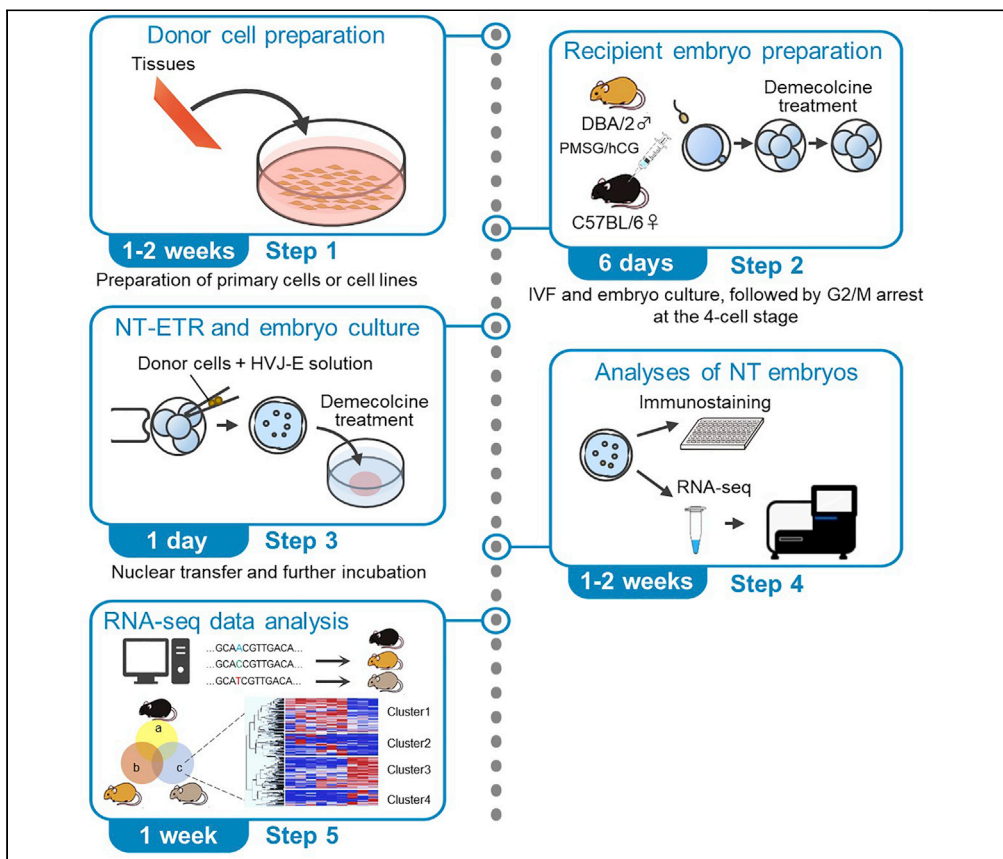


Protocol

Nuclear transfer system for the direct induction of embryonic transcripts from intra- and cross-species nuclei using mouse 4-cell embryos



Reprogramming of somatic nuclei toward the embryonic state has been studied using nuclear transfer (NT) to an oocyte at the metaphase II (MII) stage. In this NT, a somatic nucleus transplanted into an MII oocyte of the same species undergoes DNA replication and cell division before activating embryonic genes. Here, we describe a direct NT protocol using 4-cell stage mouse embryos that enables reprogramming of intra- and cross-species nuclei to express embryonic genes without requiring DNA replication and cell division.

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Highlights

Protocol to induce embryonic transcription from mammalian differentiated cells

Nuclear transfer to mouse 4-cell embryos arrested at G2/M phase

Cross-species nuclei are transcriptionally reprogrammed in mouse embryos

Detection of newly transcribed genes from multi-species and -strains samples

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Protocol

Nuclear transfer system for the direct induction of embryonic transcripts from intra- and cross-species nuclei using mouse 4-cell embryos

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SUMMARY

Reprogramming of somatic nuclei toward the embryonic state has been studied using nuclear transfer (NT) to an oocyte at the metaphase II (MII) stage. In this NT, a somatic nucleus transplanted into an MII oocyte of the same species undergoes DNA replication and cell division before activating embryonic genes. Here, we describe a direct NT protocol using 4-cell stage mouse embryos that enables reprogramming of intra- and cross-species nuclei to express embryonic genes without requiring DNA replication and cell division.

For complete details on the use and execution of this protocol, please refer to Tomikawa et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for Nuclear Transfer to Elicit embryonic Transcriptional Reprogramming (NT-ETR). NT-ETR offers a means to (1) induce embryonic transcripts from mouse somatic cells in mouse embryos arrested at the 4-cell stage and to (2) induce embryonic transcripts from cross-species somatic cells, such as those from endangered animals, in mouse embryos at the 4-cell stage (Tomikawa et al., 2021).

This protocol uses mouse 4-cell stage embryos synchronized to G2 phase as recipients for NT. We use 4-cell stage embryos because such embryos retain a degree of totipotency (Boiani et al., 2019; Maemura et al., 2021), have high transcriptional activity including zygotic transcripts, and begin to express pluripotency factors (Suzuki et al., 2015). In fact, the reprogramming ability of the 4-cell embryo allows induction of embryonic genes from the transplanted somatic nuclei within a day, and even cross-species nuclei are transcriptionally reprogrammed by NT-ETR. In conventional NT, a donor cell nucleus is introduced to an unfertilized, enucleated oocyte. This conventional approach is referred to as the Honolulu cloning technique in the case of mouse (Wakayama et al., 1998), and is still widely used to produce cloned mice. Other NT methods have also been reported (Li et al., 2012; Kang et al., 2014) and are useful for obtaining cloned animals or NT-derived embryonic stem (ES)



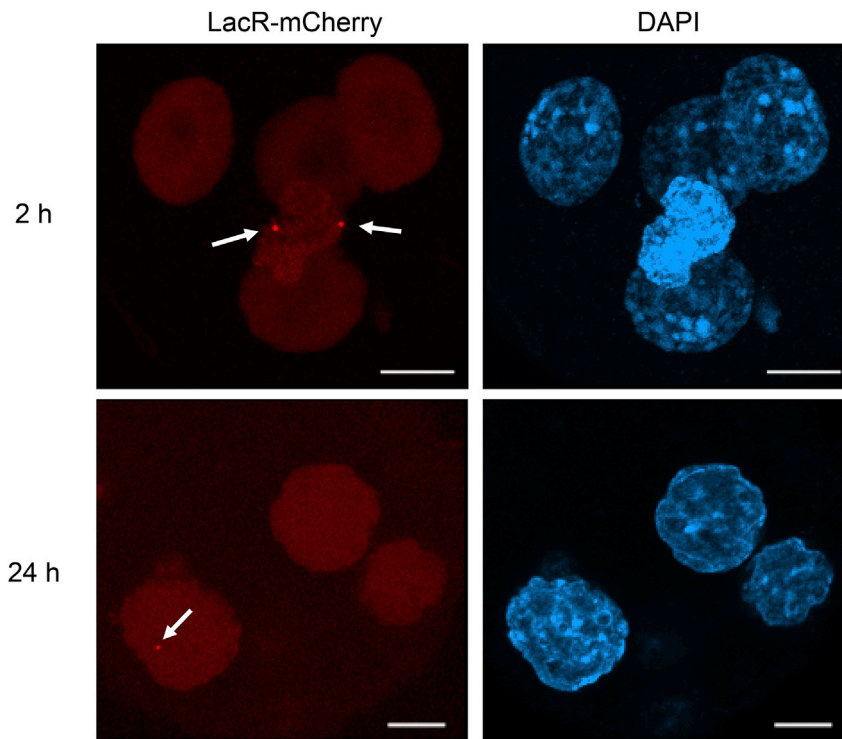


Figure 1. Identification of the injected nuclei by utilizing the transgene

Accumulation of LacR-mCherry onto the transgene site of donor nuclei marks the injected nuclei after NT-ETR (red dots; white arrows). The comparison between 2 h and 24 h after NT-ETR clearly shows the swelling of donor nuclei in the arrested mouse 4-cell embryos, finally resembling the morphology of embryonic nuclei. DNA was stained with DAPI. Scale bars, 10 μ m.

cells, but are often incapable of supporting activation of embryonic transcripts in cross-species cases. In contrast NT-ETR is capable of inducing transcription in a cross-species context, but we have not yet established protocols to support embryos to later stages or for deriving ES cells. Thus, NT-ETR is currently used to induce transcriptional reprogramming processes of intra- and cross-species nuclei and to investigate functional genomic information of various species, including those that are extinct in the wild. In this system, nuclear remodeling processes after NT can be studied by tracing the injected nuclei using, for example, lac operator/repressor-based visualization system (Janicki et al., 2004) and by computationally separating out genome-specific contributions from RNA-seq data (see [step-by-step method details](#) and [Figure 1](#)).

