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Protocol for the production of viable bimaternal mouse embryos

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A reliable nuclear transfer method was first reported in 1983; it provided definite evidence that parthenogenetic embryos are lethal at early postimplantation in mammals. Subsequently, nuclear transfer has been extensively used as an important and versatile tool for investigating embryo and somatic-cell cloning and nucleo-cytoplasmic interactions. Further development of this technique has enabled the generation of bimaternal embryos containing two haploid sets of maternal genomes from female germ cells of different origins. By using a 2-d nuclear transfer system for oocyte reconstruction, viable mice can be produced solely from maternal genomes, without the participation of the paternal genome. This oocyte reconstruction system, as described in this protocol, could provide valuable guidelines for exploring the potential endowments of gametes and for conferring novel properties to them.

INTRODUCTION

Germ cells are a unique source of totipotent genomes from which individuals are generated by the fusion of male and female gametes. An extremely small number of primordial germ cells (PGCs), that is, cells from which germ cells originate, first differentiate from pluripotent epiblast cells at around day 6.5 of gestation in mice[1]. The PGCs continuously proliferate and migrate into the genital ridge by day 11 of gestation. Following several mitotic divisions, the female PGCs enter the prophase of the first meiosis and become oogonia. Contrastingly, male PGCs entering the prospective testis continue to proliferate until mitotic arrest occurs at approximately day 14 of gestation. At the commencement of puberty, the oocytes, which are 15–20 μm in diameter and surrounded by cells that form the follicular envelope at birth, begin to grow in response to hormonal signals from the hypothalamus/pituitary. During growth, the oocytes attain an impressive 200-fold increase in volume and a final diameter of 75–80 μm. Oocyte growth is accompanied by an increase in the size of the nucleus (germinal vesicle (GV)) to a diameter of approximately 20 μm; furthermore, ultrastructural changes in the nucleolus and extranucleolar bodies are observed. Chromatin exists in the form of highly diffuse bivalent chromosomes that condense around the nucleolus at about the time the oocyte reaches a diameter of 60 μm. Subsequently, the oocyte becomes capable of reentering the cell cycle but is maintained in meiotic arrest by the follicular environment. The final stage of oogenesis is oocyte maturation wherein, under hormonal control, the oocyte is stimulated to resume the first meiotic cell cycle and to undergo the first meiotic division before its arrest at the metaphase of the second meiosis (MII).

During gametogenesis, germ cells are dynamically reprogrammed by epigenetic modifications, and the germ-cell genome confers the properties necessary for totipotency only on completion of the epigenetic reprogramming[2,3]. The parent-of-origin-specific epigenetic modifications in the chromatin are entirely nullified by the time the PGCs complete their migration into the genital ridge[4]. The PGCs subsequently acquire epigenetic modifications that are differentially imposed during oogenesis and spermatogenesis[5–8]. In particular, DNA methylation imprints at differentially methylated regions (DMRs) serve as a major epigenetic signal for the regulation of imprinted gene expressions[7,9–11]. This leads to decisive functional differences between the maternal and paternal genomes; thus, both parental genomes are required for normal development to term in mammals[12]. It is known that the DMRs in the female and male germ lines are differentially methylated during the oocyte growth phase and in the prospermatogonia before birth, respectively. The methylation imprints are maintained throughout the lifetime of the offspring. Of the *de novo* methyltransferases (Dnmts) identified thus far, Dnmt3a and Dnmt3L are known to be essential for germ line-specific *de novo* methylation imprinting[7,13–17].

How can the function of the germ line-specific methylation imprints during embryo development be elucidated? It is impossible to directly assess the genome competence of non-growing (ng) and growing-stage oocytes to support development[18,19]. This is because, at these stages, the oocytes are entirely incompetent and are unable to mature to the MII stage, undergo fertilization and develop. In the first series of experiments that aimed to explore the role of methylation imprinting during oocyte growth, we established serial nuclear transfer as a reliable procedure for reconstructing oocytes containing two haploid sets of maternal genomes by using oocytes obtained from different sources[20]. In the first nuclear transfer, an ng oocyte or a karyoplast from growing-stage oocytes was fused with a fully grown GV-stage (GV-fg) oocyte from which the GV was removed previously. In the second nuclear transfer, the resultant MII spindle derived from the ng oocyte was transferred into an intact MII oocyte. Following artificial activation, the reconstructed embryos became diploid, forming two pronuclei and polar bodies, and began to develop. The embryos that were derived solely from maternal genomes were designated as bimaternal embryos to clearly distinguish them from parthenogenetic/gynogenetic embryos. This method enables the assessment of

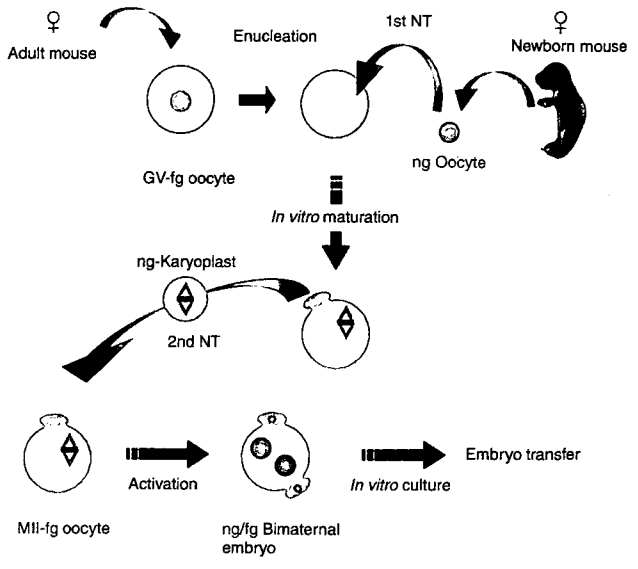

Figure 1 | Schematic diagram of production process for bimaternal embryos, which contain two sets of haploid genome derived from a non-growing oocyte (green) and an MII-stage fully grown oocyte (red). The first nuclear transfer is conducted to resume the first meiotic division and to form haploid chromosomes. The second nuclear transfer is conducted to form diploid bimaternal embryos.

the nuclear properties of ng and growing-stage oocytes. Furthermore, by using this serial nuclear transfer system, we have demonstrated several lines of direct evidence that the epigenetic modifications occurring during oocyte growth exert a decisive effect on mammalian development.

Mouse embryos carrying two sets of haploid genomes derived from ng and fully grown MII-stage (MII-fg) oocytes that are referred to as bimaternal embryos are able to grow up to 13.5 d of gestation with a well-developed placenta (**Fig. 1**) (ref. 20). In these embryos, the paternally expressed genes *Peg1/Mest*, *Peg3* and *Snrpn* are activated in the alleles derived from the ng oocyte genome, whereas the maternally expressed genes *Igf2r* and *p57Kip2* are silent[21]. However, the expression of genes regulated by paternal methylation imprinting was not altered to the paternal expression pattern. To address whether paternal methylation imprinting obstructs parthenogenesis, we further modified the imprinted genes by using mice wherein the paternal methylation regions were deleted[22–27]. The regulation of *H19* expression from the biallelic to the monoallelic mode enhances the survival of bimaternal embryos; $ng^{H19\text{-}KO}$/fg bimaternal embryos harboring a deletion of the *H19* transcription unit successfully developed as live fetuses for 17.5 gestation days[22]. On modifying the expressions of both *Igf2* and *H19*, the bimaternal embryos developed into viable mice[23]. This particular pattern of development was observed due to the appropriate expression of the *Igf2* and *H19* genes along with the *Dlk1* and *Gtl2* genes, which was facilitated by the unexpected methylation of the DMR at chromosome 12 (ref. 28). To confirm this peculiar result, an experiment was performed using ng oocytes obtained from mice harboring double mutations in the *H19* DMR and the *Dlk1-Dio3* intergenic germline-derived DMR. The results clearly revealed that the bimaternal embryos develop into viable pups with a high success rate equivalent to the rate of development achieved by *in vitro* fertilization (IVF) of manipulated control oocytes[25]. These bimaternal mice subsequently developed into fertile adults with a normal life span. The results provide conclusive evidence that the imprinted genes, regulated by these two paternally methylated imprinting control regions, are the only obstacles preventing the normal development of bimaternal mouse fetuses.

