) ES cell microinjection into blastocysts (Okumura et al., 2019). The microinjected 8-cell stage embryos in that study were incubated *in vitro* until the blastocyst stage and then transferred into the mouse uteri. The authors showed that the highest contribution of the rat cells in the chimeric animals occurred with ES cell microinjections into the 8-cell stage embryo, and reported a success rate of 18% in obtaining chimeric pups by microinjections into 8-cell stage embryos. With our protocol, we have a success rate of 22% in obtaining chimeric pups by microinjection of rat ES cells into 8-cell stage mouse embryo (Table 1). Additionally, Okumura et al. have reported a success rate of 29% in obtaining chimeric pups by microinjections into blastocysts followed by uterus transfer, with this method resulting in low degrees of chimerism in some organs (particularly liver). Although we did not do the uterus transfers into mouse fosters,

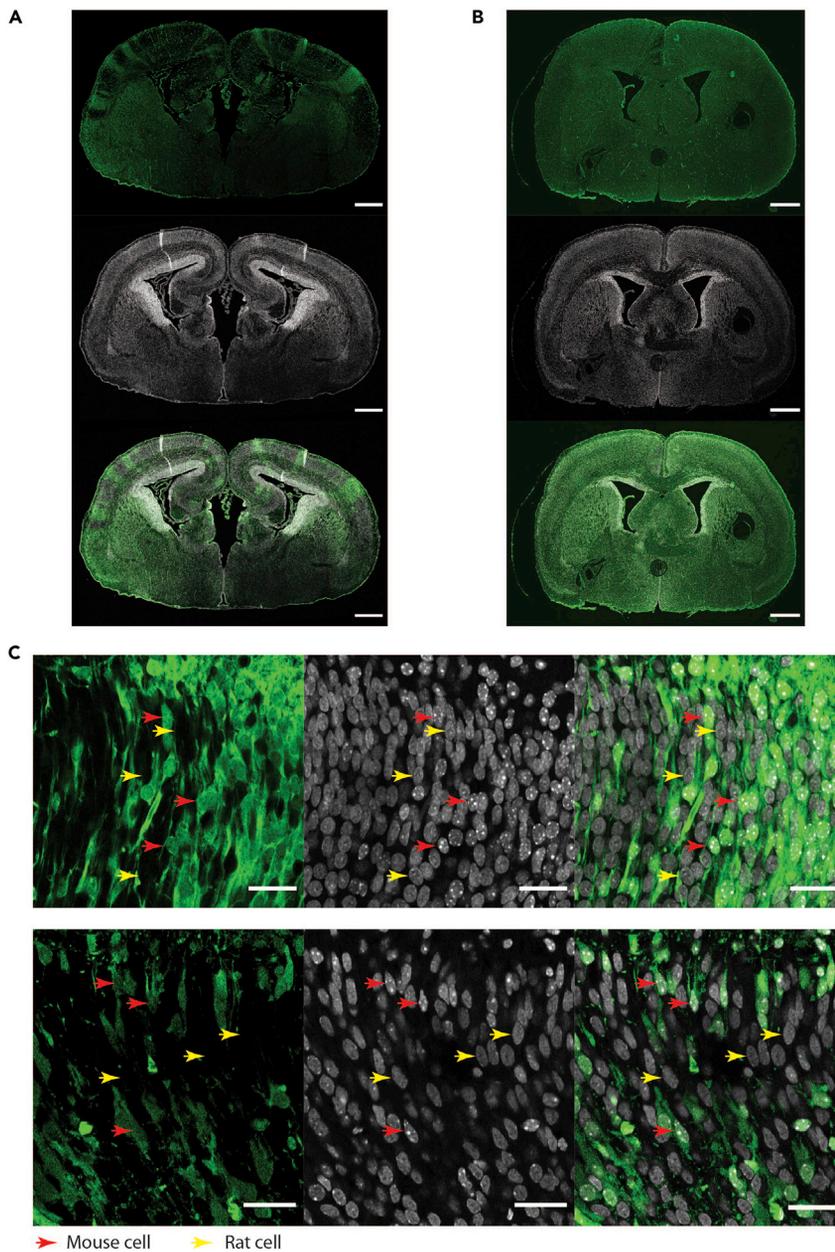


Figure 6. Mouse-rat chimeric embryonic neocortex

(A and B) Coronal sections of the E19.5 telencephalon of (A) mouse→rat, and (B) rat→mouse chimeric brains. Coronal sections were subjected to GFP immunofluorescence (green) combined with DAPI staining (white). Note the presence of dark (rat, non-fluorescent) and green (mouse, EGFP-expressing) regions showing radially oriented cortical wall segments.