Culture media are prepared following the recipes described in the [materials and equipment](#) section. A complete list of materials and equipment used in this study is shown in the [key resources table](#). This study was carried out in strict accordance with the recommendations in the Guidelines of Kindai University for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Committee on the Ethics of Animal experiments of Kindai University (Permit Number: KABT-31-003). All mice were sacrificed by cervical dislocation and all efforts were made to minimize suffering and to reduce the number of animals used in the present study. Muscle tissues of a female *Oryx dammah* were collected from a carcass for necropsy at Adventure World Zoo (Wakayama, Japan), and frozen until use. The animal welfare and ethics were in accordance with guidelines adopted by the Japanese Association of Zoos and Aquariums.

Maintenance of donor cells

© Timing: 1–2 weeks

1. Three different types of donor cells for NT-ETR are maintained as follows. All cells are cultured in a humidified incubator at 37°C under 5% CO₂ in air.
 - a. Grow ES cells on 0.1% gelatin/PBS pre-coated culture dishes in the ES cell medium. Split ES cells 1:10 when 70%–80% confluent. Four days before NT, NeoR5 cells with 50% confluency on a 60-mm dish are cultured with 1 μM retinoic acid-containing differentiation medium in a feeder-free condition for inducing differentiation. Differentiation medium is changed every 2 days and cells are not passaged during the 4-day of differentiation process.
 - b. Grow C2C12 cells in the C2C12 cell medium. Split C2C12 cells 1:6 when 70%–80% confluent.
 - c. Grow primary cells derived from muscles of post-mortem female *Oryx dammah* in the *Oryx dammah* cell medium. Split *Oryx dammah* cells 1:3 when 70%–80% confluent. *Oryx dammah* cells should be passaged up to three times because primary cells have a limited life span.

Note: Different ES cell media can be used as long as pluripotency can be maintained and ES cells can react to the retinoic acid-mediated differentiation.

Preparation of mRNA solution

⌚ Timing: 1 day

2. Approximately 5 μg of linearized pCS2-LacR-mCherry vectors (Miyamoto et al., 2011) are used for mRNA production using mMACHINE SP6 Kit (Thermo Fisher Scientific, AM1340) according to the [manufacturer's protocol](#).
3. The produced mRNAs are subjected to polyA tailing (Thermo Fisher Scientific, AM1350).
4. PolyA-tailed mRNA solution is aliquoted with the concentration at 1 μg/μL and stored at –80°C before use. Avoid thawing and freezing and frozen aliquots can be stored for a year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-RNA Polymerase II H5 monoclonal antibody, Unconjugated (1:100 IF)	Covance	Cat# MMS-129R; RRID: AB_10143905
Goat anti-Mouse IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:2,000 IF)	Thermo Fisher Scientific	Cat# A-21042; RRID: AB_2535711
Chemicals, peptides, and recombinant proteins		
Demecolcine	Sigma-Aldrich	Cat# D7385
α-amanitin	Merck	Cat# A2263-1MG
Retinoic acid	Merck	Cat# R2625-50MG
Tyrode's Solution	Sigma-Aldrich	Cat# T1788
G-MEM with L-Glutamine and Phenol Red	Wako	Cat# 078-05525
DMEM, high glucose, GlutaMAX™ Supplement	Thermo Fisher Scientific	Cat# 10566016
KnockOut Serum Replacement	Thermo Fisher Scientific	Cat# 10828028
Fetal Bovine Serum	Cosmo Bio/Funakoshi	Cat# S-FBS-NL-015/ S1820
Stemolecule CHIR99021	REPROCELL	Cat# 04-0004
Stemolecule PD0325901	REPROCELL	Cat# 04-0006
Sodium Pyruvate	Thermo Fisher Scientific	Cat# 11360-070
2-Mercaptoethanol	Merck	Cat# M3148-100ML
Non-essential amino acids	Thermo Fisher Scientific	Cat# 11140-050
Leukemia Inhibitory Factor Protein, Recombinant mouse	Merck Millipore	Cat# LIF2010
Penicillin G Potassium Salt	Nacalai	Cat# 26239-84

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Streptomycin Sulfate	Nacalai	Cat# 32237-14
Amphotericin B Suspension (Fungizone)	FUJIFILM	Cat# 019-23891
KSOMaa medium	ARK Resource	N/A
M2 medium	ARK Resource	N/A
HTF medium	ARK Resource	N/A
Mineral oil	Merck	Cat# M8410-1L
Liquid paraffin	Nacalai Tesque	Cat# 26137-85
Bovine serum albumin	Merck	Cat# A3311-50G
PBS	Thermo Fisher Scientific	Cat# 10010049
Hyaluronidase from bovine testes	Merck	Cat# H3884-100MG
Trypsin-EDTA solution	Merck	Cat# T4049-100ML
Pregnant mare serum gonadotrophin (PMSG)	ASKA Pharmaceutical Co.	Cat# 879412
Human chorionic gonadotropin (hCG)	ASKA Pharmaceutical Co.	Cat# 8772413
GenomeONE™-CF, Hemagglutinating virus of Japan envelope (HVJ-E) solution	ISHIHARA SANGYO KAISHA	Cat# CF004
VECTASHIELD Mounting Medium with DAPI	Funakoshi	Cat# H-1200
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Fisher Scientific	Cat# 28906
Critical commercial assays		
PicoPure™ RNA Isolation Kit	Thermo Fisher Scientific	Cat# KIT0204
SMART-seq v4 Ultra Low Input RNA Kit	Takara	Cat# 24888N
Nextera DNA Sample Preparation Kit	Illumina	Cat# FC-131-1024
mMESSAGE mMACHINE® SP6 Transcription Kit	Thermo Fisher Scientific	Cat# AM1340
Poly(A) Tailing Kit	Thermo Fisher Scientific	Cat# AM1350
Deposited data		
RNA-seq	Tomikawa et al., 2021	GEO: GSE162345
Experimental models: Cell lines		
Mouse ES cell line; NeoR5	Miyamoto et al., 2011	N/A
Mouse C2C12 cell line	Miyamoto et al., 2011	N/A
<i>Oryz dammah</i> cell	Tomikawa et al., 2021	N/A
Experimental models: Organisms/strains		
C57BL/6 mice, female, 8 wk~	CLEA Japan (Tokyo, Japan)	N/A
DBA/2 mice, male, 8 wk~	CLEA Japan (Tokyo, Japan)	N/A
Oligonucleotides		
LacR-mCherry mRNA	Miyamoto et al., 2011	N/A
Recombinant DNA		
pCS2-LacR-mCherry vector	Miyamoto et al., 2011	N/A
Software and algorithms		
ZEN	Carl Zeiss	N/A
Trimmomatic	Bolger et al., 2014	http://www.usadellab.org/cms/?page=trimmomatic
trim_galore 0.4.2	Babraham institute	http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
cutadapt 3.1	Martin, 2011	https://cutadapt.readthedocs.io/en/stable/
STAR 2.5.4b	Dobin et al., 2013	https://github.com/alexdobin/STAR