Furthermore, by using the serial nuclear transfer method for oocyte reconstruction, a new application has been developed for incompetent untapped germ cells; for example, viable offspring were produced using incompetent small oocyte genomes following oocyte reconstruction and IVF in mice[18] and cows[29]. In addition, by combining this method with an *in vitro* culture (IVC) system for 3 weeks, viable mice were produced from premeiotic female germ cells derived from mouse fetuses at 12.5 d postcoitum (dpc) (refs. 6,30). In humans, nuclear transfer into GV oocytes has also been used to reduce the frequency of aneuploidy in aged oocytes by replacing the cytoplasm with that from younger oocytes[31,32]. As mentioned above, our serial nuclear transfer method for oocyte reconstruction and bimaternal embryo production is a powerful tool for extensive studies on gamete function and embryo development; however, a detailed and precise description of this method has not been published to date. Here, we describe in detail our most up-to-date procedure for reconstructing oocytes by serial nuclear transfer. This protocol will aid researchers who would like to use this technique for advanced studies to explore the potential endowments of gametes and to confer novel properties to them.

MATERIALS

REAGENTS

- Mice: B6D2F1(C57BL/6 × DBA/2) female mice, 2–3 months old, for the collection of MII and fully grown GV-stage oocytes; B6D2F1 or ICR (CD-1) mice, 2–6 months old, as recipients, foster mothers and vasectomized males; and *H19*-DMR and *Dlk1-Dio3* intergenic germline-derived (IG)-DMR double-knockout mice, 1-d-old female newborn, as the donors of ng oocytes
  ! CAUTION Experiments involving live rodents must conform to appropriate national and institutional regulations.
- Equine chorionic gonadotrophin (eCG or PMSG; Aska Pharmaceutical Co. Ltd., cat. no. S938)
- Human chorionic gonadotrophin (hCG; Aska Pharmaceutical Co. Ltd., cat. no. S012)
- M2 medium (Sigma, cat. no. M7167)
- M16 medium (Sigma, cat. no. M7292)
- Alpha-minimum essential medium (α-MEM; GIBCO/BRL, cat. no. 12000-022)
- Hyaluronidase (Sigma, cat. no. H4272)
- Cytochalasin B (CB; Sigma, cat. no. C6762)
- Colcemid (or demecolcine; Wako Chemicals, cat. no. 049-16961)
- Dibutyryl cyclic-AMP (dbcAMP; Sigma, cat. no. D0260)
- Dimethyl sulfoxide (DMSO; Sigma, cat. no. D2650)
- Collagenase (Wako Chemicals, cat. no. 034-10533)
- Paraffin liquid (Nacalai Tesque, cat. no. 26137-85)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; Sigma, cat. no. E6635)
- $SrCl_2 \cdot 6H_2O$ (Sigma-Aldrich, cat. no. 28-5240-5)
- NaCl (Nakalai Tesque, cat. no. 31333-45)
- KCl (Nakalai Tesque, cat. no. 28538-75)
- KH_2PO_4 (Nakalai Tesque, cat. no. 28736-75)
- $MgSO_4 \cdot 7H_2O$ (Nakalai Tesque, cat. no. 21003-75)
- $NaHCO_3$ (Nakalai Tesque, cat. no. 31213-15)
- Sodium lactate, 60% (wt/wt) syrup (Sigma, cat. no. L7900)
- Sodium pyruvate (Sigma, cat. no. P4562)

- D-glucose (Nakalai Tesque, cat. no. 16806-25)
- Bovine serum albumin (BSA; Sigma, cat. no. A8806)
- Fetal bovine serum (FBS; SAFC Biosciences, cat. no. 12303C)
- Penicillin–streptomysin sulfate mixed solution (P-S solution; Nakalai Tesque, cat. no. 26253-84)
- Phenol red (Sigma, cat. no. P3532)
- Trypsin–EDTA solution (GIBCO/BRL, cat. no. 25300-054)
- 70% ethanol
- Chilled freezing medium (cell banker containing FBS, Nippon Zenyaku Kogyo Co. Ltd., cat. no. ZCB-101)
- β-Propiolactone (Wako, cat. no. 166-21012)
- Nonidet P-40 (NP-40; Nakalai Tesque, cat. no. 23640-94)
- Inactivated Sendai virus (HVJ) ▲ **CRITICAL** Preparation procedure is described in Steps 8–24.

EQUIPMENT

- Inverse microscope with differential interference device and Hoffman optics (TE2000-U; Nikon)
- Stereomicroscope (SMZ800; Nikon)
- Micromanipulator (MMN-1; Narishige)
- Injector for oocyte enucleation and nuclear transfer (IM-5A)
- Injector for oocyte holding (IM-5B)
- Microforge with micro-measure (MF-900; Narishige)
- Micropipette puller (P-97/IVF; Sutter Instrument)
- Fiber illuminator (C-F1115; Nikon)
- CO_2 gas incubator (MTP-3159; Sanyo)
- Multiple-gas incubator (HERAcell150; Kendro)
- Bio-clean bench (VST-1000; NK-system)
- Pipetman (P1000, P200, P10 and P2; Gilson)
- Plastic Petri dish (Falcon, cat. no. 35-1008)
- 50-μm disposable pipette (1-000-0500; Drummond)
- 10-μm Drummond microdispenser 100 replacement tube (3-000-210-G; Drummond)
- Tweezers (INOX 5, FONTAX)
- Glass tube (GD-1; Narishige)
- Cryotube (IWAKI, cat. no. 2711-001, Corning or Nunc)
- BICELL (bio-freezing vessel) (Nihon Freezer Co. Ltd.)
- Holding pipette: outer diameter (OD) = ~80 μm, with a narrow end of inner diameter (ID) of ~10 μm, and processed from a glass tube (GD-1) ▲ **CRITICAL** Processing of micromanipulation tools should be performed using a micropipette puller and microforge.
- Micropipette: OD/ID = 20–25 μm for the GV-stage oocyte and 15–20 μm for the MII-stage oocyte, with a right-angled end, and processed from a glass tube (10-μm Drummond microdispenser 100 replacement tube)
- Glass needle: thickness = 8–10 μm, length = 150–200 μm, and processed from a glass tube (10-μm Drummond microdispenser 100 replacement tube)

REAGENT SETUP

Stock solutions and media These must be prepared as described in **Tables 1** and 2. Supplement all media with antibiotics (e.g., 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomysin) in this protocol. ▲ **CRITICAL** Developmental ability of the reconstituted embryos depends on the medium quality. The storage period of each medium or the quality of basal distilled water could be also important factors for experimental success.

