Protocol

Generation of 2C-like mouse embryonic stem cells *in vivo* to evaluate developmental potency

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Highlights

A detailed protocol for inducing 2C-like mESCs with 2-Deoxy-Glucose

Conditions for enriching 2C-like mESCs with flow cytometry

A protocol to assess expanded potential of 2C-like mESCs

This protocol describes an optimized workflow for generating 2-cell (2C) stage-like mouse embryonic stem cells (mESCs) for microinjection into 8-cell stage mouse embryos to evaluate the developmental potency of these cells. We detail the following steps: 1) chemical suppression of glycolysis to induce a 2C-like state in mESCs, 2) flow cytometry to enrich for 2C-like cells, 3) embryo microinjection of 2C-like mESCs into 8-cell stage mouse embryos, and finally, 4) immunofluorescence staining of the chimeric blastocysts.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Generation of 2C-like mouse embryonic stem cells *in vivo* to evaluate developmental potency

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SUMMARY

This protocol describes an optimized workflow for generating 2-cell (2C) stage-like mouse embryonic stem cells (mESCs) for microinjection into 8-cell stage mouse embryos to evaluate the developmental potency of these cells. We detail the following steps: 1) chemical suppression of glycolysis to induce a 2C-like state in mESCs, 2) flow cytometry to enrich for 2C-like cells, 3) embryo microinjection of 2C-like mESCs into 8-cell stage mouse embryos, and finally, 4) immunofluorescence staining of the chimeric blastocysts.
For complete details on the use and execution of this protocol, please refer to Hu et al. (2020).

BEFORE YOU BEGIN

This protocol was used in a recent publication where we assessed the developmental potential of 2C-like mESC expressing high levels of NELFA (NELFA^high). We previously established that NELFA is a maternal driver of the 2C-like state and whose heterogeneous expression in mESCs is coupled to 2C gene upregulation and expanded developmental potential *in vivo* (Hu et al., 2020). Using CRISPR-mediated genomic engineering, we generated a NELFA-Strep-HA-P2A-EGFP reporter mESC line. Other 2C-like reporters such as MERVL and Zscan4c have also been reported (Macfarlan et al., 2012; Choi et al., 2017; Yang et al., 2020). Before proceeding, it is imperative to validate that these 2C-reporter mESC lines have a normal karyotype and express the appropriate markers of the 2C-like state. For the purpose of microinjection into mouse embryos, we have generated a double reporter cell line in which (1) NELFA is genetically labeled with EGFP and (2) mCherry is constitutively expressed (Figure 1). This dual reporter enables the tracking of both NELFA-EGFP-positive and NELFA-EGFP-negative mESCs post-injection in the blastocysts. All relevant regulatory and ethical approvals for animal experimentation must be obtained prior to the start of the experiments. Prior training for flow cytometry, rodent handling, embryo microinjection and confocal imaging are also required.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Cdx2 (1:250)	Abcam	Cat# ab157524
Anti-mCherry (1:450)	Abcam	Cat# ab167453
		(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 594 Donkey Anti-Rabbit IgG (H+L) (1:250)	Thermo Fisher Scientific	Cat# A-22107
Alexa Fluor 647 Rabbit Anti-Mouse IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat# A-21239
Chemicals, peptides, and recombinant proteins		
Knockout DMEM	Thermo Fisher Scientific	Cat# 10829018
M2 embryo media	Sigma-Aldrich	Cat# M7167
KSOM Media	Sigma-Aldrich	Cat# MR-106-D
ES Cell qualified Fetal Bovine Serum	Thermo Fisher Scientific	Cat# 1886334
Phosphate buffered saline pH 7.4	Thermo Fisher Scientific	Cat# 10010031
Penicillin-streptomycin	Thermo Fisher Scientific	Cat# 15140163
L-Glutamine	Sigma-Aldrich	Cat# G7513-100ML
Non-essential amino acids	Sigma-Aldrich	Cat# M7145-100ML
β-Mercaptoethanol	Sigma-Aldrich	Cat# M3148-25ML
Trypsin-EDTA 0.25%	Thermo Fisher Scientific	Cat# 25200072
Leukocyte inhibitory factor (LIF)	Sigma-Aldrich	Cat# ESG1107
PD0325901	Sigma-Aldrich	Cat# PZ0162-25MG
CHIR99021	Sigma-Aldrich	Cat# SML1046-25MG
2-Deoxy-Glucose	Sigma-Aldrich	Cat# D8375-5G
Triton-X100	Sigma-Aldrich	Cat# T8787-250ML
Paraformaldehyde	Sigma-Aldrich	Cat# 252549-100ML
DAPI (1:1000)	Sigma-Aldrich	Cat# 10236276001
Albumin Bovine Serum (BSA), Fraction V	GoldBio	Cat# A-420-10
Experimental models: Cell lines		
NELFA-EGFP; mCherry dual reporter mESCs	This study	N/A
Experimental models: Organisms/strains		
Mouse Embryo from ICR or C57BL/6 N strains	Charles River	N/A