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DESeq2 1.22.2	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Kallisto software 0.45.0	Bray et al., 2016	https://github.com/pachterlab/kallisto/releases
Sleuth 0.30.0	Pimentel et al., 2017	https://github.com/pachterlab/sleuth
Homer	Heinz et al., 2010	http://homer.ucsd.edu/homer/
Bedtools v2.26.0	Quinlan and Hall, 2010	https://bedtools.readthedocs.io/en/latest/
deepTools 3.1.3	Ramírez et al., 2016	https://deeptools.readthedocs.io/en/develop/
Custom Source Code	Github Folder:s Miyamoto-lab/NT-ETR	https://github.com/Miyamoto-lab/NT-ETR ; https://doi.org/10.5281/zenodo.5547988
Other		
XYClone laser system	Hamilton Thorne	Cat# 271348
Flaming/Brown Micropipette Puller	Sutter Instrument	Cat# P-97
EM MYSTAR Hematocrit Capillary Plain Type	AS ONE	Cat# 2-454-20
Microforge	NARISHIGE	Cat# MF-900
Microinjector	Eppendorf	Product name: Femtojet 4i
Dissecting needle	TGK	Cat# 454-50-75-01
Surgical scissors	Natsume Seisakusho Co.,Ltd.	Cat# B-12
Benchtop centrifuge	Bio Medical Science	Cat# BSR-Mini6000KS
Silicon tips for bulb assembly	Drummond	Cat# 1-000-9003
60-mm dish	Falcon	Cat# 35-1007
60-mm dish for tissue culture	IWAKI	Cat# 3010-060
35-mm dish	Falcon	Cat# 35-1008
Thermo plate	Tokai Hit	Cat# Tpi-W
Inverted microscope	Olympus	IX73-SLICSI
Micromanipulators	NARISHIGE	IM-11-2
BOROSILICATE GLASS	SutterInstrument	Cat# B100-75-10
Micro loader	Eppendorf	Cat# 5242-956-003
Femtojet 4i	Eppendorf	Cat# 5252000021
Femtotip	Eppendorf	Cat# 930000035
Glass capillary, EM MYSTAR Hematocrit Capillary Plain Type	AS ONE	Cat# 9100175

MATERIALS AND EQUIPMENT

Culture media preparation

ES cell medium		
Reagent	Final concentration	Amount
G-MEM with L-Glutamine and Phenol Red	n/a	153.63 mL
KnockOut Serum Replacement (KSR)	20%	40 mL
Sodium pyruvate (100 mM)	1 mM	2 mL
2-mercaptoethanol (2-ME)	0.1 mM	2 mL
Non-essential amino acids (NEAA)	1/100 of the stock	2 mL
Recombinant mouse leukemia inhibitory factor (LIF)	1 ng/mL	20 μ L
Stemolecule CHIR99021 (3 mM stock)	3 μ M	200 μ L
Stemolecule PD0325901 (2 mM stock)	0.5 μ M	50 μ L
Penicillin/Streptomycin stock	20 U/mL/20 μ g/mL	100 μ L
Total	n/a	200 mL

Store at 4°C for 3 weeks.

ES cell differentiation medium

Reagent	Final concentration	Amount
G-MEM with L-Glutamine and Phenol Red	n/a	155.88 mL
Fetal Bovine serum (FBS)	20%	40 mL
Sodium pyruvate (100 mM)	1 mM	2 mL
Non-essential amino acids (NEAA)	1/100 of the stock	2 mL
Retinoic acid (10 mM)	1 μ M	20 μ L
Penicillin/Streptomycin stock	20 U/mL/20 μ g/mL	100 μ L
Total	n/a	200 mL

Store at 4°C for 1 month.

C2C12 cell medium

Reagent	Final concentration	Amount
DMEM, high glucose, GlutaMAX™ Supplement	n/a	177.9 mL
Fetal Bovine serum (FBS)	10%	20 mL
Sodium pyruvate (100 mM)	1 mM	2 mL
Penicillin/Streptomycin stock	20 U/mL/20 μ g/mL	100 μ L
Total	n/a	200 mL

Store at 4°C for 1 month.

Oryx dammah cell medium

Reagent	Final concentration	Amount
DMEM, high glucose, GlutaMAX™ Supplement	n/a	173.9 mL
Fetal Bovine serum (FBS)	10%	20 mL
Sodium pyruvate (100 mM)	1 mM	2 mL
Penicillin/Streptomycin stock	20 U/mL/20 μ g/mL	100 μ L
Fungizone	5 μ g/mL	4 mL
Total	n/a	200 mL

Store at 4°C for 1 month.

Demecolcine preparation

Demecolcine comes as powder. Dissolve all powder with ethanol to be a final concentration of 1 mg/mL, make 10 μ L aliquots and store at -20°C . Aliquots can be stored for up to a year.

α -Amanitin preparation

α -Amanitin comes as powder. Dissolve all powder with sterile water to be a final concentration of 1 mg/mL, sterile filter, make 10 μ L aliquots and store at -20°C . α -Amanitin powder is stable for more than a year, but it is recommended to use promptly after dissolving in water. We routinely prepare the α -Amanitin every 6 month.

Retinoic acid preparation

Retinoic acid comes as powder. Dissolve all powder with DMSO to a final concentration of 10 mM, make 20 μ L aliquots and store at -20°C . Protect from exposure to light during the storage and use the aliquots within a year.

Inactivated hemagglutinating virus of Japan envelope (HVJ-E) solution preparation

Suspend freeze-dried HVJ-E powder in 260 μ L ice-cold HVJ suspending buffer according to the [manufacturer's instruction](#). Aliquot 5 μ L of HVJ-E solution and store at -80°C before use. We routinely prepare the HVJ-E solution every 6 month.

Glass capillary for transferring embryos

Pull the heated glass capillary (AS ONE, EM MYSTAR Hematocrit Capillary Plain Type) to achieve ~150 μm in diameter. This glass capillary is connected to embryo-handling mouth pipette using silicon tips for bulb assembly (Drummond, 1-000-9003).

Microinjection pipettes

Pull injection pipettes (Sutter Instrument, Thin Wall Borosilicate Glass) using P-97 micropipette puller. We use the following parameter (Heat, 750; Pull, 235; Velocity, 120; Time, 70). Cut the tip of the pulled glass pipette by gently and quickly attaching to the weakly heated glass bead on the platinum wire by Narishige MF-900 microforge (heater setting at level ~50) (Methods video S1). Inside diameter of cell injection pipettes should be around 20 μm , while that of holding pipettes is around 80–100 μm . Microforge is used to heat the tip of the capillary for thickening the inside of the holding pipette. Heating is stopped when the inside diameter of the holding pipette is around 20–30 μm . The diameter can be judged by the scale on ocular lens (Methods video S1).

Note: The working condition for micropipette puller can be affected by many factors, and therefore it is important to test various parameters at your institute. Please also refer to other detailed protocols for making injection pipettes and microinjection (Kishigami and Wakayama, 2009; Percharde et al., 2021).

STEP-BY-STEP METHOD DETAILS

Preparation of recipient mouse embryos

⌚ Timing: 6 days

Here, we detail preparation of recipient embryos for NT by performing *in vitro* fertilization (IVF) of mouse oocytes and sperm and collection of 4-cell stage embryos arrested at G2 phase with demecolcine. Refer to Manipulating the Mouse Embryo: A Laboratory Manual (Behringer et al., 2014) for the standard methods of mouse embryo manipulation.

1. Superovulation is induced by injecting 7.5 U of pregnant mare serum gonadotrophin (PMSG) to 3 or 4 C57BL/6 female mice (>8 weeks of age) and then 48 h later, 7.5 U of human chorionic gonadotropin (hCG). We normally perform the injection at 5:00–7:00 PM.
2. On the next day at 7:30–9:30 AM, euthanize a male mouse by cervical dislocation.
 - a. For collecting sperm from a DBA/2 fertile male mouse (>8 weeks of age), hold the cauda epididymis by forceps, cut the ductus epididymis with a small surgical scissors (Natsume Seisakusho Co., Ltd., B-12), collect sperm with a fine needle (TGK, 454-50-75-01), transfer the sperm with the same needle to 200 μL drop of human tubal fluid (HTF) medium in a 35-mm dish covered with liquid paraffin on a heated Thermo plate.
 - b. Preincubate it for 1.5 h at 37°C under 5% CO_2 in air for capacitation.
3. During the sperm preincubation, euthanize female mice by cervical dislocation.
 - a. Collect cumulus-oocyte complexes (COCs) from the excised oviducts of sacrificed C57BL/6 female mice 15–16 h after hCG injection. For this COCs collection, transfer the excised oviducts in a 35-mm dish covered with liquid paraffin on a Thermo plate.
 - b. Tear off the membrane of the ampulla of fallopian tube by a fine dissecting needle (TGK, 454-50-75-01) and pull COCs using the same needle out of the fallopian tube.
4. Recover COCs into pre-equilibrated HTF medium (200 μL drop) on a Thermo plate.
5. Soon after the recovery of COCs, add the preincubated sperm suspension prepared in step 2 to the COCs-containing HTF medium to be the final concentration of 2×10^2 sperm/ μL using P10 Pipetman, and further incubate at 37°C under 5% CO_2 in air for 2 h.
6. For removing cumulus cells, add 12 μL of hyaluronidase (10 mg/mL) to the fertilized zygotes-containing HTF medium at 2 h post insemination (hpi) and incubate at 37°C under 5% CO_2 in air for 5 min.