PROCEDURE

Preparation of micromanipulation tools

1| Pull out 10-μl Drummond microdispenser 100 replacement tubes by using a puller. The appropriate distance between the shoulder and tip of the pipette should be ~8–10 mm.

▲ **CRITICAL STEP** In the absence of any indication otherwise, all steps should be performed at room temperature (~25 °C).

2| For the cutting knife, break the tip of a pulled tube, and once again pull the tip of the broken tube by contact with heated glass by using a microforge (**Fig. 2a,b**).

3| For the blunt enucleation pipette, break the tip of a pulled tube such that the ID is ~20–25 μm (**Fig. 2a**); next, rinse the tip with dilute (10%, vol/vol) hydrofluoric acid and 10–20 times with deionized water.

4| For the blunt transfer pipette, break the tip of a pulled tube using the microforge and rinse the tube with dilute hydrofluoric acid and deionized water (interior diameter (ID) required: 15–20 μm for ng-oocyte transplantation, 25 μm for GV removal and MII-spindle exchange) (**Fig. 2a**).

5| Bend the cutting knives and all the pipettes ~2 mm from the tip by using the microforge (**Fig. 2c**).

6| Rinse the enucleation and transfer pipettes with 100% Nonidet P-40 (NP-40) and 10–20 times with deionized water.

! **CAUTION** As hydrofluoric acid and NP-40 are toxic for oocytes, ensure that the manipulation pipettes are washed thoroughly.

7| Bend the cutting knives and all the pipettes at ~15–20 mm from the tip by placing them near a flame (**Fig. 2d**). Use above glass tools in combination with the injection system described in Steps 27–29 (**Fig. 2e**).

▲ **CRITICAL STEP** If all the pipettes are washed by pumping hot water through them using a syringe, they can be reused several times.

Preparation of the hemagglutinating virus of Japan

8| Prepare 50–100 10-d-old embryonated chicken eggs.

! **CAUTION** All steps should be performed in a safety cabinet regardless of HVJ inactivation. As far as possible, all manipulations involving HVJ should be performed on ice. All waste must be treated with freshly prepared 1% (vol/vol) sodium hypochlorite solution for more than 6 h.

9| Puncture a hole in each eggshell by using a 21-gauge (G) needle. Via this hole, inoculate 0.1 ml of fresh HVJ suspension into the chorioallantoic cavity by using a 26-G needle on a 1-ml syringe. Incubate the eggs for 3 d at 37 °C and then overnight at 4 °C.

10| Harvest the chorioallantoic fluid (usually 6–10 ml per egg) from the cavity by using an 18-G needle attached to a 10-ml syringe.

TABLE 1 | Preparation of stock solutions.

Stock solution	Basal solution	Components	Volume	Concentration	Note
CB stock solution					Storable indefinitely at −20 °C
	DMSO		5 ml	—	
		CB	5 mg	1 mg ml^{-1}	
Colcemid stock solution					Storable indefinitely at −20 °C
	DMSO		10 ml	—	
		Colcemid	1 mg	0.1 mg ml^{-1}	
Ca^{2+}- and BSA-free M16 medium					Filter with a 0.22-μm filter after preparation. Storable for 3 m at 4 °C
	DW		100 ml	—	
		NaCl	553.3 mg	94.66 mM	
		KCl	35.6 mg	4.78 mM	
		KH_2PO_4	16.2 mg	1.19 mM	
		$MgSO4 \cdot 7H_2O$	29.3 mg	1.19 mM	
		$NaHCO_3$	210.1 mg	25.0 mM	
		Sodium lactate	570 μl	23.28 mM	
		Sodium pyruvate	3.6 mg	0.33 mM	
		Glucose	100 mg	5.56 mM	
		P-S solution	500 μl	Penicillin: 50 U ml^{-1} Streptomycin: 50 μg ml^{-1}	
		Phenol red	1.0 mg	—	
Ca^{2+}-free BSS buffer					
	DW		1,000 ml		Dissolve the reagents in DW, and then adjust the pH to 7.5 using HCl. Finally, autoclave the solution at 120 °C for 20 min
		NaCl	8 g	137 mM	
		KCl	0.4 g	5.4 mM	
		KH_2PO_4	0.06 g	0.44 mM	
		$Na_2HPO_4 \cdot 2H_2O$	0.06 g	0.34 mM	
		Tris (hydroxymethyl) aminomethane-HCl (pH 7.5)	1.57 g	13 mM	
200 mM $CaCl_2 \cdot 2H_2O$ solution					
	DW		100 ml		
		$CaCl_2 \cdot 2H_2O$	2.94 g		
BSS buffer	Ca^{2+}-free BSS buffer	1,000 ml			
		200 mM $CaCl_2 \cdot 2H_2O$ solution	10 ml	2 mM	
EDTA solution					Filter with a 0.22-μm filter after preparation. Storable for 6 m at room temperature
	DW		100 ml		
		EDTA	0.02 g		
		NaCl	0.8 g		
		KCl	0.02 g		
		Na_2HPO_4	0.115 g		
		KH_2PO_4	0.02 g		
		Glucose	0.02 g		
		Phenol red	0.001 g		

TABLE 2 | Preparation of media for oocyte manipulation.

Medium	Basal medium	Components	Volume	Final concentration	Note
Handling medium for GV-stage oocytes					Prepare and filter with a 0.22-μm filter before use. Storable for several months at 4 °C
	M2 medium		9.5 ml	—	
		dbcAMP stock solution	1 mg	200 μM	
		BSA	40 mg	4.0%	
Nuclear transfer medium for GV-stage oocytes					Prepare immediately before use
	M2 medium		9.5 ml	—	
		CB stock solution	100 μl	10 μg ml^{-1}	
		Colcemid stock solution	10 μl	0.1 μg ml^{-1}	
		dbcAMP stock solution	1 mg	200 μM	
		BSA	40 mg	4.0%	
Nuclear transfer medium for MII-stage oocytes					Prepare immediately before use
	M2 medium		950 μl	—	
		CB stock solution	50 μl	50 μg ml^{-1}	
In vitro maturation medium for reconstituted GV-stage oocytes					Prepare immediately before use
	α-MEM		950 μl	—	
		FBS	50 μl	5%	
Activation medium					Prepare and filter with a 0.22-μm filter before use
	Ca^{2+}- and BSA-free M16 medium		10 ml	—	
		$SrCl_2 \cdot 6H_2O$	26.7 mg	10 mM	
		PVA	10 mg	0.1%	
Collagenase solution					Filter with a 0.22-μm filter after preparation. Storable indefinitely at −20 °C
	BSA-free M16 medium		100 ml	—	
		Collagenase	100 mg	1 mg ml^{-1}	
Hyaluronidase solution					Filter with a 0.22-μm filter after preparation. Can be stored indefinitely at −20 °C
	M2 medium		100 ml		
		Hyaluronidase	30 mg	300 U ml^{-1}	

11| Transfer the fluid obtained from each egg into a separate 15-ml tube, and centrifuge the tubes at 200*g* and 4 °C for 10 min. Exclude hemolyzed and clear (not well harvested) chorioallantoic fluid samples at this step.