MATERIALS AND EQUIPMENT

mESC medium		
Reagent	Final concentration	Amount
Knockout DMEM	–	425 mL
ES Cell qualified Fetal Bovine Serum	15% (v/v)	75 mL
Penicillin-streptomycin (100×)	1×	5 mL
L-Glutamine (100×)	1×	5 mL
Non-essential amino acids (100×)	1×	5 mL
β-Mercaptoethanol (14.3 M)	0.05 mM	1.75 μL
Leukocyte inhibitory factor (LIF) 10^7 U/mL	1000 U/mL	50 μL
10 mM PD0325901 stock	1 μM	50 μL
10 mM CHIR99021 stock	3 μM	150 μL

Filter sterilize through a 0.22 μm filter unit before use and store sterile medium at 4°C for up to 1 month.

0.1% Gelatin solution		
Reagent	Final concentration	Amount
2% Gelatin (Sigma# G1393-100ML)	0.1% (w/v)	45 mL
Sterile water	–	855 mL

Filter sterilize through a 0.22 μm filter unit before use and store at 4°C for up to 1 month.

Immunofluorescence (IF) blocking buffer

Reagent	Final concentration	Amount
Bovine Serum Albumin	1% (w/v)	0.5 g
10× Phosphate buffered saline	1×	5 mL
10% (v/v) Triton-X 100	0.1% (v/v)	0.5 mL
ddH$_2$O	N/A	44 mL
Total	N/A	50 mL

Buffer can be stored in 4°C for up to 3 months.

Flow cytometry buffer

Reagent	Final concentration	Amount
Fetal Bovine Serum	2% (v/v)	1 mL
10× Phosphate buffered saline	1×	5 mL
ddH$_2$O	N/A	44 mL
Total	N/A	50 mL

Filter sterilize before use and buffer can be stored in 4°C for up to 1 month.

Equipment

Type	Manufacturer	Catalog
CO$_2$ Incubator	Thermo Fisher Scientific	Cat# 51030301
Cell sorter	Sony Biotechnology	Cat# Sy3200
Micropipette puller	Sutter Instrument, USA	Cat# P-97
Microforge	NARISHIGE, Japan	Cat# MF-900
Micromanipulator	PRIME TECH, Japan	Cat# PMAS-CT150
Inverted microscope	Olympus, Japan	Cat# IX71
Stereomicroscope	Olympus, Japan	Cat# SZX16
Confocal microscope	ZEISS, Germany	Cat# LSM 700
35 mm Glass bottom dish	Thermo Fisher Scientific	Cat# 150682

STEP-BY-STEP METHOD DETAILS
2-DG treatment to induce NELFA[high] 2C-like mESCs

⏱ **Timing: 5 days**

In our protocol, we use 2-deoxy-glucose (2-DG) treatment, which inhibits glycolysis, to boost the NELFA[high] 2C-like population in culture. We previously determined that 2-DG treatment for 4 days can lead to an 8-10-fold increase in the 2C-like population. Accordingly, we have determined the optimal seeding density to be 1.2×10^5 mESCs/well for a 6-well plate. This ratio can be scale up or down according to the culture dish size. Unless otherwise stated, the protocol described here details the instructions for a single well of a 6-well plate.

1. Seed NELFA reporter mESCs at 1.2×10^5 per well of a 6-well plate in 3 mL of standard mESC medium containing LIF and 2i (1 µM PD0325901 and 3 µM CHIR99021).

   Note: LIF is a cytokine that is commonly used to maintain mESCs in an undifferentiated state, and 2i refers to the combination of two inhibitors - PD0325091; MEK inhibitor, and CHIR99021; GSK3 inhibitor, that further blocks mESC differentiation.