(C) Higher magnifications of the neocortical wall (coronal section) of a mouse→rat (upper panel) and a rat→mouse (lower panel) chimeric brain. The presence of GFP and of nuclear speckles in the mouse cells can be used to easily distinguish them from the non-fluorescent and nuclear speckles-less rat cells. Scale bars, (A and B) 500 μm , (C) 25 μm .

the uterus transfer into rats using our protocol had a success rate of 36% in obtaining chimeric pups, and the chimerism in all tissues analyzed, including brain (the tissue not assessed by Okumura et al., 2019) and liver, was readily detectable by standard PCR in all the chimeras obtained using our protocol.

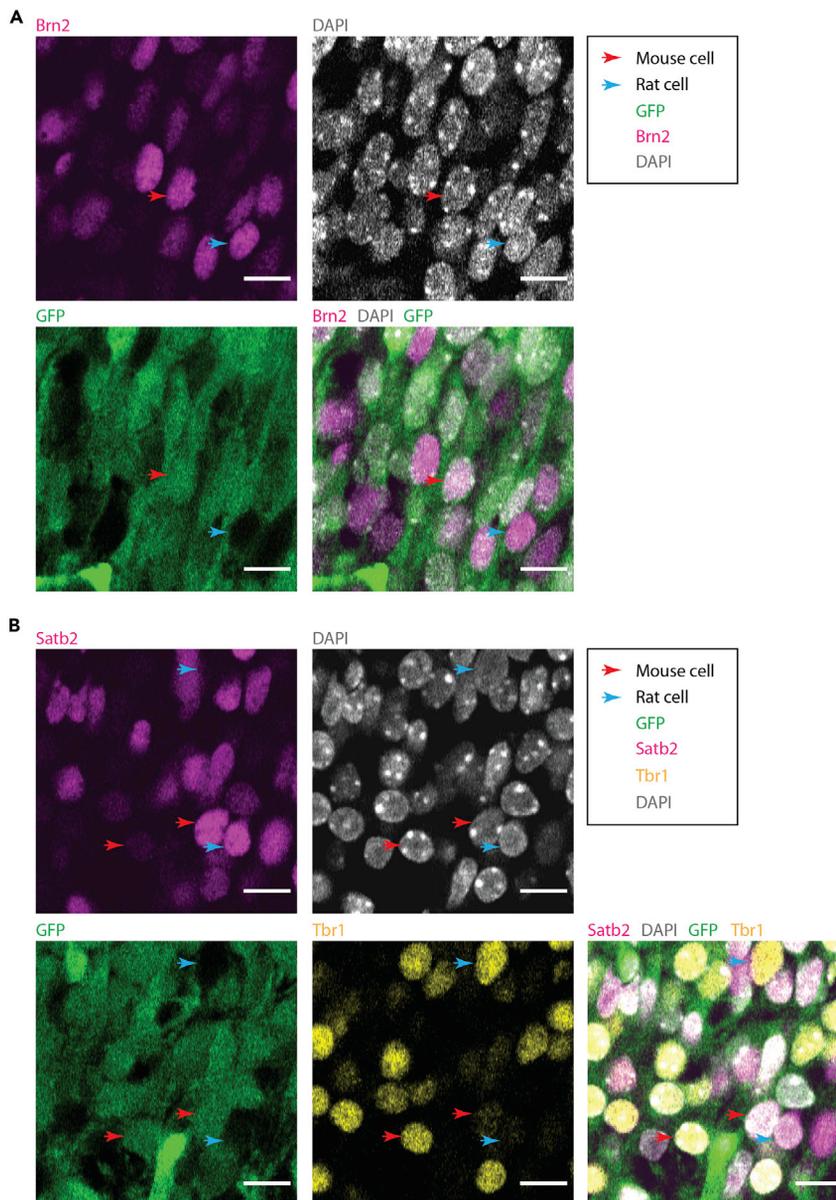


Figure 7. Regions of the cortical plate of the E19.5 neocortex of a mouse→rat chimeric brain

(A) Coronal sections were subjected to GFP (green) and Brn2 (magenta) double immunofluorescence combined with DAPI staining (white).