12| Transfer the supernatant into fresh tubes, and centrifuge at 20,000*g* and 4 °C for 30 min.

13| Suspend the pellet in 1 ml of Ca^{2+}-free BSS per tube, and incubate for 24 h at 4 °C.

14| Collect the suspensions from all tubes into a 15-ml tube.

15| Centrifuge at 20,000*g* and 4 °C for 30 min, and suspend the pellet in 1 ml of SSC.

16| Centrifuge at 200*g* and 4 °C for 10 min, and transfer the supernatant into a fresh tube.

17| For HVJ inactivation, add 1% (vol/vol) β-propiolactone solution to the suspension at a final concentration of 0.025%.

18| Mix gently and incubate at 4 °C for 24 h.

19| To inactivate the β-propiolactone in the HVJ suspension, incubate the suspension for 20 min at 37 °C.

20| Dispense the HVJ suspension into cryotubes and preserve it in LN_2. For cell fusion, dilute the HVJ suspension in BSS after thawing from LN_2. Subsequently, confirm the suspension titer of HVJ by using oocytes or embryos even though the titer required for cell fusion is 2,700 HAU ml^{-1}.

HVJ titer check

21| Perform a hemagglutination (HA) test for determining the titer of the HVJ suspension. This requires a 500-fold dilution of the suspension by using SSC. First take 50 μl SSC into 11 wells (nos. 2–12) of a 96-well plate (**Fig. 3a**).

22| Prepare a serial dilution of the HVJ samples as follows. Add 50 μl of the 500-fold-diluted HVJ suspension into well no. 1 of the 96-well plate (**Fig. 3b**). Add 50 μl of the 500-fold-diluted HVJ suspension into the next well (no. 2) and mix; subsequently, add 50 μl of this sample mixture into the third well (no. 3). Repeat this manipulation sequentially for all the wells up to well no.11 (**Fig. 3b**).

23| Add 50 μl of 0.5% (vol/vol) chicken red blood cell (RBC) suspension to all the wells (nos. 1–12) and mix (**Fig. 3c**). Incubate the plate for 1 h at room temperature.

24| Assess the position of the RBCs. Agglutinated RBCs will coat the wells uniformly, whereas the nonagglutinated RBCs will form a dense cluster at the bottom of the wells. The maximum dilution at which RBC agglutination is observed is considered the titer of the virus suspension and is expressed as HA units (HAU) per ml. For example, if the maximum dilution at which agglutination is observed is 64, then the titer of the HVJ suspension is 6.4×10^5 ($64 \times 20 \times 500$) HAU ml^{-1}.

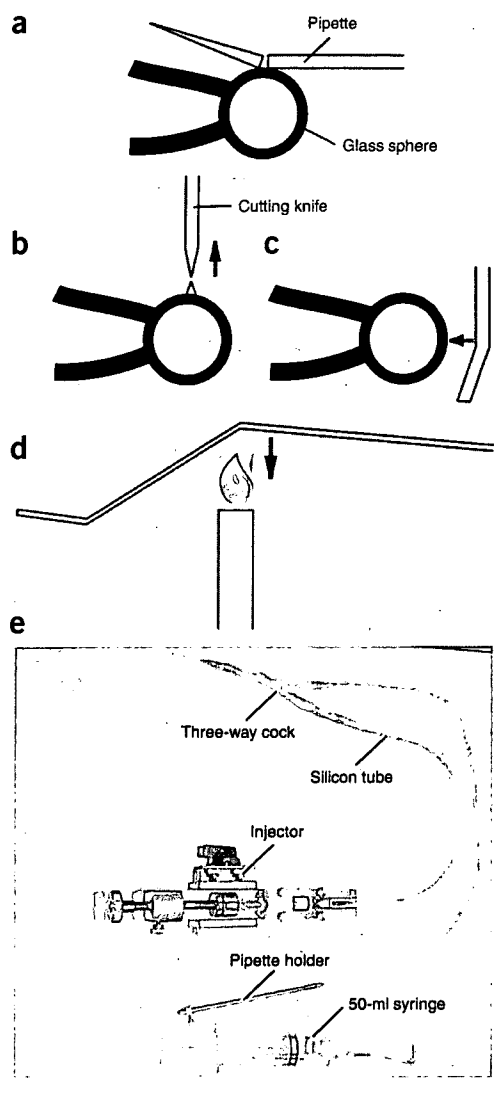

Figure 2 | Preparation of micromanipulation tools using a microforge. (**a**) Break the tip of the pulled tube such that the ID is appropriately sized (refer to Steps 1–7). (**b**) To prepare the cutting knife, pull the tip of the broken tubes once again by maintaining contact with heated glass. (**c**) Bend the cutting knives and all the pipettes at ~150–200 μm from the tip. (**d**) Bend the cutting knives and all the pipettes ~15–20 mm from the tip by placing them near a flame. (**e**) Construction of the injection system. Ensure that air leakage from the tube seams is blocked: and use the 18-G needle blunted at the interface between the silicon tube and a 50-ml plastic syringe.

Oocyte collection

25| Collect oocytes. ng oocytes, GV-fg oocytes and MII-fg oocytes are all required and should be collected as described in options A, B or C, respectively.

(A) Fresh ng oocytes

(i) Collect the ovaries of 1- to 3-d-old mice under a stereoscopic microscope. The pair of ovaries is located dorsolaterally in the abdominal cavity toward the posterolateral poles of the kidneys. They are enclosed within a thin membrane called the bursa. Remove the bursa and uterus by using tweezers, and separate the clump comprising the ovary, oviduct and uterus and immerse it in 2.5 ml of M2 medium in a 35-mm dish (**Table 2**).

(ii) After isolating the ovaries, incubate them in 2.5 ml of EDTA solution (**Table 2**) in a 35-mm dish at 37 °C for 12 min. The EDTA treatment is effective for dispersing ng oocytes from an ovarian tissue[1].

(iii) Transfer the ovaries to 2.5 ml of M2 medium in a 35-mm dish and gently puncture them using 27-G needles mounted on 1-ml syringes. The ng oocytes and follicle cells should flow out.

(iv) Collect the ng oocytes using a glass pipette drawn from a 50-μm disposable pipette.

(v) Culture the ng oocytes in 15- to 20-μl M2 medium droplets containing 10 μg ml^{-1} cytochalasin B and 0.1 μg ml^{-1} colcemid for at least 10 min before the nuclear transfer.