2. Culture cells on gelatin coated (0.1% w/v, 24 h) 6-well plates in an incubator at 37°C with 5% CO$_2$.
3. Discard spent medium the following day and transfer 3 mL of fresh medium containing 4 mM 2-DG to the reporter cells. This is day 1 of treatment.
   a. 2-DG is prepared fresh at a final concentration of 1 M in sterile water.
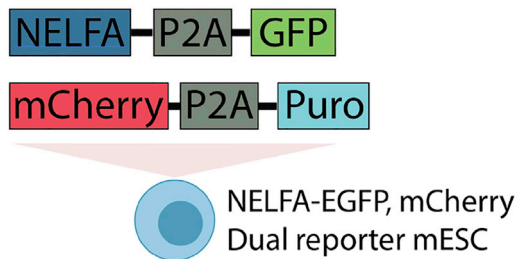
Figure 1. Schema of NELFA-EGFP; mCherry dual reporter mESCs

Note: User would require 4 μL of 2-DG solution (1 M stock) per mL of culture medium. For example, if 3 mL of ESC medium is used, 12 μL of 2-DG 1 M stock solution is added fresh daily. To prepare the 2-DG solution (1 M stock), dissolve 2-DG powder in sterile water and adjust concentration to 1 M. Make 50 μL single use aliquots and store in −20°C for up to 6 months. We recommend discarding thawed leftovers, and to avoid freeze-thawing of 2-DG stock solution.

4. Discard spent medium the following day and transfer 3 mL of fresh medium containing 4 mM of 2-DG. Repeat this step until cells have been treated for a total of 4 days.

   ⚠ CRITICAL: Monitor reporter cells under a fluorescence microscope daily to determine if treatment is effective. 2-DG-treated cells tend to grow slower than untreated controls and exhibit a more 'flattened' cell morphology. In a successful experiment, there should be a gradual increase in the number of 2C-like cells that is most apparent on day 4 of treatment (Figure 2A).

5. Prepare cells for flow cytometry after 4 days of 2-DG treatment.
   a. Discard culture medium by aspiration.
   b. Wash 2C-reporter mESCs carefully twice with 2 mL of sterile PBS.
   c. Discard PBS after second wash.
   d. Add 0.3 mL of room temperature (22°C–25°C) 0.25% trypsin to dislodge the cells.
   e. Incubate cells at 37°C for 2 min during trypsin treatment.
   f. Add 0.7 mL of mESC medium to quench trypsin.
   g. Collect cells by centrifugation at 200 g for 5 min at room temperature (22°C–25°C).
   h. Discard supernatant and resuspend cells in 1 mL of mESC medium containing freshly prepared 4 mM 2-DG.
   i. Strain cells through a 40 μm cell strainer and place cells on ice until flow cytometry commences.

Flow cytometry enrichment of 2C-like cells

⏱ Timing: 2–3 h

We used a Sony Sy3200 cell sorter for our experiment but other cell sorters with the appropriate lasers can also be used. Should phenol-red present in the culture medium interferes with the cell sorting process, alternative cell medium such as indicator-free culture medium, or the commonly used flow medium (1× PBS, 2% (v/v) FBS) can be used. It is important to perform the entire experiment under sterile conditions to ensure the cells do not become contaminated.