(B) Coronal sections were subjected to GFP (green), Satb2 (magenta) and Tbr1 (yellow) triple immunofluorescence combined with DAPI staining (white). The presence of GFP and of nuclear speckles in the mouse cells can be used to distinguish them from the non-fluorescent and nuclear speckle-less rat cells. Note that the progeny cells of the injected mouse ES cells are positive for markers of both deep-layer (Tbr1) and upper-layer (Satb2 and Brn2) neurons, confirming that they were able to *in-vivo* differentiate into neurons and integrate into the relevant tissue. Scale bars 10 μ m.

LIMITATIONS

This protocol optimizes the number of live embryos and embryos with detectable interspecies chimerism obtained using ES cell microinjection into 8-cell stage embryos. The chimeric embryos obtained displayed chimerism in all of the tissues/organs tested (samples obtained from liver, lung, heart, kidney, brain stem, eye, intestine, stomach, spleen, skin, front and hind paws, and

tail), as assessed by PCR genotyping. However, the degree of chimerism among different embryos and/or among different tissues that were tested within an embryo was variable and not easily predictable. A high degree of chimerism (approaching 50%) is desirable but difficult to obtain, likely due to its adverse developmental effects. Increasing the initial number of ES cells microinjected per embryo also did not seem to increase the overall chimerism, likely due to early death during development. Such adverse developmental effects have been described before for interspecies chimeras generated using morula aggregation (Bozyk et al., 2017); however, the reasons for this are still unclear. Therefore, obtaining a sufficient number of embryos with a high degree of chimerism and no developmental abnormalities may require a substantial increase in the number of embryo transfers, and/or optimizing the number of microinjected ES cells or the developmental stage of the microinjected embryos (morula vs. blastocyst).

TROUBLESHOOTING

Problem 1

Foster females are not pregnant, and there are no visible signs of embryo implantation (step 10b).

Potential solution

The problem may be related to issues arising at any of the following steps: (1) initial low embryo number or quality; (2) embryo damage during the microinjection procedure or the subsequent steps; (3) loss of embryos during transfer; or (4) failure of the chimeric embryos to implant into the uterus.

Make sure you use a sufficient number of animals for mating to obtain a sufficient number of embryos. Please note that even with optimal mating conditions, 10%–20% of the plugged females may not be pregnant due to the mating occurring at the wrong time of the estrous cycle. Moreover, animals that are too young, too old or that come from lines with reduced fertility may also produce fewer and/or low-quality embryos. Choose only good-quality embryos for microinjection and transfer. There should be no visible damage to the blastomeres or zona pellucida. Avoid any mechanical damage to the cells during the microinjection procedure and minimize the embryo incubation time between the different steps, particularly between embryo isolation and microinjection. To test their viability, some of the embryos can be set aside before and after microinjection and allowed to develop for 18–24 h *in vitro* in KSOM medium in a cell culture incubator (Labotect, C16), in a humidified atmosphere of 5% CO₂ / 95% air at 37°C. Healthy embryos should continue to develop to the blastocyst stage under these conditions. The number of such healthy embryos can be taken as an indicator of the quality of the embryos transferred to the foster mothers.

Embryos can also be lost during the transfer into a foster female if the transfer needle is inserted incorrectly (the tip is outside the oviduct or faces in the wrong direction – towards the ovary). For oviduct transfers, checking for the presence of air bubbles inside the ampulla serves to ensure that the transfer occurred in the right place.

Failure of the embryos to implant can also be linked to the lack of pseudo-pregnancy despite the presence of a copulation plug. Make sure to only use females with a swollen ampulla and the presence of a corpus luteum.

After surgery, place the females in a calm and quiet area of the animal room and minimize handling, as excessive stress can lead to lack of implantation or early termination of the pregnancy.