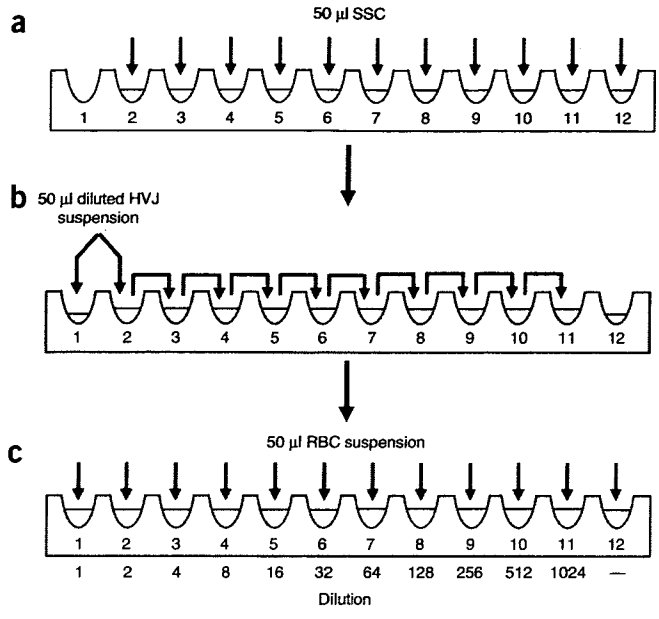

Figure 3 | Hemagglutination test. (**a**) Add 50 µl of SSC into 11 wells (nos. 2–12). (**b**) Add 50 µl of diluted HVJ suspension into well nos. 1 and 2. Mix the suspension in well no. 2 thoroughly, and add 50 µl of this mixture to well no. 3. Repeat this manipulation sequentially for all the wells up to well no. 11. (**c**) Add 50 µl of RBC suspension to all the wells (nos. 1–12). Mix the suspension in all the wells thoroughly. Evaluate hemagglutination following incubation at room temperature for 1 h.

(B) GV-fg oocytes

(i) Inject adult female mice with 5 IU of eCG.
▲ **CRITICAL STEP** Hybrid mice such as B6D2F1 are recommended for this experiment. Certain outbred strains such as the ICR strain are not suitable for this experiment, as their ooplasm at the GV stage is extremely fragile.

(ii) Collect the ovaries at 44–48 h after the eCG injection.

(iii) To obtain GV-fg oocytes, transfer the ovaries to 2.5 ml of M2 medium containing dbcAMP in a 35-mm dish.

(iv) Puncture the large antral follicles by using 27-G needles mounted on 1-ml syringe barrels.

(v) Collect the GV-fg-stage oocytes tightly connected with polyptychial cumulus cells by using a glass pipette drawn from a 50-µm disposable pipette, and transfer them to 2.5 ml of fresh M2 medium containing dbcAMP in a 35-mm dish.

(vi) Denude the oocytes from the cumulus cells by pipetting using a glass pipette (ID ~75 µm) drawn from a 50-µm disposable pipette.

(vii) To create a perivitelline space between the zona pellucida and the oocyte, culture the denuded oocytes in a 50-µl α-MEM droplet containing dbcAMP and 5% FBS at 37 °C for 1 h in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 before the nuclear transfer.

(C) MII-fg oocytes

(i) Inject adult female mice with 5 IU of eCG and 5 IU of hCG at 48 h apart.
▲ **CRITICAL STEP** Hybrid mice are recommended for this experiment as described in Step B(i).

(ii) Collect the oviducts at 14–16 h after the hCG injection. Separate the ovary, oviduct and end of the uterus from the mesometrium with fine forceps. Following cutting end of the uterus near the oviduct, additionally cut between the oviduct and ovary with fine scissors.

(iii) To obtain MII-fg oocytes, transfer the oviducts to 1 ml of M2 medium containing 300 µg ml^{-1} of hyaluronidase in a 35-mm dish.

(iv) Puncture the oviductal ampullae using 27-G needles mounted on 1-ml syringe barrels. The oocyte-cumulus cell complexes (COCs) should flow out.

(v) Incubate these COCs in the M2 medium containing hyaluronidase for a few minutes at room temperature (~25 °C).

(vi) Transfer these COCs to 2.5 ml of fresh M2 medium in a 35-mm dish.

(vii) Denude the oocytes from the complexes by pipetting using a glass pipette (ID ~75 µm).

(viii) Wash the oocytes three times in fresh M2 medium.

Cryopreservation of the ng oocytes

26| If desired, freeze the oocytes as described in option A. Frozen oocytes should be thawed as described in option B.

(A) Freezing of the ng oocytes

(i) Incubate a maximum of 10 ovaries obtained from 1–3 d newborn mice in 200 µl of BSA-free M2 medium containing 1 mg ml^{-1} collagenase at 37 °C in a 1.5-ml tube.

(ii) After 20 min, centrifuge the tubes at 5,200*g* for several seconds at room temperature and remove the supernatant.

(iii) Add 200 µl of trypsin–EDTA solution and incubate the tubes at 37 °C for 10 min.

(iv) Add 200 µl of M2 medium and mix by vigorous pipetting using a Pipetman.

(v) Centrifuge the tubes at 5,200*g* for several seconds at room temperature and throw away the supernatant.

(vi) Add 100 µl of chilled freezing medium per ovary and mix by gentle pipetting.

(vii) Transfer 100 µl of cell suspension to each 2-ml cryotube and stand on ice for 5 min.

(viii) Store the vials wrapped in BICELL (bio-freezing vessel) at −80 °C.

(ix) On the following day, transfer the cryotubes into a tube container at −80 °C in a freezer or −196 °C in liquid nitrogen (LN_2).

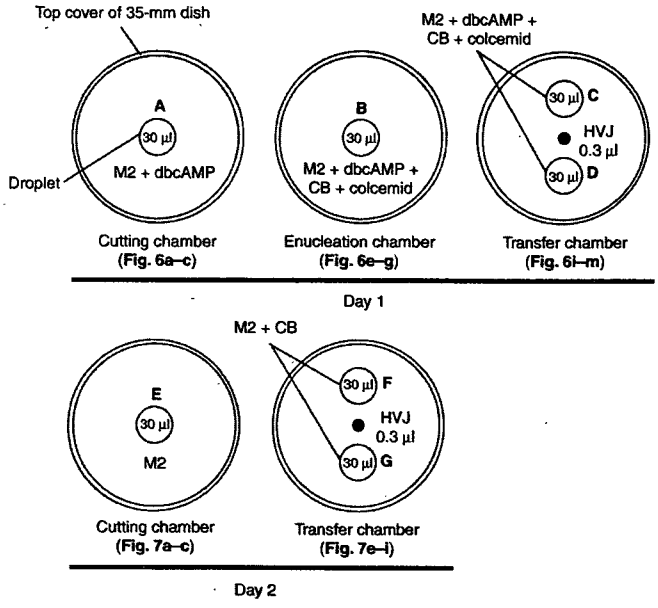

Figure 4 | Setup of the manipulation chamber. Two days are required to complete all the steps for oocyte reconstruction by serial nuclear transfer. On day 1, prepare the chamber for the three types of manipulations, namely cutting, enucleation and transfer of the ng oocyte to the top of a 35-mm dish. Similarly, on day 2, prepare the chamber for two types of manipulations, namely cutting and transfer of the ng oocyte-derived MII plate. Cover all droplets on the manipulation chamber with paraffin oil. A–G correspond to the description for serial nuclear transfer in **Figures 6** and **7**.

(B) Thawing the ng oocytes

(i) Heat α-MEM supplemented with 20% FBS in a heat block at 37 °C.

(ii) Thaw the frozen cryotube containing the ng oocytes in a heat block at 37 °C as rapidly as possible.

(iii) Add 1 ml of the warm α-MEM supplemented with 20% (vol/vol) FBS per cryotube and mix gently.

(iv) Centrifuge the tube at 5,200*g* for several seconds at room temperature and throw away the medium.

(v) Add 100 µl of M2 medium containing 10 µg ml^{-1} cytochalasin B and 0.1 µg ml^{-1} colcemid and mix by gentle pipetting.

Setup of the injector equipped with the micromanipulator

27| Combine silicon tubes for constructing the injection system as shown in **Figure 2e**. We use air, not paraffin, to fill the microinjector; however, the material used can depend on the experimenter's preference, as it does not affect the outcome of the experiment.