6. Begin by defining the negative and positive sorting gates (Figure 3).

   Note: In our study, we have used a double EGFP and mCherry reporter. Our gene of interest, NELFA, is reported by EGFP while mCherry is constitutively expressed to mark the injected
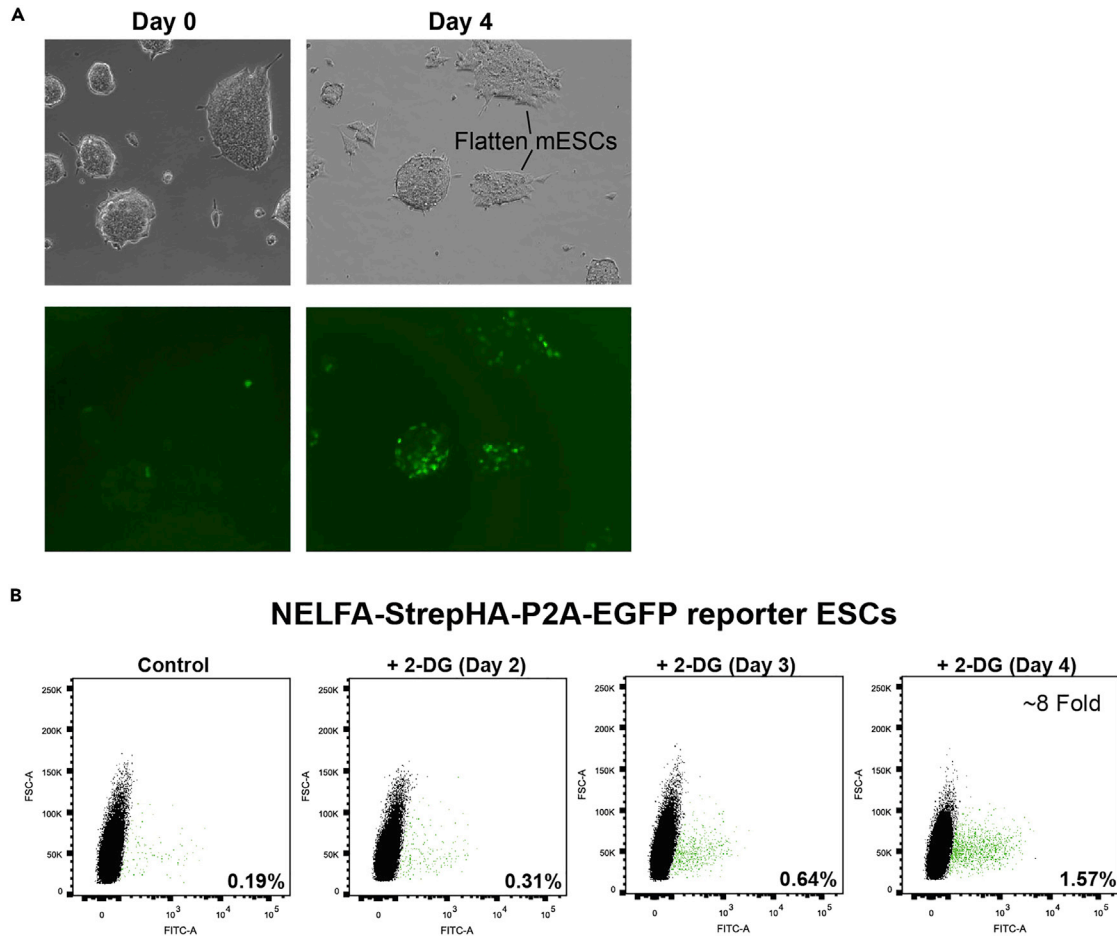
Figure 2. Representative fluorescence images and FACS analysis
(A) Representative brightfield and fluorescence images of reporter mESCs undergoing 2-DG treatment.
(B) Representative FACS plot of NELFA-EGFP reporter ESCs following 2-DG treatment for 4 days.

cells. The latter will allow us to track the localization and fate of both EGFP-positive and EGFP-negative mESCs in the early embryos after injection.

⚠ CRITICAL: It is important to adopt a strict gating boundary for every flow cytometry setup by including negative control i.e., cells with no EGFP or mCherry to define boundaries for the positive cells.

a. Fine tune the plate voltages and laser intensities to obtain clear separation of singlets and doublets in the forward and side scatter plots.
b. Define boundaries for cells that are EGFP negative in the control group and record an average of 10,000 events for reference.
c. Unload the control cells and rinse the cell sorter with sterile water for 2 min.
d. Load 2-DG treated mESCs.
e. Record an average of 10,000 events for reference using the same voltages and laser intensity.
f. From this plot, define boundaries of EGFP positive cells that show clear separation from the bulk EGFP negative population.
g. We typically bias for the top 2% brightest EGFP cells. Once the gates are determined, the sorting can begin.
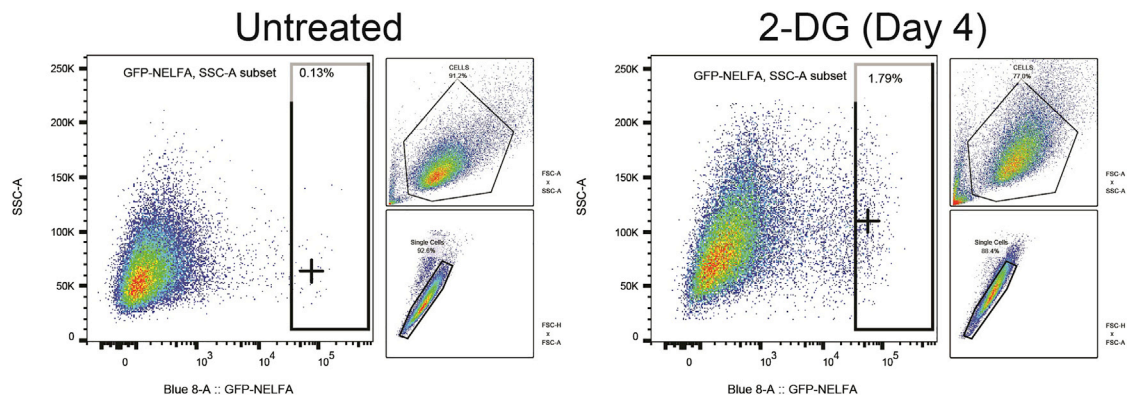
Figure 3. Representative gating strategy for NELFA EGFP; mCherry double reporter ESCs during flow cytometry

7. Cells are sorted at the slowest speed setting using a 100 μm nozzle for ensuring maximal cell viability.
   a. Depending on the downstream applications, we typically sort 1×10^4 to 3×10^5 EGFP-positive and EGFP-negative cells respectively.