To ensure that the microinjection and transfer procedures were optimal, we suggest performing control microinjections and transfers. In this case non-injected embryos, mock-injected embryos and embryos microinjected with ES cells from the same species (intraspecies chimeras) can be transferred to additional fosters to determine which step in the protocol requires optimization.

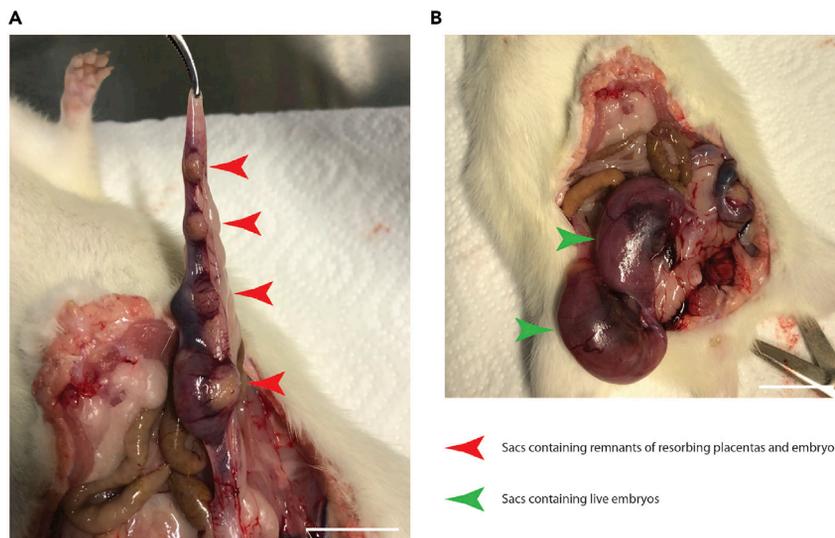


Figure 8. Rat uteri with implanted embryos after foster rat dissections at E18.5

(A) Uterine horn with multiple implantation sites but no live embryos (red arrowheads).

(B) Uterine horn with two alive, fully developed embryos (green arrowheads). Note the differences in size and the pale color of the amniotic sacs containing the remnants of the placenta and embryos undergoing resorption in A. These embryos died at different stages of development. Scale bars, 25 mm.

The site of embryo transfer, i.e., oviduct transfer or uterus transfer, can also affect the efficiency of implantation. As shown in [Table 1](#), we observed a higher pregnancy in rat fosters using the embryo transfer to the uterus instead of the oviduct.

Problem 2

Most or all transferred embryos die and get completely or partially resorbed before the desired dissection time point. This problem is illustrated in [Figure 8A](#), which shows the dissection of a foster rat uterus with implanted but dead embryos undergoing resorption. In contrast, a foster rat uterus containing two fully developed live embryos is shown in [Figure 8B](#) (step 10b).

Potential solution

Premature loss of embryos is usually linked to two main factors.

First, suboptimal caging conditions and excessive stress in the foster females may cause a termination of pregnancy. To avoid this, move the foster females immediately after surgery to a quiet and calm area of the animal room and minimize the handling thereafter. Avoid housing single females as it can be stressful to pregnant females, in particular in the case of rats.

Second, another potential reason for embryo loss may be due to defects in development that could be related to either the microinjection and transfer procedure or the presence of foreign-species cells. If the control transfers (see the problem and potential solution above) develop normally, then the negative effect of foreign-species cells is the most likely problem. This is particularly expected if the degree of chimerism in the embryo is very high. Reducing the number of microinjected ES cells or using a different embryonic stage (blastocyst instead of 8-cell stage embryo) may reduce the degree of chimerism and increase the chance of obtaining live embryos.

Problem 3

The embryos obtained are alive and morphologically correctly developed, however, no interspecies chimerism is detectable, neither by low-sensitivity methods such as visual inspection using a

fluorescent microscope, nor by more sensitive methods such as PCR-based genotyping or immunofluorescence on the sectioned tissues (step 10c).

Potential solution

The lack of chimerism could result from either lack of integration/early loss of the microinjected ES cells or a very low contribution of such cells to the developing embryos such that they cannot be easily detected by standard methods.

Make sure the ES cell line retains pluripotency throughout the whole procedure. Perform control microinjections and transfers to ensure that these cells are capable of efficiently producing intraspecies chimeras with a high degree of chimerism.