28| Ensure that air leakage from the tube seams is blocked. If necessary, plug the holes in the tubes by using instant adhesive and/or parafilm.

29| Use an 18-G needle blunted at the interface between the silicon tube and the 50-ml plastic syringe.

30| Once the enucleation and transfer pipettes are equipped with holders and entered into the operation droplet, place the pipette beside the droplet B 2–3 mm apart (**Fig. 4**).

31| Suction paraffin oil into the extended part of the pipette using the 50-ml syringe.

32| Enter the pipette into the droplet B and adjust the internal pressure in the pipette by pumping the 50-ml syringe. Constrict the silicon tube ahead of the syringe tightly by using a forceps.

CAUTION Ensure that the front of the oil droplet at the tip of the pipette is at a standstill. The internal pressure of the pipette should be finely controlled by using the injector.

Construction of bimaternal embryos by serial nuclear transfer: day 1

33| Prepare the droplets for oocyte manipulation (**Fig. 4**) and *in vitro* maturation (IVM) of the reconstructed oocytes. For oocyte manipulation, place 30-µl droplets (droplets A–D) of manipulation medium on top of a 35-mm dish as described in **Figure 4** and then cover with mineral oil. In addition, for IVM culture and washing oocytes, prepare 50-µl droplets of α-MEM containing 5% (vol/vol) FBS with or without dbcAMP on a 35-mm dish, respectively.

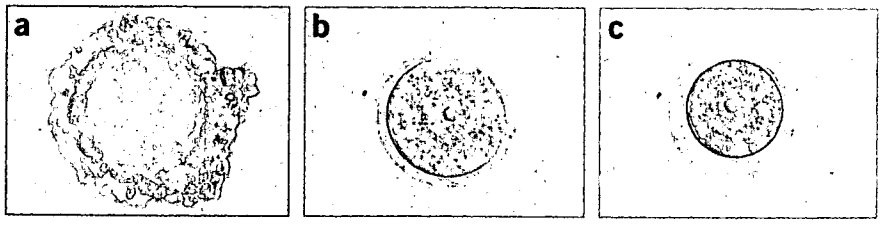

Figure 5 | Creating a perivitelline space in the GV oocytes. (**a**) An oocyte–cumulus cell complex obtained from a large antral follicle. (**b**) A denuded GV-stage oocyte before incubation. (**c**) A GV-stage oocyte with a perivitelline space.

34| Place the denuded GV-fg oocytes into α-MEM droplets containing dbcAMP and 5% FBS, and incubate at 37 °C for 1 h in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 (**Fig. 5a,b**).

△ **CRITICAL STEP** Creating a perivitelline space in the GV-fg oocytes by incubation is extremely important for avoiding damage caused to the zona pellucida with a glass knife.

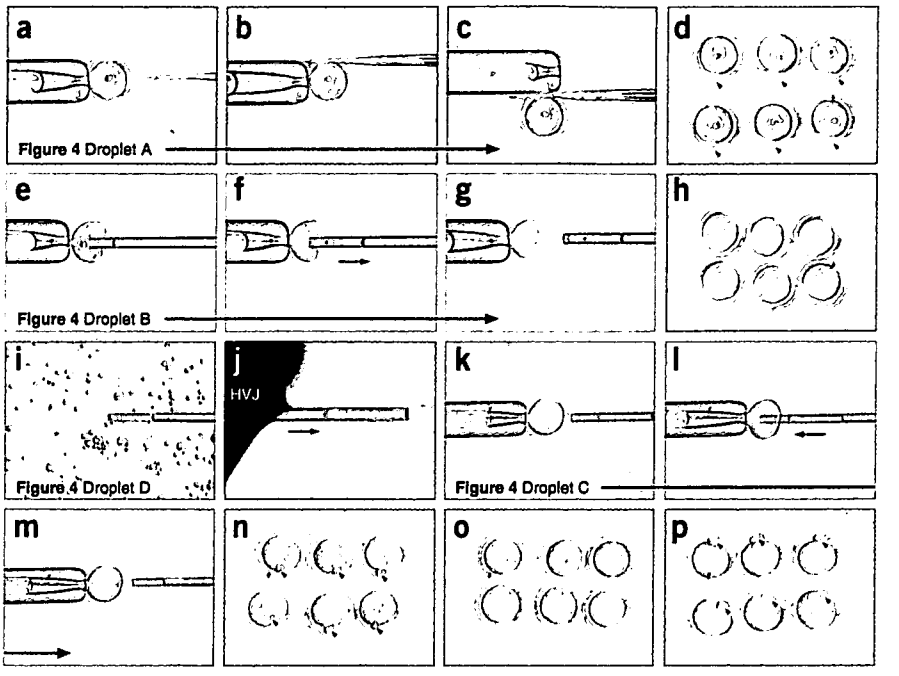

Figure 6 | Sequential protocol for the first nuclear transfer. (**a**) Hold a GV oocyte containing a perivitelline space. (**b**) Insert a cutting knife into the perivitelline space. (**c**) Dissect only the zona pellucida without rupturing the oolemma. (**d**) Thus, the GV oocytes exhibit a slit (arrowhead). (**e**) Insert the enucleation pipette into the oocyte via the slit, and maneuver it adjacent to the GV. (**f**) Suction the GV into the enucleation pipette and (**g**) slowly withdraw the pipette. (**h**) Enucleated GV oocytes. (**i**) Draw several ng oocytes into the transfer pipette by suction. (**j**) Next, draw up a small volume of inactivated HVJ solution. (**k**) Hold an enucleated GV oocyte. (**l,m**) Place the ng oocyte under the zona of the enucleated recipient GV oocyte containing inactivated HVJ. Following (**n**) incubation of the enucleated recipient GV oocyte with the ng oocyte (arrowhead), (**o**) confirm cell fusion and cultivate the reconstructed oocytes for IVM. (**p**) The reconstructed oocytes are arrested at MII after IVM (arrowhead; first polar body).

35| After incubation, transfer the GV-fg oocytes forming a perivitelline space into droplet A in the micromanipulation chamber (**Figs. 4** and **5c**).

36| Rotate the GV-fg oocyte such that the perivitelline space is positioned at 6 or 12 o'clock and attach it onto the holding pipette (**Fig. 6a**).

37| Insert a cutting knife into the perivitelline space and dissect only the zona pellucida without rupturing the oolemma (**Fig. 6b,c**).
△ **CRITICAL STEP** If the cutting knife is too small, use a larger one. An appropriately sized knife will facilitate the cutting of the zona pellucida.
? TROUBLESHOOTING

38| Place the GV-fg oocytes having slits in the zona pellucida into M2 droplets containing colcemid, CB and dbcAMP (**Fig. 6d**) and incubate them at 37 °C for 10–20 min in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

39| Transfer the GV-fg oocytes into droplet B in **Figure 4**.

40| Insert the enucleation pipette into the oocyte through the slit, and maneuver it so that it is adjacent to the GV (**Fig. 6e**).

41| Suction the GV into the enucleation pipette, and slowly withdraw the pipette (**Fig. 6f–h**).
? TROUBLESHOOTING

42| Place ~10–20 enucleated GV-fg oocytes into droplet C for ng-oocyte transplantation (**Fig. 4**).