   Note: EGFP-positive and negative cells should be sort enriched separately to reduce chances of cross contamination during flow cytometry.

8. Sort cells into 5 mL collection tubes containing 1 mL of ESC medium supplemented with 4 mM 2-DG. The sorting chamber is chilled at 4°C throughout the whole sorting process.
9. Flush the cell sorter briefly with FACS buffer, and analyze the sorted cells on the cell sorter to determine sort purity.

   ⚠ CRITICAL: It is crucial to perform post-sort purity checks on the sorted cells to verify that there is minimal cross contamination between the EGFP-positive and negative populations, and that all cells are mCherry-positive.

   a. Once the intended cell numbers for each population are obtained and placed on ice, set up a second sort to obtain another set of EGFP negative and positive populations using the same gating parameters.
   b. Flush cell sorter with sterile water for 2 min and the respective enriched cell populations from the second sort is loaded iteratively into the cell sorter to determine sorting purity.
   c. Record an average of 10,000 events and obtain ~95% pure EGFP positive and populations.
   d. If the post-sorting purity is sub-optimal i.e., less than 80%, the user should consider recalibrating the cell sorter's laser with fluorophore standards and repeat the cell sorting procedure with more stringent gating i.e., defining the EGFP positive boundary even further away from the EGFP negative population.
10. Flow sorted cells are transported on ice promptly for embryo microinjection.

   ⚠ CRITICAL: In the same batch of sorted cells for embryo injection, we typically set aside some cells for gene expression profiling with qPCR to ensure that the prototypic 2C restricted transcripts such as *MERVL, Dux,* and *Zscan4c* (Macfarlan et al., 2012; Hendrickson et al., 2017; Hu et al., 2020; Akiyama et al., 2015) are upregulated in the EGFP-positive cells compared to the EGFP-negative population. Additionally, a subset of the sorted cells can be re-cultured on gelatin-coated plates to assess cell viability and sterility postsorting.
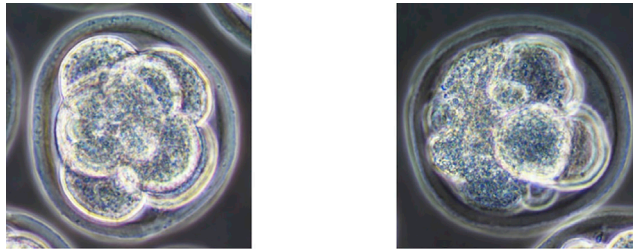
Figure 4. Representative images showing the healthy 8-cell embryo (left) and abnormal 8-cell embryo (right)

Embryo microinjection

⏲ **Timing: 2 days**

We inject either NELFA^{high} or NELFA^{low} mESCs with good survival status into mouse 8-cell stage embryos to generate chimeric blastocysts. The injected embryos are cultured for 2 days before immunofluorescence staining.

11. Collect flow cytometry enriched mESCs via centrifugation at 200 g for 3 min at 4°C.
12. Discard FACS buffer supernatant and carefully resuspend the cells with 500 μL of M2 culture medium. Re-suspended cells are kept at 4°C until injection.

> *Note:* We typically sort 1×10^4 to 3×10^5 EGFP-positive (NELFA^{high}) and EGFP-negative (NELFA^{low}) cells respectively during each flow cytometry session and resuspend the cells in 500 μL of M2 medium. Care is taken to resuspend cells gently to maintain cell viability and to obtain uniform suspension of single cells to facilitate downstream injection.

13. Prior to harvesting embryos for injection, pre-warm M2 and KSOM media in a 37°C incubator supplemented with 5% CO_2.
14. Mouse embryo harvesting.
    a. Sacrifice the donor ICR female mice by cervical dislocation at 2.5 days post-coitum (dpc) (Behringer et al., 2014).
    b. Immediately dissect out the fallopian tubes.
    c. Use a sterile 3 mL syringe with 27G needle to flush pre-warmed M2 medium into the fallopian tube to harvest the 8-cell stage embryos.
    d. Use a mouth pipette to transfer the embryos to a petri dish filled with pre-warmed KSOM medium.
    e. In a separate petri dish, wash the harvested embryos 5 times in drops (50 μL per drop) of pre-warmed KSOM medium iteratively.
        i. Carefully mouth pipette the embryos to the first drop of KSOM.
        ii. Transfer the embryos from the first drop to second drop of KSOM medium.
        iii. Steps i-ii are repeated until the embryos are transferred to the 5th drop of KSOM medium.
    f. Pick the healthy 8-cell stage embryos from the pool of washed embryos, and set aside in a separate petri dish containing pre-warmed KSOM medium (Figure 4).
    g. Place healthy embryos in a 37°C incubator supplemented with 5% CO_2 until injection.