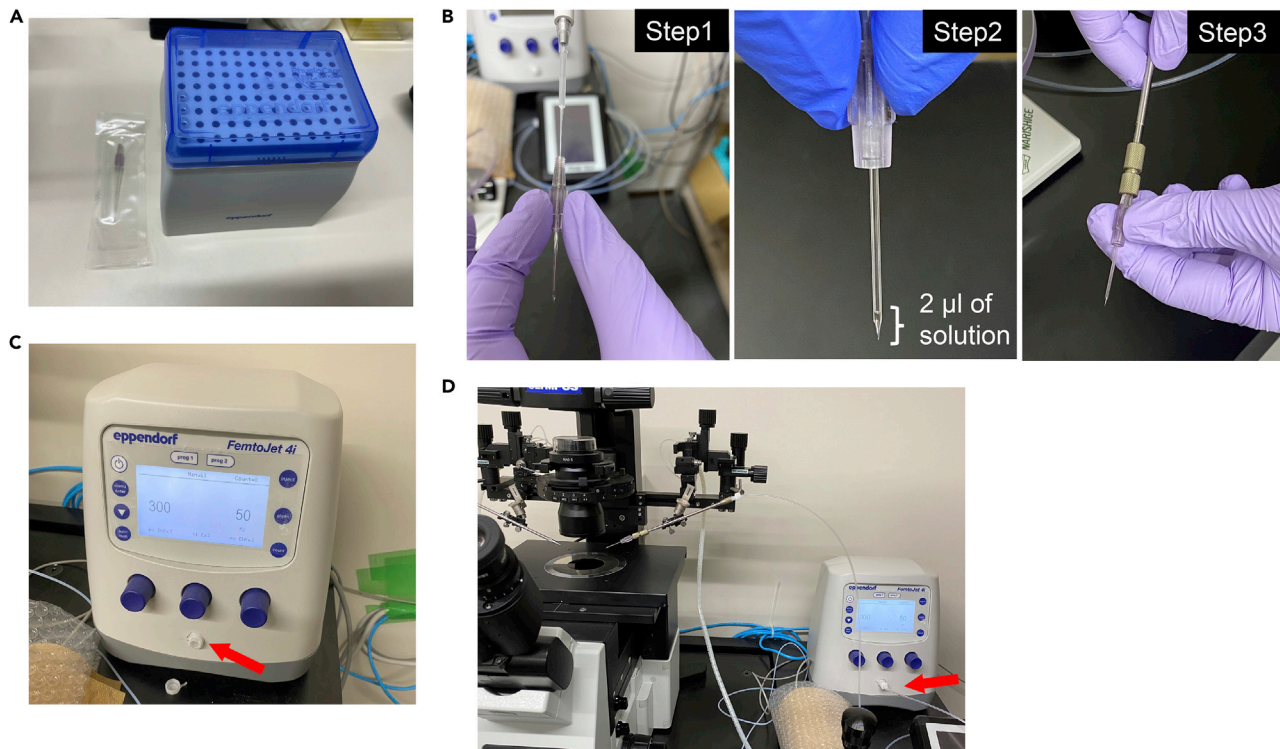


Figure 2. RNA injection into fertilized eggs using the Eppendorf Femtojet 4i system

- (A) A Femtotip (left) and micro tips (right, Eppendorf) to load mRNA or siRNA solution to Femtotips.
 (B) The process of loading solution to Femtotip using a micro loader tip (steps 1 and 2) and the Femtotip loaded with 2 μL of solution is connected to Eppendorf Femtojet 4i injector (step 3).
 (C) Femtojet 4i system set for mRNA injection, placed on the right of Figure 2D. Rid is open and injector is not connected (red arrow).
 (D) Insertion of the filled Femtotip to the Femtojet 4i system (red arrow) and our manipulation system for RNA/DNA injection to eggs/embryos.

7. After denudation, wash the zygotes several times in pre-equilibrated potassium simplex optimized medium (KSOMaa), transfer the washed zygotes to drops of KSOMaa (35 μL , covered with liquid paraffin) in a 60-mm dish, and incubate at 37°C under 5% CO_2 in air.
 - a. At 6 hpi, check the morphology of zygotes under the microscope and remove abnormal embryos such as parthenogenetically activated, polyspermy, degenerated, and fragmented embryos.
 - b. Further culture normally fertilized embryos in drops of KSOMaa (35 μL , covered with liquid paraffin) until reaching the 4-cell stage at 40 hpi.
 - c. (Optional) Injection of mRNA or siRNA into the IVF-derived BDF1 embryos is performed from 6 hpi and completed within 2 h. If the transgenic mouse ES cell line, termed NeoR5 (Miyamoto et al., 2011) containing 20–30 copies of Lac operator repeats, is used as donor cells, the injection of 200 ng/ μL Lac repressor mCherry (LacR-mCherry) mRNA enables us to trace the injected nuclei due to the specific accumulation of LacR-mCherry at the transgene Lac operator repeats (Figure 1, arrows).
 - i. mRNA or siRNA solution is spun down by benchtop centrifuge (Bio Medical Science, BSR-Mini6000KS) at 22°C for 1 min.
 - ii. Load 2 μL of mRNA or siRNA solution to Femtotip using micro loader (Eppendorf) (Figures 2A and 2B).
 - iii. Insert the filled Femtotip to injector (Figure 2B).
 - iv. Turn on Femtojet 4i. Make sure that the injection tube is disconnected when turning on (Figure 2C). After the calibration, insert the injection tube (Figure 2D) and apply clean function to remove air bubbles. Choose the appropriate pressure setting; in our hands,

- pi: 300 hPa and pc: 50 hPa are used (Figure 2C). Injection time is controlled by foot pedal in order to achieve an equal volume of injection (typically, 0.05–0.2 s).
- v. Inject 5–10 pl of mRNA or siRNA solution to the cytoplasm of zygotes during 6–8 hpi in M2 medium. Microinjection is performed at 22°C.
- vi. The injected zygotes are cultured in KSOMaa drops (35 µL, covered with liquid paraffin).

Note: The drops of media should be prepared under liquid paraffin 1 day before sperm/oocyte collection and equilibrated for 12–16 h. After collection of the cauda epididymis and oviduct from male and female mice respectively, all steps were performed under liquid paraffin.

Note: When loading mRNA or siRNA solution into Femtotip, bubbles are often contaminated at the tip of Femtotip. In that case, pipetting using micro loader can remove the bubble. It is important to remove as much bubbles as possible from the tip.

Note: LacR-mCherry protein stored in embryos is imported into nuclei and can mark the place of nuclei. Furthermore, the specific accumulation of LacR-mCherry at the transgene Lac operator repeats distinguishes injected NeoR5 and other embryonic nuclei. Alternatively, transgenic donor cells such as Histone H2B-GFP-expressing cells could be used for tracing the injected nuclei. However, it might be difficult to distinguish the injected nuclei and embryonic nuclei if fluorescent proteins are expressed from injected nuclei and finally accumulated in embryonic nuclei.

△ CRITICAL: After the injection, leave the injected embryos at 22°C for more than 10 min for recovery. If embryos are returned to the incubator soon after injection, embryo lysis is often observed. Embryos should not be kept at 22°C more than 30 min.

8. During 40–42 hpi, prepare KSOMaa drops containing 0.1 µg/mL demecolcine (35 µL, covered with liquid paraffin) and transfer to the incubator for equilibration (at least 30 min). Prepare M2 medium containing 0.1 µg/mL demecolcine while waiting for the equilibration of KSOMaa with demecolcine and store the M2 medium with demecolcine on ice until use. Transfer the 4-cell stage embryos to the equilibrated KSOMaa drops containing 0.1 µg/mL demecolcine before 43 hpi and further culture until 46 hpi at 37°C under 5% CO₂ in air.