43| Transfer plenty of the ng oocytes (obtained from Step 26A) into droplet D by using a glass pipette (**Fig. 4**).

44| Suction a few ng oocytes into the transfer pipette, followed by a small volume of the inactivated HVJ solution (**Fig. 6i,j**).
? TROUBLESHOOTING

45| Push the pipette into the central position of the enucleated GV-fg oocyte in droplet C (**Fig. 4**).

46| Gently inject the ng oocyte with inactivated HVJ solution into the hollow and slowly withdraw the pipette (**Fig. 6k–m**).
△ **CRITICAL STEP** Pressing the ng oocyte toward the recipient GV-fg oocyte for a while will enhance the fusion rate.
‼ **CAUTION** Following ng-oocyte transplantation, incubate the manipulated oocytes as quickly as possible.
? TROUBLESHOOTING

47| Transfer the enucleated GV-fg oocytes with ng oocytes into a 50-μl α-MEM droplet supplemented with dbcAMP and 5% (vol/vol) FBS in a 35-mm dish, and incubate them at 37 °C for 30–60 min in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 (**Fig. 6n**).

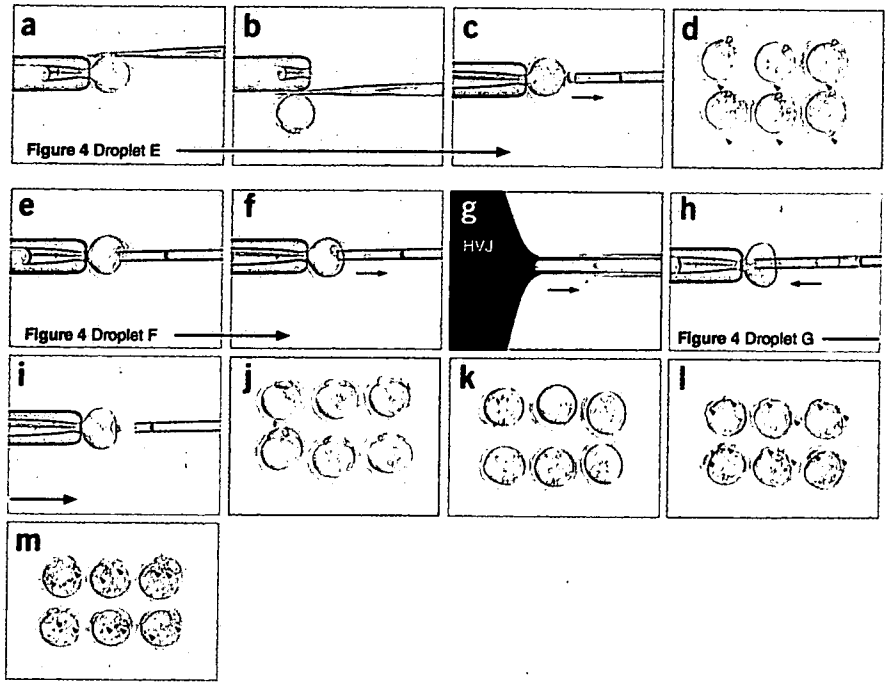

Figure 7 | Sequential protocol for the second nuclear transfer. (**a**) Insert a cutting knife into the perivitelline space opposite the MII spindle. (**b**) Dissect only the zona pellucida without rupturing the oolemma. (**c**) Slowly suction the first polar body from the fg oocytes. (**d**) Thus, the fg oocytes without the first polar body exhibit a slit (black arrowhead, slit; white arrowhead, MII spindle). (**e**) Insert the transfer pipette into the MII oocytes reconstructed with ng oocytes, and (**f**) suction the MII plate along with a small volume of ooplasm. (**g**) Next, draw up a small volume of inactivated HVJ solution. (**h,i**) Place the MII plate under the zona pellucida of the fg oocyte with inactivated HVJ. (**j**) After incubating the fg oocyte with the ng oocyte-derived MII spindle, (**k**) confirm cell fusion and activate the reconstructed oocytes. (**l**) Following activation, confirm the presence of two polar bodies (arrowheads). (**m**) Thus, a reconstructed ng/fg bimaternal embryo that forms two female pronuclei (arrowheads) is created.

48| Confirm the fusion of enucleated GV-fg oocytes with ng oocytes (**Fig. 6o**), and culture the reconstructed oocytes for 14 h in α-MEM droplets containing 5% (vol/vol) FBS. More than 95% of the reconstructed oocytes exclude the first polar body and form MII plate derived from ng oocyte chromatin (**Fig. 6p**).
? TROUBLESHOOTING

49| Prepare the M16 droplets for IVC.

Construction of bimaternal embryos by serial nuclear transfer: day 2

50| Prepare the droplets for oocyte manipulation (**Fig. 4**) and artificial activation.

51| Transfer the MII-fg oocytes into droplet E for cutting the zona pellucida (**Fig. 4**).

52| Rotate the MII-fg oocyte to position the spindle at 6 or 12 o'clock and hold it onto the holding pipette.
? TROUBLESHOOTING

53| Insert a cutting knife into the perivitelline space at the opposite end of the MII plate and dissect only the zona pellucida without disrupting the oolemma (**Fig. 7a,b**).

54| Place the MII-fg oocytes having slits in the zona pellucida into M2 droplets containing CB and incubate them at 37 °C for 5–10 min in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

55| Place 10–20 reconstructed MII oocytes that were produced on day 1 and 10–20 MII-fg oocytes having slits in the zona pellucida in the droplets F and G, respectively (**Fig. 4**).

56| Before nuclear transplantation, remove the first polar body of the MII-fg oocyte to subsequently assess cell fusion (**Fig. 7c,d**).

57| Insert the transfer pipette into the reconstructed MII oocytes and suction the ng oocyte-derived MII plate along with a small volume of ooplasm (ng-karyoplast) into the pipette (**Fig. 7e,f**).
? TROUBLESHOOTING

58| Suction a small volume of the inactivated HVJ solution into the pipette (**Fig. 7g**).
? TROUBLESHOOTING

59| Push the pipette into the central position of the MII-fg oocyte and gently inject the ng-karyoplast with inactivated HVJ solution into the hollow, and slowly withdraw the pipette (**Fig. 7h,i**).

▲ **CRITICAL STEP** Pressing the ng-karyoplast toward the MII-fg oocyte will enhance the fusion rate. Furthermore, placing the ng-karyoplast in the opposite direction of the MII plate of recipient oocytes is significant for forming the two sets of second polar bodies and pronuclei.
? TROUBLESHOOTING

60| Transfer the manipulated oocytes into 50-μl M16 droplets in 35-mm dish and incubate them at 37 °C for 30–60 min in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 (**Fig. 7j**).
▲ **CRITICAL STEP** To accelerate cell fusion, every 15 min, transfer the manipulated oocytes into an M16 droplet in 35-mm dish and incubate them as quickly as possible.