> *Note:* It is important to pre-warm the KSOM medium in the 37°C incubator for at least 30 min in advance.

15. Embryo microinjection.
    a. Prepare the holding and injection pipettes using the micropipette puller and microforge. Thereafter, setup and adjust the holding and injection pipettes (Figure 5A).
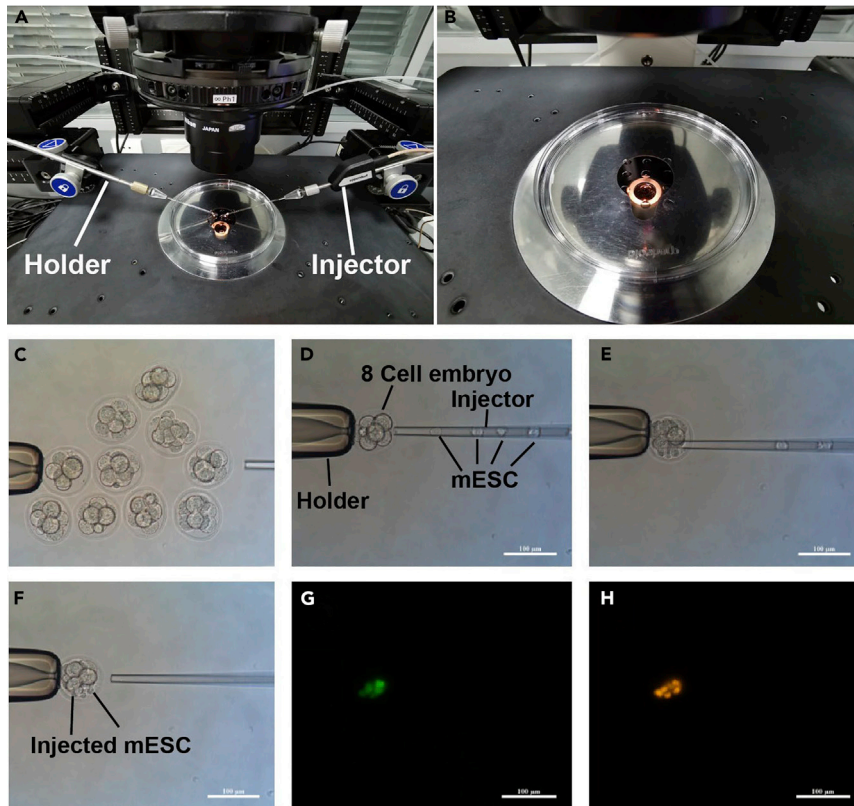
Figure 5. Schematic representation of the embryo microinjection setup
Scale bars are at 100 μm.
(A) Mounting of the holding pipette and injection needle onto the microinjector.
(B) Injection dish with of 50 μL drops of M2 medium.
(C) Placement of the 8-cell embryos near to the holding pipette in the M2 droplet.
(D) Injection pipette is loaded with either NELFA^{high} or NELFA^{low} mESCs.
(E and F) Injection of loaded mESCs into the cell space of an 8-cell stage embryo held in place by the holding pipette.
(G and H) Representative EGFP and mCherry fluorescence images of NELFA^{high} mESC injected in the embryos

b. Prepare multiple 50 μL drops of M2 medium in a clean petri dish cover. (Figure 5B).
c. Cover 50 μL of M2 medium drops completely with 200 μL of mineral oil.
d. Carefully mouth pipette 8–10 embryos into each M2-mineral droplet (Figure 5C).
e. Retrieve the FACS sorted mESCs from 4°C and gently pipette to mix.
f. Carefully mouth pipette the sorted cells into a glass capillary, ensuring that there are no bubbles (Figure 5D).
g. For each droplet containing the embryos, release a small number of mESCs into each droplet.
h. Place the prepared petri dish on the stage of an inverted light microscope.
i. Carefully transfer the holding and injection pipettes into the droplets containing the embryos and mESCs.
j. Inject 5–7 NELFA^{high} or NELFA^{low} mESCs into the cell space of 8-cell stage embryos by piercing through the zona pellucida (Figures 5E and 5F).
k. Transfer carefully the injected chimeric embryos into a fresh petri dish containing KSOM medium and place in an incubator at 37°C with 5% CO_2 for 48 h before immunofluorescence staining (Figure 6).