△ CRITICAL: To avoid a potential change in demecolcine concentration in the embryo culture medium, prepare culture drops including 0.1 µg/mL demecolcine just before the NT experiment.

Nuclear transfer to elicit embryonic transcriptional reprogramming (NT-ETR)

⌚ Timing: 2–3 h

This section describes the NT process in which donor cells are fused with a G2-arrested mouse 4-cell stage embryo. We named this NT system as NT-ETR, and include a schematic of the process of NT-ETR in Figure 3.

9. At 45 hpi, detach the donor cells (differentiated ES, C2C12 or *Oryz dammah* cells) from PBS-pre-washed culture dishes by the treatment with 0.25% Trypsin-EDTA solution for 1–2 min at 37°C under 5% CO₂ in air. The Trypsin-EDTA solution containing cells are mixed with each culture medium and cells are collected by centrifugation (300 g, 5 min). Resuspend the pellets in each culture medium at 0.1–2 × 10⁵ cells/mL.
 - a. To collect the differentiated ES cells, ES cells cultured in the differentiation medium for 4 days are collected for NT.

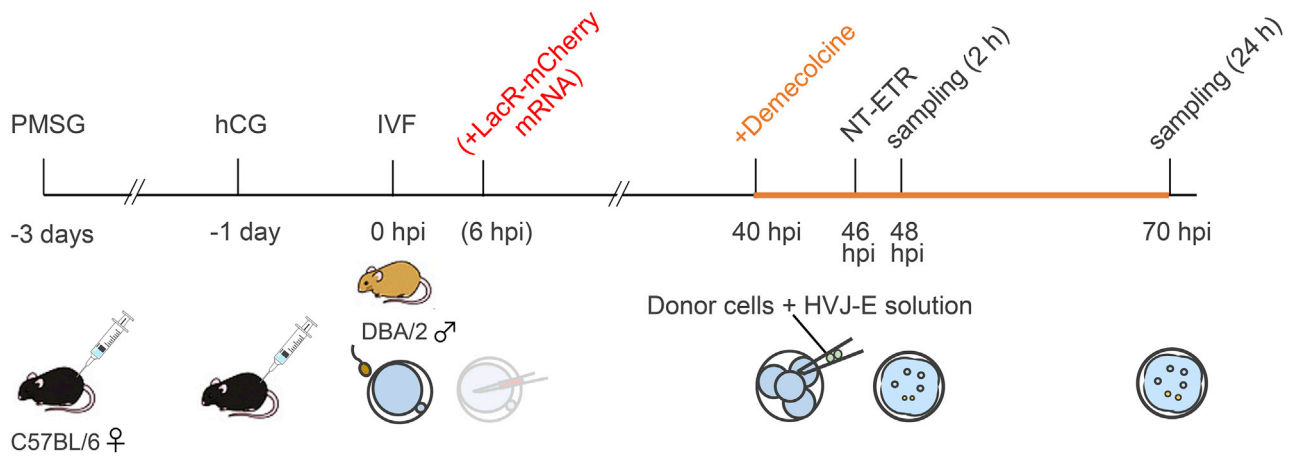


Figure 3. The whole process of NT-ETR

IVF-derived BDF1 mouse embryos are injected with or without LacR-mCherry mRNA solution at 6 hpi and cultured in KSOMaa medium containing 0.1 $\mu\text{g}/\text{mL}$ demecolcine from 40 hpi. At 46 hpi, NT of differentiated donor cells is performed. The NT embryos are further cultured for 2 or 24 h and subjected to immunofluorescence staining, qRT-PCR or RNA-seq analyses. IVF represents *in vitro* fertilization. Figure 3 is modified from the original Figure 3 of Tomikawa et al. (2021).

- b. Monolayers of C2C12 cells at approximately 60%–70% confluency (semi-confluent) are collected and used for NT.
- c. *Oryz dammah* cells after three passages are used for NT when cells reach at 60%–70% confluency.

Note: We have not rigorously synchronized the cell cycle of donor cells. It might be possible that rigorous cell cycle synchronization, for example by using demecolcine for donor cell cycle synchronization as well, would further improve the reprogramming efficiency.

10. Thaw 5 μL of the frozen HVJ-E solution on ice. Then, mix with 20 μL of the diluted Cell Fusion Buffer from the kit (GenomeONE™-CF kit, dilute the original buffer solution in the kit with ultra-pure water by 20 times).
11. Prepare each reagent drop (10 μL for M2 medium and donor cell suspension, and 5 μL for HVJ-E solution) aligned on a 10-cm dish lid, which is covered with mineral oil as shown in Figure 4A. Keep the rest of the prepared HVJ-E solution (15 μL should be left) on ice.
12. Set up the manipulation system on microscope. Our setting is shown in Figure 4B.
13. Transfer the 4-cell embryos incubated in the demecolcine-containing KSOMaa medium to a M2 medium drop with 0.1 $\mu\text{g}/\text{mL}$ demecolcine at 46 hpi just before NT.
14. Aspirate HVJ-E solution into the injection pipette, and pick up two donor cells with a minimal volume of cell suspension medium (see troubleshooting 1). Relatively small cells are picked up.

Note: A single nucleus can be also fused with the arrested 4-cell embryos. We have confirmed that at least 2 nuclei can be reprogrammed in a 4-cell embryo. The advantage of injecting multiple nuclei is that it can increase the chance of detecting newly transcribed genes from the transplanted nuclei.

15. Transfer donor cells to a HVJ-E solution drop and aspirate the cells together with the two to three times more volume of HVJ-E solution than donor cells (Figure 4C).
 - a. Anchor an embryo with holding pipette, and make a small hole in the zona pellucida by the XYClone laser system (laser power: 220) (Figure 4D).
 - b. Normally, one or two treatments with laser illumination is enough to make a hole on zona pellucida for injection pipette to penetrate.