61| Following incubation for 30–60 min, confirm the fusion of the MII-fg oocytes with the ng-karyoplasts and transfer the fused oocytes into 50-μl M16 droplets containing $SrCl_2$ in a 35-mm dish (**Fig. 7k**).
? TROUBLESHOOTING

62| Following incubation for 3–4 h, culture the reconstructed ng/fg bimaternal embryos that form two second polar bodies and two female pronuclei (**Fig. 7l,m**) in M16 droplets in a 35-mm dish at 37 °C for 3.5 d in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.
? TROUBLESHOOTING

63| Transfer the ng/fg bimaternal embryos that develop to the blastocyst stage into the uterine horns of recipient female mice at 2.5 d of pseudopregnancy.
? TROUBLESHOOTING

○ **TIMING**
Steps 1–24 and 27–31 (preparations of micromanipulation tools and HVJ, and setup of the injector equipped with the micromanipulator) should be finished before starting oocyte manipulation.

For oocyte reconstruction by the serial nuclear transfer method, 2 d are required to complete all the steps. We here show our usual time course of the serial nuclear transfer. During CB and colcemid treatment of the GV-fg oocytes (Step 38), the ng oocytes are prepared from newborn female mice or cryopreserved stocks.

On day 1:
9:30 Preparation of the microdroplets for oocyte manipulation and IVM (Step 33)
12:30 Collection of the GV-fg oocytes (Step 25B)
13:30 Incubation of the collected GV-fg oocytes (Step 34)
14:20 Preparation of the micromanipulation (Step 32)
14:30 Cutting of the perivitelline space of the GV-fg oocytes (Steps 35–37)
15:00 Transfer of the GV-fg oocytes having slits into M2 droplets with cytoskeletal inhibitors, and incubation of them (Step 38)
15:15 Enucleation of the GV-fg oocytes (Steps 39–41)
16:45 Injection of the ng oocytes with HVJ into the enucleated GV-fg oocytes (Steps 42–47)
18:30 End of injection
19:30 Confirmation of cell fusion between the ng and the GV-fg oocytes, and starting cultivation of the reconstructed oocytes for IVM (Step 48)
19:40 Preparation of M16 IVC droplets in a 35-mm dish (Step 49)
On day 2:
8:00 Preparation of the microdroplets for oocyte manipulation and activation (Step 50)
8:30 Collection of the MII-fg oocytes (C in Step 25)
9:10 Preparation of the micromanipulation (Step 32)
9:15 Cutting of the perivitelline space of the MII-fg oocytes (Steps 51–53)
9:45 Transfer of the MII-fg oocytes having slits into M2 droplets supplemented with CB, and incubation of them (Step 54)
9:50 Injection of the ng-karyoplasts with HVJ into the MII-fg oocytes (Steps 54–60)
11:20 End of injection
12:20 Confirmation of cell fusion between the ng-karyoplasts and the MII-fg oocytes, and transferring the fused oocytes into activation droplets (Steps 61)
15:50 Starting the cultivation of the reconstructed ng/fg bimaternal embryos that form two second polar bodies and two female pronuclei in IVC droplets for 3.5 d (Step 62)

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
37 and 57	Difficulty in cutting the zona pellucida	Small cutting knife	Use a larger cutting knife
		Inaccurate positioning of the holding pipette	Ensure that the holding pipette is oriented horizontally and is firmly attached to the pipette holder
52	Difficulty in locating the oocyte spindle	Inappropriate microscope settings	Adjust the focus and illumination of the microscope. A protrusion on the oolemma, which contains a small transparent sphere, indicates the presence of the MII spindle. During trials, Hoechst staining may be used to detect the MII plate; this may improve technical skills
41, 44, 46, 57–59	Difficulty in controlling inner pressure in pipette	Air leakage from the tube seams	Plug the holes in the tubes by using an instant adhesive and/or parafilm. The presence of even a few holes hinders the control of the injector
		Insufficient constriction with forceps	Ensure tight constriction of the silicon tube by using forceps
		Inadequate pressure adjustment in the 50-ml plastic syringe	Attempt to readjust the pressure in the syringe by pumping
	Difficulty in suctioning nuclei into and releasing them from the pipette	Dirty pipette	Replace the pipette with a new one
		Inappropriate adjustment of inner pressure in the pipette by the injector	Check for air leakage from the tube seams, and check the constriction in the injector
48	Low maturation rate of the reconstructed oocytes	Insufficient washing after manipulation	Avoid IVM medium contamination with the cytoskeletal inhibitor or dbcAMP; wash the manipulated oocytes thoroughly
		Unreliable confirmation of cell fusion	Check for cell fusion and degradation under a high-power microscope
48 and 61	Unsuccessful fusion with HVJ	Inferior HVJ stock solution	Prepare fresh HVJ solution
		Insufficient karyoplast insertion	On placing the ng-karyoplast in the perivitelline space, press it toward the recipient oocyte rapidly and carefully for a while
		Small pipette	Use a larger pipette
		Inappropriate temperature conditions	Incubate the oocytes receiving the ng oocyte/ng-karyoplast with HVJ as quickly as possible
41, 46 and 59	Death of oocytes following enucleation and nuclear transplantation	Insufficient CB treatment	Prolong the CB treatment period, particularly for GV-fg oocytes. If the treatment period is too short, the oolemma of the GV-fg oocyte may become uneven
		Inappropriate pipette size	Use pipettes with an internal diameter ranging from 20 to 25 μm
62	No extrusion of the two polar bodies from the oocyte	Inappropriate site of fusion with ng-karyoplast and the MII-fg oocyte	As shown in **Figure 6**, inject the ng-karyoplast with inactivated HVJ solution at the opposite site of the recipient oocyte MII plate
	Failure of bimaternal embryo development to the blastocyst stage	Unfavorable culture conditions	Check the quality of all reagents used for the culture
63	Failure of bimaternal embryo development to term, despite appropriate steps involved in embryo manipulation	Inappropriate embryo-transfer recipients	Mate oestrous female mice with vasectomized males on the day after the second nuclear transfer. At 2.5 d of pseudopregnancy, transfer the bimaternal embryos into the vascularized uterine horns

ANTICIPATED RESULTS

It is commonly believed that serial nuclear transfer is a complex procedure. With regard to technical skills, however, all that is required is that certain points pertaining to manipulation of the GV-stage oocytes, fusion with HVJ and transplantation of the MII plate be kept in mind. However, a great deal of practice is required to develop the requisite skills. We recommend that readers begin by exchanging GVs and MII-plates between oocytes from different populations. Each set of experiments will improve the experimenter's basic technique for performing serial nuclear transfer. In addition, producing IVF embryos following MII oocyte reconstruction by serial nuclear transfer could help experimenters improve their basic manipulation skills[20,25]. In order to apply reconstructed MII oocytes for IVF, the use of hyaluronidase must be minimized; therefore, handling sticky oocytes may be more difficult than manipulating ng/fg bimaternal embryos by the typical serial nuclear transfer method. Additionally, the amount of HVJ used for cell fusion should be minimized because HVJ could induce oocyte activation (data not shown), resulting in the inhibition of sperm penetration. When reconstructed IVF embryo production is performed by a highly skilled person, 40–50% of the blastocyst-stage embryos transferred into the uterine horns of recipient females will develop to full term. The greatest challenge that experimenters may face is with regard to fusing the ng oocyte-derived MII spindles and the fg oocytes during the second nuclear transfer. As described above, minimizing the amount of HVJ used during the first nuclear transfer may solve this problem.

Unlike other oocyte manipulation techniques such as somatic cell nuclear transfer, the serial nuclear transfer method for ng/fg bimaternal embryo production is definitely time consuming and complex. However, with regard to the need for constant practice, serial nuclear transfer does not differ considerably from other methods.

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