Note: We typically perform injection in batches of 10 embryos during each session to minimize the time that the harvested embryo spends outside the incubator as unfavourable conditions will affect embryo development post-injection.
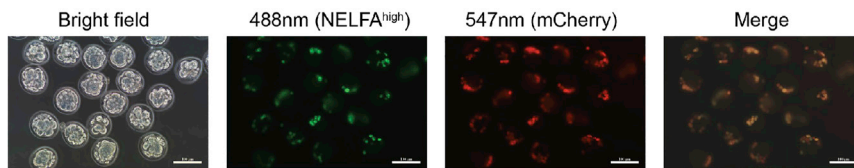
Bright field   488nm (NELFA^high)   547nm (mCherry)   Merge

Figure 6. Representative images of mouse embryos after injection of NELFA^{high} mESCs. Scale bar are at 100 μm.

Immunofluorescence (IF) staining on injected embryos

© Timing: 2 days

It is important to minimize disruption of the embryos post-injection. Do not remove the embryos from the incubator frequently as this will cause development to arrest.

16. Remove the injected embryos from the incubator when developmental timepoint reaches approximately late E4.5 stage.
17. Carefully remove the microinjected chimeric embryos using a mouth pipette and wash the embryos 3–4 times in consecutive 100 μL drops of PBS with 1% BSA.
18. Fix the embryos with 4% paraformaldehyde (PFA) at room temperature (22°C–25°C) for 15 min.

   Note: It is important to wash the embryos in PBS containing BSA to prevent the embryos from sticking to the glass capillary and ensure that the PFA solution covers the embryos entirely.

19. Carefully transfer the embryos using a mouth pipette into a 4-well dish containing PBS with 1% BSA. Aspirate and discard the PFA as per institutional safety regulations.
20. Dispense sufficient PBS + 1% BSA (∼0.5 mL) such that it covers the embryo completely and incubate at room temperature (22°C–25°C) for 5 min.
21. Step 5 is repeated twice for a total of 3 wash steps. The addition of BSA in the wash buffer prevents the embryos from sticking to the glass capillary during transfer.

   Note: Embryos may be stored in sterile PBS + 1% BSA at 4°C for several days until the next step. Ensure that there is sufficient liquid covering the embryos to prevent dry up. For longer storage, cover the PBS containing the embryos with a layer of mineral oil to prevent evaporation.

22. Permeabilize and block the embryos with IF blocking buffer for 1 h at room temperature (22°C–25°C).
23. During the blocking step incubation, prepare primary antibody dilutions in IF blocking buffer.
    a. Determine the optimal primary antibody dilutions empirically. For a start, follow the recommended IF dilution by the antibody manufacturer.
    b. Setup IF staining for both mCherry (1:400) and Cdx2 (1:250), a trophectoderm marker (Hu et al., 2020).
24. Gently aspirate and remove the blocking buffer from the well and add the primary antibody solution to the embryos.
25. Incubate the primary antibody for 12 h at 4°C.

   Note: We fill up any unused wells on the culture dish with 1× PBS to reduce evaporation of the primary antibody solution.

26. Next day, carefully transfer the embryos using a mouth pipette into a 4-well dish containing IF blocking buffer and incubate at room temperature (22°C–25°C) for 5 min. Aspirate and discard the primary antibody solution.
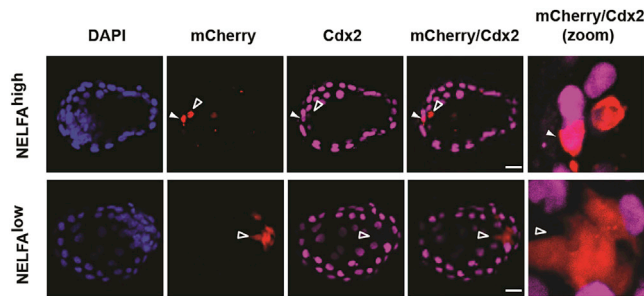
Figure 7. Representative images showing immunostaining of chimeric blastocysts injected with mCherry-labelled NELFA^{high} and NELFA^{low} mESCs following 2-DG treatment. NELFA^{high} chimeric blastocysts showed integration of injected cells in both the inner cells mass (ICM) (open arrows) and trophectoderm (filled arrows) whereas all NELFA^{low} chimeric blastocysts contributed exclusively to the ICM. Trophectoderm is marked by Cdx2 (magenta). DAPI nuclear staining is shown in blue. Scale bars: 20 μm.