Problem 4

The embryos obtained are chimeric as assessed by genotyping of tissue samples, but no fluorescence signal can be detected despite using an ES cell line or recipient embryos that should express fluorescent markers (step 10c).

Potential solution

The problem could be caused by silencing of the expression during the cell differentiation process or a genetic loss of the fluorescent marker.

When using an ES cell line expressing a fluorescent protein marker, make sure that the ES cells continue to express this marker during the *in vitro* culture prior to the embryo microinjection. During the microinjection procedure, select only brightly fluorescent cells for the microinjection (see: [Methods video S2](#)). Regular genotyping to ensure that the transgene is intact is also advisable.

When using embryos from a donor strain that expresses a fluorescent protein marker, selecting only hemi- or homozygous embryos is preferable. Select the embryos under a fluorescent microscope and pick only the ones with a high expression of the fluorescent protein marker. Genotype your animal stock regularly.

Using two fluorescent markers (one for the ES cells and a different one for the recipient embryo) may also be helpful. Alternatively, in some cases mouse and rat cells can be distinguished visually due to the different pattern of nuclear chromatin speckles, e.g., in the developing neocortex ([Figure 6C](#) and [Stepien et al., 2020](#)).

Problem 5

No embryos or only a low number of embryos (to be subjected to microinjection of ES cells) can be collected from the donor females, or the collected embryos are at the wrong developmental stage (step 1 in [before you begin](#)).

Potential solution

In order to collect a large number of embryos at the right developmental stage for microinjection, consider a pilot breeding experiment to optimize 1) the age and number of animals used for mating, and 2) the timing of embryo collection.

To obtain a large number of embryos, use the animals of an appropriate age – 8–12 week-old females for mouse, 10–16 week-old females for rat, and 10–18 week-old males for either species. The choice of a particular strain, especially in case of in-bred or genetically modified strains, can also affect the frequency of mating, the number of embryos recovered per female, and the course of development of the embryo. Although in most cases, the females in the appropriate phase of the estrous cycle mate readily when placed together with a male, females from difficult breeder strains may be required to be placed together with males continuously for a few days before they

start mating. The number of embryos that can be collected can also be negatively affected by using the same male for continuous mating. In our experience, the mating frequency for a male of once per week is optimal for obtaining a high embryo yield.

Most mice and rats mate close to the middle of the dark phase of the standard 12/12 light-dark cycle, and 8-cell stage embryos can reliably be collected from the oviducts 14–15 h from the start of the dark phase. At time points later than 14–15 h, the embryos can enter the uterine horn. Since the preferred timing of mating can be different for different strains and can additionally be affected by other conditions (e.g., temperature and humidity) of a particular breeding facility, consider a pilot embryo collection to check the developmental stage and the localization of the embryos (in oviducts vs in uterine horns). Avoid changing the breeding conditions during the experiment.

In case the embryos collected are too young (2- or 4-cell stage), they can be incubated *in vitro* until they have reached the 8-cell stage. Older embryos (in the blastocyst stage) can also be used to increase the number of embryos available for transfer into foster mothers; however, it should be noted that the embryonic stage can affect the level of chimerism.

If possible, we recommend obtaining the donor females from commercial sources to avoid breeding excess animals and to reduce the workload. In such a case, make sure the animals are delivered at the right time to collect the embryos at the appropriate developmental stage and perform pilot collection experiments to estimate the number of females needed to obtain enough good-quality embryos.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Wieland B. Huttner (huttner@mpi-cbg.de).

Materials availability

The Tg(ACTB-EGFP)D4Nagy/J-derived GFP-expressing mouse ES cell line is available upon request from the lead contact, Wieland B. Huttner (huttner@mpi-cbg.de).

Data and code availability

No unique datasets or code was generated in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100494>.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.K.S., S.V., and W.B.H.; experimental plan, B.K.S., S.V., and R.N.; methodology, B.K.S., S.V., and R.N.; formal analysis, B.K.S. and S.V.; investigation, B.K.S., S.V., R.N., and A.H.; writing – original draft, B.K.S., S.V., and R.N.; writing – review and editing, B.K.S., S.V., and W.B.H.; visualization, B.K.S., S.V., and R.N.; funding acquisition, W.B.H.; supervision, W.B.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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