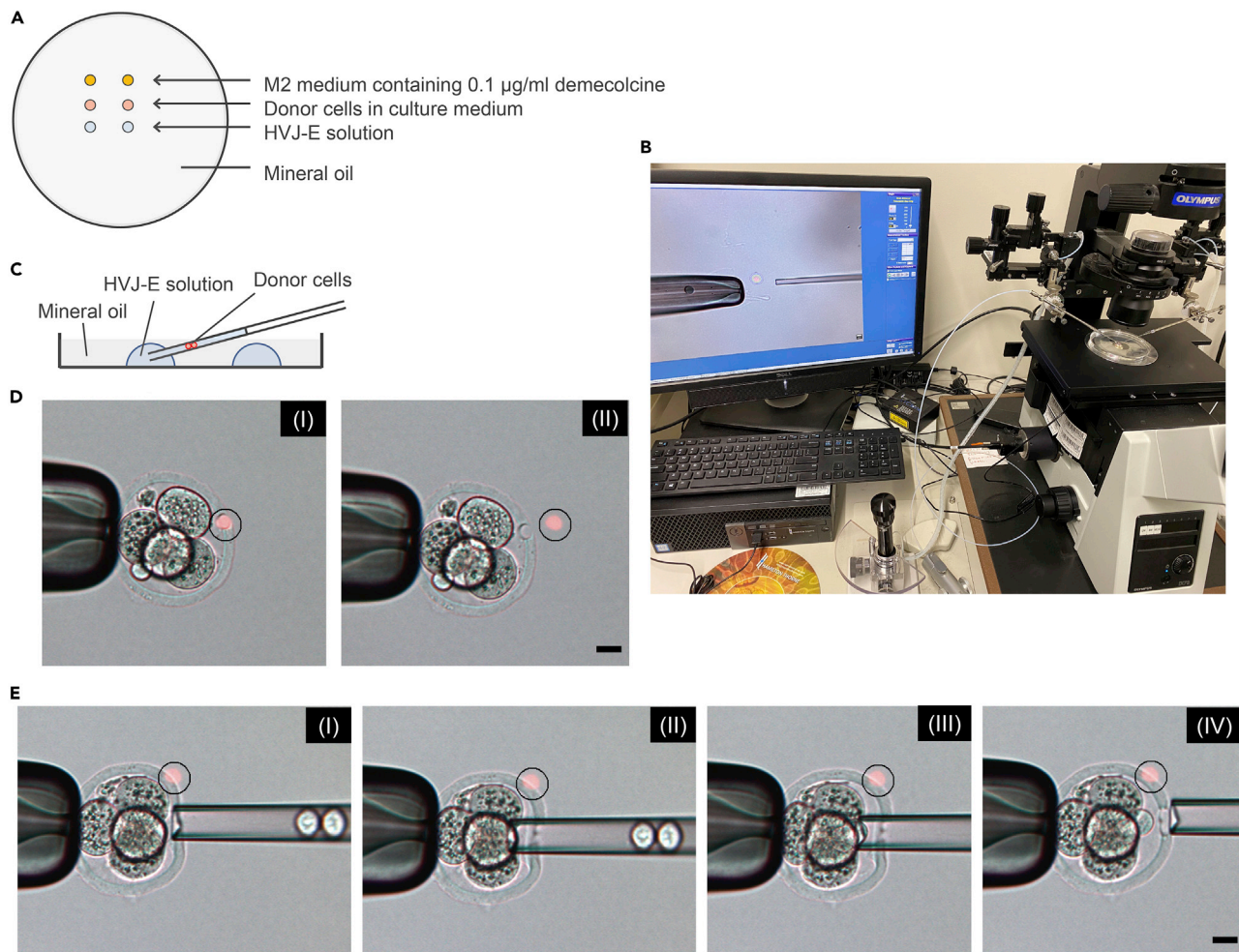


Figure 4. Technical points during NT-ETR procedure

- (A) Drops of reagents for NT-ETR, set on the 10 cm dish lid.
 (B) Our manipulation system for NT-ETR. Holding and injection pipettes are set on the manipulator, equipped with the XYClone laser system.
 (C) Aspiration of two donor cells together with HVJ-E solution just before injection of the cells to a 4-cell embryo. Two to three times more volume of HVJ-E solution than donor cells is needed for successful fusion of donor cells with a 4-cell embryo.
 (D) The process of making a hole in the zona pellucida of a recipient 4-cell embryo by the XYClone laser system (I and II). Red pointer in the black circle marks the place for laser illumination. Scale bar, 20 μm .
 (E) Injection of two donor cells and HVJ-E solution into the perivitelline space of a recipient 4-cell embryo through the hole of zona pellucida, produced by XYClone laser illumination (I–IV). Scale bar, 20 μm .

△ CRITICAL: Avoid illuminating with the laser more than two times as it can damage embryos.

Note: In case the XYClone laser system is not available, a piezo micromanipulator can be also used to make a hole in the zona pellucida to deliver donor cells to the perivitelline space of recipient 4-cell embryos.

16. Insert the injection pipette containing cells and HVJ-E solution into the perivitelline space of a recipient 4-cell embryo and place the tip of the pipette between two blastomeres (Figure 4E). Then, donor cells are ejected with HVJ-E solution (Figure 4E).
17. Leave the manipulated embryo in a M2 medium drop for 5 min at 22°C.

⚠ **CRITICAL:** The activity of the HVJ-E solution decreases significantly after returning to 22°C. If the manipulation time exceeds more than 1 h, prepare the HVJ-E solution drops again. In this case, the remaining HVJ-E solution prepared at step 10 should be kept on ice and such ice-cold solution can be used for making HVJ-E drops for NT-ETR. If this condition is kept, the efficiency of cell fusion should be more than 80% (see [troubleshooting 2](#)).

18. Transfer the manipulated embryos to equilibrated KSOMaa medium containing 0.1 µg/mL demecolcine and culture for 2 and 24 h (see [troubleshooting 3](#)). When identifying newly transcribed genes, NT embryos are cultured in the demecolcine-containing KSOMaa medium with or without 25 µg/mL α -amanitin (final concentration) for the comparison.
19. Use the NT embryos for immunofluorescence, reverse transcription-polymerase chain reaction (RT-PCR) or RNA-sequencing (RNA-seq) analyses.
 - a. If NT embryos are subjected to immunostaining, fix embryos in 4% paraformaldehyde and follow the protocol described in [Tomikawa et al. \(2021\)](#).
 - b. If NT embryos are used for RT-PCR, 3–5 embryos are extracted using Arcturus PicoPure RNA isolation kit as described in [Tomikawa et al. \(2021\)](#).
 - c. If NT embryos are subjected to RNA-seq, 3–5 embryos are transferred to lysis buffer as follows.
 - i. Treat the NT embryos with Tyrode's solution for removing zona pellucida, followed by three times washes with 0.1% BSA/PBS (see [troubleshooting 4](#)).
 - ii. Fill a glass capillary with 1 µL of 0.1% BSA/PBS by capillary action.
 - iii. Pick up 3–5 embryos and the minimal volume of BSA/PBS to the BSA/PBS-filled glass capillary.
 - iv. Transfer the NT embryos in 1 µL of 0.1% BSA/PBS to 9.5 µL of 1× Reaction buffer from SMART-seq v4 Ultra Low Input RNA Kit. SMART-seq library preparation was performed using [SMART-seq v4 Ultra Low Input RNA Kit](#) and [Nextera DNA Sample Preparation Kit](#) according to the manufacturer's instruction.

⏸ **Pause point:** Fixed embryos can be stored for a week at 4°C. When snap-freezing of lysed embryos in liquid nitrogen is performed, such frozen lysates can be stored at –80°C up to a month.

Note: For detecting embryonic transcription from the injected cells, NT embryos are incubated for 24 h after NT-ETR. NT embryos collected 2 h after NT-ETR are used as a negative control.

Note: For RNA-seq analyses, paired-end sequencing (50 bp + 25 bp) was performed by the NextSeq platform (Illumina). More than 10 million reads per sample are recommended to obtain.

Detection of newly transcribed genes from the injected nuclei

⌚ Timing: 1–4 h for steps 20–23

⌚ Timing: 1–2 h per sample for steps 24–27

⌚ Timing: 1–2 h per sample for steps 28–36

⌚ Timing: 1–4 h for steps 37–39

⌚ Timing: 3–4 h per sample for steps 40 and 41

⌚ Timing: 1–4 h for steps 42–46

For well annotated species with available cDNA references, a combination of kallisto (Bray et al., 2016) and sleuth (Pimentel et al., 2017) was used to quantify transcriptional abundances and identify newly transcribed genes (steps 20–36). This pipeline was developed for NT-ETR using three strains of mice (Tomikawa et al., 2021). For organisms with less well annotated genomes or where cDNA references are not available, the alternative pipeline based on STAR aligner (Dobin et al., 2013) and DESeq2 (Love et al., 2014) was used (steps 37–46). This second pipeline was used for the *Oryz dammah* data from Tomikawa et al. (2021). In general, pipelines based on kallisto/sleuth will be faster than those based on STAR/DESeq2.

Reference genome preparation

20. Transcriptomes must be downloaded. For the three mouse strains used for NT-ETR in Tomikawa et al. (2021), cDNA references were available at the Sanger Mouse Genomes Project: <https://www.sanger.ac.uk/data/mouse-genomes-project/>
21. Generate a combined reference genome by concatenation of the cDNA fasta file. This may be achieved using cat at the command line:

```
cat genome1.cdna.fa genome2.cdna.all.fa genome3.cdna.all.fa > merged.cdna.fa
```

Trimming of adapter sequences and quality control (recommended)

22. Initial quality control (QC) checks can be done with fastqc, available from the Babraham bioinformatics site: <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

```
fastqc -o OutputDir -t Sample1_R1.fastq.gz Sample1_R2.fastq.gz
```

23. Reads were trimmed of adapter sequences using TrimGalore! available from Babraham: https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

```
trim_galore -paired -o OutputDir Sample1_R1.fastq.gz Sample1_R2.fastq.gz
```

The result of trimming will generate new pair of fastq files trimmed of adapter sequences with file-names following the convention Sample1_1_val_1.fq.gz and Sample1_2_val_2.fq.gz for read 1 and read 2, respectively.

Transcript quantification

24. Download and install kallisto. Full installation instructions are available at the Patcher lab GitHub repository: <https://pachterlab.github.io/kallisto/>
25. Index the newly generated merged cDNA reference file using kallisto:

```
kallisto index -i merged.cdna.idx merged.cdna.fa
```

26. To align reads to the transcriptome, use the quant function with the trimmed fastq files:

```
kallisto quant -i merged.cdna.idx -o OutputDir Sample1_1_val_1.fq
Sample1_2_val_2.fq -b 100
```

This will generate a file of transcript abundances with maximum likelihood estimated abundancies stored in the tsv file and bootstrapped samples stored in the h5 file (see [troubleshooting 5](#)).