27. Repeat step 26 for a total of 3 washes.
28. Prepare the secondary antibody dilutions. We have used 1: 500 dilution for Alexa Fluor 594 (for staining mCherry) and 1:250 dilution for Alexa Fluor 647 (for staining Cdx2). Additionally, we co-stain DAPI (1:1,000) simultaneously with the respective secondary antibodies.
    a. For 1 well of embryos, add 1 μL of Alexa Fluor 594 Donkey Anti-Rabbit IgG (H+L), 2 μL of Alexa Fluor 647 Rabbit Anti-Mouse IgG (H+L) and 0.5 μL of DAPI solution into 0.5 mL of IF blocking buffer.
29. Perform secondary antibody incubation at room temperature (22°C–25°C) for 1 h, protected from light.
30. After incubation, discard the secondary antibody solution carefully and transfer the embryos into another well containing IF blocking buffer.
31. Incubate at room temperature (22°C–25°C) for 5 min.
32. Discard the IF blocking buffer and repeat steps 15 and 16 for a total 3 washes.
33. Wash the embryos once with 1× PBS + 1% BSA.
34. In preparation for confocal imaging, we typically make a microdrop of PBS on the glass surface of a 35 mm glass-bottom dish and cover with mineral oil. Carefully transfer the embryos into the microdrops on the imaging dish using a mouth pipette.
35. Acquire the images using the appropriate confocal microscope configurations.

Note: The fixed embryos may be kept in the imaging dish covered with mineral oil to prevent evaporation and stored at 4°C for several weeks. Ensure that PBS is sterile and/or add sodium azide to prevent bacterial growth and contamination.

EXPECTED OUTCOMES

With viable and pure cells obtained from flow cytometry, coupled with robust induction of the 2C program (based on reporter cell activation), we should observe a higher proportion of the 2C-like mESCs localizing to the outer compartment of the late blastocysts, and co-expressing Cdx2, a key trophectoderm marker (Figure 7), highlighting the expanded developmental potency of 2C-like mESCs (Macfarlan et al., 2012). To unambiguously ascertain the expanded potential of these cells, it is important to include different makers of the epiblast and trophectoderm lineages in the immunostaining assessment, rather than depend solely on the relative inner versus outer positions of the injected cells in the blastocyst (Posfai et al., 2021).

LIMITATIONS

In this protocol, the robust generation and isolation of 2C-like mESCs are crucial for a successful experiment. Additionally, a skilled operator is required to perform the microinjection experiment.

STAR Protocols
Protocol

Nonetheless, the overall success rates may vary between different laboratories. We have listed some of the common problems we have faced and the respective solutions in the section below.

TROUBLESHOOTING

Problem 1
Flow sorted cells have poor viability post sorting.

Potential solution
To resolve this issue, we experimented with several different commercial cell sorters and different media for cell sorting. In general, we find that using a larger nozzle improves cell viability when sorted at a low-speed setting. We have determined that the nozzle size of 100 μm is optimal for our purpose. Alternatively, where cell sorting is not available, we have also dissociated the NELFA reporter mESCs with accutase for 3–5 min, followed by resuspension in M2 medium, for manual picking of the EGFP-positive and negative cells under the fluorescent microscope using a mouth pipette. The latter requires a skilled operator to perform the cell picking expeditiously to minimize exposure to the laser.

Problem 2
Poor cell purity resulting in the mixture of 2C-like and pluripotent ESCs.

Potential solution
Obtaining a pure population of either 2C-like (NELFA^{high}) or pluripotent cells (NELFA^{low}) is quintessential for the experiment. We have relied on EGFP to mark the 2C state. Although most cell sorter can enrich for both GFP-positive and negative population of cells simultaneously, we recommend sorting each population of cells separately to minimize cross contamination of the sort streams. Additionally, it is good practice to setup a sort purity assessment prior to embryo injection and only proceed if the cell purity is greater than 95%.

Problem 3
Poor induction of 2C-like mESCs following 2-DG treatment, resulting in a low proportion of 2C-like cells available for downstream injection.

Potential solution
Prior to attempting a full-scale experiment, the user should empirically determine the optimal starting seeding density of the reporter ESCs, the 2-DG concentration, as well as the treatment duration, as these conditions may vary between laboratories. We have also noticed that there is generally poor 2C induction when the cells are overly confluent during treatment, and should therefore be avoided.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wee-Wei Tee (wwtee@imcb.a-star.edu.sg).

Materials availability
For request of NELFA reporter mESCs, please contact lead contact.

Data and code availability
This study did not generate unique codes or new data.

AUTHOR CONTRIBUTIONS

Conceptualization – W.W.T.; Methodology – D.E.K.T., B.W., and B.S. All authors contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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