27. To reduce time spent on these stages, steps 24–26 can be run for all samples in parallel using a HPC cluster.

Differential transcript abundance and downstream processing

28. Having generated transcript abundances for all relevant samples (steps 24–27), downstream analysis relies on the R package Sleuth. Full installation instructions and help can be found at the Pachter lab GitHub repository: <https://pachterlab.github.io/sleuth/>

29. Samples are read into Sleuth and pre-processed using the function `sleuth_prep`:

```
idata <- data.frame(path=path/to/samples, sample=samples, timepoint =
c("NoAmn", "NoAmn", "NoAmn", "Amn", "Amn", "Amn"), stringsAsFactors=FALSE)
so <- sleuth_prep(idata, read_bootstrap_tpm = TRUE, extra_bootstrap_summary =
TRUE)
```

30. A linear model (full model) is fit to the abundance data with a parameter accounting the two conditions:

```
Ttome <- sleuth_fit(so, ~timepoint, 'full')
```

A second (reduced complexity) model can be fit to the data, which assumes abundancies equal in the two conditions:

```
Ttome <- sleuth_fit(so, ~1, 'reduced')
Ttome <- sleuth_fit(so, ~1, 'reduced')
```

A likelihood ratio between the full model and reduced model is then used to determine differential abundance:

```
Ttome <- sleuth_lrt(so, 'reduced', 'full')
Ttome <- sleuth_fit(so, ~1, 'reduced')
```

31. A data frame summary of the results can be generated by calling `sleuth_results` to allow export to other formats:

```
sleuth_table_gene <-
sleuth_results(Ttome, 'reduced:full', 'lrt', show_all = FALSE)
```


32. (Optional) To quantify differential abundance at the gene level rather than the transcript level, a data frame containing the mapping of transcripts to gene level annotation can be passed at sleuth prep stage:

```
Ttome <- sleuth_prep(idata, read_bootstrap_tpm = TRUE,  
target_mapping = GeneDF, extra_bootstrap_summary = TRUE)
```

33. (Optional) As an alternative to a likelihood ratio test, it is possible to calculate differential abundancies using a Wald test:

```
model <- sleuth_wt(so, 'timepointNoAmn', 'full')
```

34. (Optional) Dimensionality reduction in the form of PCA may be applied to the data using Sleuth's inbuilt function `plot_PCA`. Alternatively, individual abundance files can be read into R and other programming environments and PCA run using appropriate in-built functions. Note that due to the limited number of samples, approaches such as UMAP and tSNE are not appropriate.
35. To identify newly transcribed genes, steps 28–34 were calculated by comparing α -amanitin-treated and untreated NT samples.
36. To identify strain-specific newly transcribed genes, differential expression between α -amanitin-treated and -untreated samples were calculated using step 32 using a strain-specific mapping of transcriptome to strain-specific gene (see [troubleshooting 6](#)). This can either be done by manually generating a spreadsheet of mappings or using the `read.gtf` function of the `refGenome` R package to read the `.gtf` files and extract the "transcript_id" and "gene_name" entries and appending a genome identifier to the "gene_name" column. If genomes were downloaded from the Sanger Mouse Genomes project website, the "gene_name" column will already be appended with a genome identifier.

Reference genome preparation

37. As before, reference genomes must first be downloaded. The *Oryx dammah* genome assembly ([Dudchenko et al., 2017, 2018](#); [Humble et al., 2020](#)) and gff3 annotation was downloaded from the DNAZoo website: https://www.dnazoo.org/assemblies/Oryx_dammah.

Note: While STAR is capable of using gff3 files, it may be helpful to convert them to gtf files using the `gffread` function (<http://ccb.jhu.edu/software/stringtie/gff.shtml>):

```
gffread oryx.gff3 -T -o oryx.gtf
```

38. Genomes are first indexed for use with STAR aligner. Full installation instructions for STAR are available at the Dobin GitHub repo <https://github.com/alexdobin/STAR>. Note that both mouse and oryx genomes must be indexed, and indexing may take several hours for larger genomes. Example code for indexing the oryx genome will look like:

```
STAR --runMode genomeGenerate --limitGenomeGenerateRAM 48000000000 --  
genomeDir OryxGenomeIndexFolder --genomeFastaFiles  
oryx.genome.dna.toplevel.fa --sjdbGTFfile oryx.gtf --sjdbOverhang 149
```

Adapter trimming and QC

39. Read files should be pre-processed with fastqc and TrimGalore! as in pipeline 1 (see steps 22–23).

Transcript quantification

40. Adapter-trimmed reads are first aligned to the mouse genome to filter out mouse transcripts, with unmapped reads subsequently mapped to the oryx genome. To map to the mouse genome, the following options were used (see [troubleshooting 5](#)):

```
STAR --genomeDir MouseGenomeIndexFolder --sjdbGTFfile mouse.gtf --
outReadsUnmapped Fastx --sjdbOverhang 149 --readFilesIn Sample1_1_val_1.fq
Sample1_2_val_2.fq --quantMode GeneCounts
```

To map unmapped reads to the oryx genome, the following options were used:

```
STAR --genomeDir OryxGenomeIndexFolder --sjdbGTFfile oryx.gtf --outReadsUnmapped
Fastx --sjdbOverhang 149 --readFilesIn path/to/mouse/results/Unmapped.out.mate1
path/to/mouse/results//Unmapped.out.mate2 --quantMode GeneCount
```

41. To speed up this stage, steps 39–40 can be applied to all samples in parallel using HPC platforms.

Downstream analysis

42. The result of STAR alignment is an output folder containing reads counts, which are stored in a tab delimited file ReadsPerGene.out.tab, and a bam file containing sequence alignment. Differential expression (DE) of individual genes is calculated using DESeq2 based on the count data in ReadsPerGene.out.tab. Installation instructions for DESeq2 can be found at Bioconductor: <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>. Example code for performing DE analysis is shown below:

```
ddsHTSeq<-DESeqDataSetFromHTSeqCount(sampleTable=sampleTable,
design=~condition)
dds <- DESeq(ddsHTSeq)
res1<-results(dds, contrast=c("condition", "NT", "Am"))
```

43. (Recommended) Bigwig files can be generated from individual sample bam files to visualize mapped reads using genome browsers such as IGV (<https://software.broadinstitute.org/software/igv/>). This is recommended for less completely annotated genomes such as *Oryx dammah*. Bigwig files can be generated from bam files using DeepTools (<https://deeptools.readthedocs.io/en/develop/index.html>):

```
bamCoverage --minMappingQuality 1 --bam Sample1.bam --binSize 10 --
skipNonCoveredRegions --outFileName Sample1.bw
```

44. For identification of newly transcribed genes, steps 42–43 were calculated by comparing α -amanitin-treated and untreated NT samples.

Ortholog identification

45. Following identification of differentially expressed genes in *Oryx dammah*, orthologs to bovine gene were extracted from the extended gff3 files. To do so, a compiled list of all ortholog matches or part matches were extracted from the gff3 file e.g.,:

```
awk 'OFS="\t" {if ($3=="match") {print $1,$4-1,$5,$7,$9}}' oryx.gff3 >
oryx_matches.txt
```

A list of transcript/gene identified with partial matches could then be extracted using grep with keyword "BOVIN".

```
grep "BOVIN" oryx_match.txt > oryx_bovine_match.txt
```

The resultant tab delimited txt file will contain mapping form transcript IDs in the oryx genome to bovine gene IDs.

46. (optional) To identify other orthologs e.g., mouse or human, step 45 can be repeated using keywords "HUMAN" or "MOUSE".

Motif analysis was performed on the promoter regions of differentially expressed gene sets

⌚ Timing: 2 h per sample

Motif analysis

47. The TSS for each gene in each genome of interest was identified using GenomicFeatures package in R and exported as a tab delimited bed file:

```
refgenes <- makeTxDbFromGFF("mousegenome1.gtf", format="gtf") transcripts
<- genes(refgenes, columns=c("tx_id", "tx_name"))
tss <- resize(transcripts, width=1, fix='start')
write.table(as.data.frame(tss), file =
"MouseGenom1TSS.bed", quote=FALSE, sep="\t", col.names=FALSE)
```

48. This result of step 47 is a bed file containing the TSS location of genes. Coordinates can be extended using bedtools (<https://bedtools.readthedocs.io/en/latest/>) slop function:

```
bedtools slop -i MouseGenom1TSS.bed -g mouse.geome1.chrom.sizes -b 1000 >
Promoters.mouse.genome1.bed
```

49. Individual promoter bed files could be filtered by differentially expressed gene lists:

```
grep -f Delist1.txt MouseGenom1TSS.bed > DEMouseGenom1TSS.bed
```

For example, if `Delist1.txt` contains a list of DE genes from a given mouse strain (genome 1), the bed file would filter the TSS (+/- a specified size) coordinates of only those genes, allowing subsequent strain-specific motif analysis.

50. Genome sequences for the DE promoter regions of a given mouse genome could be extracted with bedtools function `fastaFromBed`:

```
fastaFromBed -fi mouseGenome1.fasta -bed DEMouseGenom1TSS.bed -fo  
DEMouseGenom1TSS.fasta
```

51. Finally, de novo motif analysis was performed using Homer ([Heinz et al., 2010](http://homer.ucsd.edu/homer/)). Full installation instructions are available from <http://homer.ucsd.edu/homer/>:

```
findMotifs.pl DEMouseGenom1TSS.fasta fasta OutputDir -mset vertebrates
```

52. Steps 47–51 could be repeated for each genome of interest, to generate strain-specific enrichment for NT-ETR using 3 different strains of mice and for the mouse/oryx NT-ETR.

EXPECTED OUTCOMES

We can trace the injected nuclei by using transgenic cells as donor cells. For example, when differentiated ES cell nuclei are used for NT-ETR, significant swelling of injected nuclei is observed. At 24 h after NT, the injected nuclei resemble nuclei of 4-cell embryos ([Figure 1](#)) and are indistinguishable from nuclei of recipient 4-cell embryos without marking by the transgene (in our case, we used accumulation of LacR-mCherry as a marker). Further, immunostaining with the antibody against RNA polymerase II phosphorylated at serine 2 (PollphoS2), elongating RNA polymerase II, shows that the PollphoS2 signals are observed in all nuclei of the reconstructed embryos including the injected differentiated nuclei at 24 h after NT-ETR ([Tomikawa et al., 2021](#)), indicating that 4-cell embryos at G2 phase can induce nuclear remodeling and transcriptional activation to the transplanted somatic nuclei within a day.

If a different stain of cells from both oocytes and sperm is used as a donor for NT-ETR, the newly synthesized transcripts from donor cells can be computationally discerned. We used C2C12 cells derived from C3H strain mice and identified 429 differentially expressed genes between α -amanitin-treated and untreated NT samples, which include embryonic transcripts ([Tomikawa et al., 2021](#)). Similar results are observed after NT-ETR using *Oryx dammah* cells as donor cells. *Oryx dammah* is a species of oryx that is extinct in the wild. *Oryx dammah* cell nuclei are enlarged and indistinguishable from recipient mouse 4-cell embryo nuclei within 24 h. Transcriptional activity is also observed in the *Oryx dammah* cell nuclei from the results of PollphoS2 immunostaining. Indeed, transcriptomes of each species (C57BL/6, DBA/2 mice and *Oryx dammah*) are greatly altered by blocking Poll activities with α -amanitin, indicating that RNA polymerase-dependent transcription is induced after NT-ETR also in *Oryx dammah* cell nuclei ([Tomikawa et al., 2021](#)).

To summarize, NT-ETR using mouse 4-cell embryos arrested at G2 phase can induce nuclear remodeling and genome-wide transcriptional reprogramming to both intra- and cross-species somatic nuclei within a day.

LIMITATIONS

NT-ETR system enables to induce transcription of embryonic genes from intra- and cross-species cells. One of the major limitations of the present system is that detection of genome-wide transcriptional reprogramming is applicable only to animal species harboring high-quality reference genome sequence information to separate sequencing reads bioinformatically. As another limitation, we have not shown a method to re-initiate the development of the NT embryos for propagation of the injected nuclei. Since recipient 4-cell embryos are reversibly arrested with demecolcine, future studies might enable the propagation of the injected nuclei for preserving genomic materials.

TROUBLESHOOTING

Problem 1

Cell fusion is not efficient.

Potential solution

It is important to select intact donor cells for NT-ETR (refer to step 14). Cells with broken cell membranes are not appropriate. Also, it is crucial to prepare fresh HVJ-E solution for NT.

Problem 2

After NT-ETR, blastomeres are also fused to each other.

Potential solution

When sufficient volume of HVJ-E solution is delivered to the perivitelline space with donor cells, blastomeres of 4-cell embryos fuse to each other and form 1-cell like embryos (refer to step 17 CRITICAL). For detecting transcriptional reprogramming, this is not a problem. A donor cell can be also fused with only one blastomere by controlling the volume of HVJ-E solution (lower volume of HVJ-E solution is transferred together with single donor cell adjacent to a blastomere).

Problem 3

Demecolcine treatment interferes with microtubule organization (Kapur et al., 2007) and results in developmental arrest of 4-cell embryos at G2 or M phase. In our observation, most of NT embryos stay at G2 phase until 54 hpi, and thereafter some of them start to show chromosome condensation. When embryos reach M phase, transplanted nuclei are also condensed with M phase-like structures. In such embryos, active transcription is not observed.

Potential solution

At the moment, it is difficult to control the progression to M phase. The only solution is to prepare enough NT embryos to allow selection for those at G2 phase. In general, the preparation of 30 embryos per experiment would be subsequent for analyses. The ratio of G2 phase to M phase is variable depending on the experiment. For example, in some experiments more than 80% of embryos are arrested at G2 phase, while in another experiment most embryos reach M phase. If M phase embryos are frequently observed in several sequential experiments, we recommend preparing a new demecolcine stock (refer to step 18).

Embryos at M and G2 phase can be distinguished by the presence or absence of nuclei and nucleoli structures. Alternatively, when LacR-mCherry is expressed, accumulation of LacR-mCherry in nuclei can mark nuclear areas.

Problem 4

During the Tyrode's treatment, embryos tightly attach to the bottom of the culture dish and are often broken.

Potential solution

Tyrode's solution quickly removes zona pellucida and, after removal, embryos attach to the culture dish. It is therefore important to carefully observe the process of dissolving zona pellucida and pick

up the treated embryos immediately after zona pellucida removal. Excess exposure to Tyrode's solution results in lysis of embryos (refer to step 19c).

Problem 5

STAR or kallisto fails to run or fails to produce an output.

Potential solution

Assuming that the software is correctly installed, a common reason for failure of either STAR or kallisto to run in this pipeline is due to incorrect merging or genomic/cDNA fasta files or gene annotation files. In the case of genomic/cDNA fasta files, it is important that the individual genome/transcriptomes possess unique identifiers for the chromosomes/scaffolds/transcripts e.g., ensure that there are not two Chr1 in the merged genome files but instead append unique IDs such as speciesAChr1, speciesBChr1. For genome strains downloaded from the Sanger Mouse Genomes project, coordinate locations will typically already be appended with the strain ID. It is important to ensure that chromosome information is also updated in the corresponding gene annotation files. When merging gene annotation files, it is important to ensure that they are of the correct type, e.g., do not merge gtf with gff3 (refer to steps 26 and 40).

Problem 6

Failure to identify strain specific transcripts with Sleuth.

Potential solution

A failure to identify strain specific transcripts may represent a genuine biological observation if there is no strain specific activation, or a technical limitation if genomes are too similar when weighed against the depth of sequencing. In some cases, a failure may be down to an incorrectly prepared mapping of transcripts to genes at step 36, and it is recommended to check tables manually by printing to screen or visualizing as a separate csv file (refer to step 36).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kei Miyamoto (kmiyamo@waka.kindai.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The dataset generated using this system is available in the Gene Expression Omnibus (GEO) public repository under accession GEO: GSE162345.

The code generated during this study is available at GitHub: <https://github.com/Miyamoto-lab/NT-ETR>.

All the other data are available from the Lead Contact upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101284>.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.M.; methodology, J.T., C.A.P., and K.M.; investigation, J.T., C.A.P., R.H., and K.M.; writing, J.T., C.A.P., and K.M.

DECLARATION OF INTERESTS

The authors declare no competing of interests.

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