Embryonic diapause is a naturally occurring strategy in mammals that determines successful rates of gestation under unfavorable conditions. This dormant state can be captured in the form of quiescent mouse embryonic stem cells (ESCs). Here, we present a step-by-step protocol to derive quiescent ESCs that naturally exist in culture by harnessing the heterogeneity of mitochondrial activity. The derived quiescent ESCs with low mitochondrial activity can be utilized as a surrogate to study stages of early embryonic development.

HIGHLIGHTS

Ground-state ESCs are heterogeneous with regard to mitochondrial membrane potential ($\Delta \Psi_m$)

A step-by-step protocol for capturing ground-state ESCs with distinct levels of $\Delta \Psi_m$

Low $\Delta \Psi_m$ ground-state ESCs exhibit defined features of quiescent state

Low $\Delta \Psi_m$ quiescent ESCs retain their differentiation capacity in vitro

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Protocol

**In Vitro Derivation of Quiescent Mouse Embryonic Stem Cells Based on Distinct Mitochondrial Activity**

Le Tran Phuc Khoa¹,³,* and Yali Dou¹,²,⁴,*

¹Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
²Present address: Department of Medicine, Department of Biochemistry and Molecular Medicine, University of Southern California, Los Angeles, CA 90033, USA
³Technical Contact
⁴Lead Contact
*Correspondence: lekh@med.umich.edu (L.T.P.K.), yalidou@usc.edu (Y.D.)

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**SUMMARY**

Embryonic diapause is a naturally occurring strategy in mammals that determines successful rates of gestation under unfavorable conditions. This dormant state can be captured in the form of quiescent mouse embryonic stem cells (ESCs). Here, we present a step-by-step protocol to derive quiescent ESCs that naturally exist in culture by harnessing the heterogeneity of mitochondrial activity. The derived quiescent ESCs with low mitochondrial activity can be utilized as a surrogate to study stages of early embryonic development.

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

**BEFORE YOU BEGIN**

© Timing: 1–2 days

This protocol includes methodologies of ground-state embryonic stem cell (ESC) culture, cell labeling, fluorescence-activated cell sorting (FACS) and *in vitro* embryoid body (EB) differentiation. Before starting this protocol, all reagents and the ESC line should be purchased and prepared in a ready-to-use mode with careful considerations.

1. Geltrex preparation
   a. Thaw the Geltrex 18 h at 4°C. Geltrex should be aliquoted (200 μL/tube), stored at −20°C and used within 3 months.
   b. Thaw an aliquot of Geltrex 18 h at 4°C. Gently mix the Geltrex with 19.8 mL of DMEM/F-12 in a 50 mL conical tube.
   c. Pre-chill 24-well or 6-well cell culture plates in 4°C for at least 15 min before use.
   d. Add 0.5 mL or 1 mL of the diluted Geltrex into the above 24-well or 6-well plates, respectively. Incubate the Geltrex-coated plates in the cell culture incubator for at least 1 h.
   e. Leave the Geltrex-coated plates in the laminar flow hood for at least 30 min but no more than 1 h.
   f. Gently aspirate the Geltrex, add Dulbecco’s Phosphate-Buffered Saline (DPBS) into the plates, and store in the cell culture incubator. The Geltrex-coated plates are ready to use and stable for 1 week.

⚠️ CRITICAL: Geltrex is very easy to gel at room temperature (RT, 25°C–27°C), researchers are highly recommended to follow the above steps to maintain the quality of Geltrex. We
strongly suggest that all tips and plastic pipettes should be pre-chilled at –20°C for at least 15 min before touching the Geltrex.

2. PD0325901 (10 mM stock): dissolve 5 mg of PD0325901 into 1.04 mL of Dimethyl Sulfoxide (DMSO). PD0325901 stock can be aliquoted at 50 μL/tube, stored at –20°C and used within 6 months.

3. CHIR99021 (30 mM stock): dissolve 25 mg of CHIR99021 into 1.79 mL of DMSO. CHIR99021 stock can be aliquoted at 50 μL/tube, stored at –20°C and used within 6 months.

4. 2i stock (PD0325901, 5 mM plus CHIR99021, 15 mM): mix an equal volume of PD0325901 and CHIR99021 from steps 2 and 3. 2i stock can be stored at –20°C and used within 6 months.

5. Preparation of culture medium: ESCs are routinely cultured in N2B27/2i/LIF media. N2B27 medium consists of a 1:1 mixture of DMEM/F12 and Neurobasal, N2 (1:100 dilution), B27 (1:100 dilution), 2 mM Glutamine, Penicillin/Streptomycin (1:100 dilution) and 0.055 mM 2-mercaptoethanol. 2i (1:5,000 dilution) and LIF (1,000 U/mL) are added as the last components.

| N2B27/2i/LIF Media (Make 40 mL) | Final Concentration | Amount  |
|-------------------------------|---------------------|---------|
| DMEM/F12                      | N/A                 | 19.274 mL |
| Neurobasal                    | N/A                 | 19.274 mL |
| N2 (100X)                     | 1 x                 | 400 μL |
| B27 (50X)                     | 0.5 x               | 400 μL |
| Glutamine (200 mM)            | 2 mM                | 200 μL* |
| Penicillin/streptomycin (10,000 U/mL) | 100 U/mL            | 400 μL |
| 2-mercaptoethanol (55 mM)     | 0.055 mM            | 40 μL |
| 2i (PD0325901, 5 mM plus CHIR99021, 15 mM) | PD0325901, 1 μM plus CHIR99021, 3 μM | 8 μL |
| LIF (107 units/mL)            | 1,000 units/mL      | 4 μL |

*Since DMEM/F12 already contains L-Glutamine, only another 200 μL is required.

⚠ CRITICAL: To maintain the stability of all chemicals and get reproducible results, the culture medium should be prepared and used within one week. In addition, each component should be routinely tested for lot-to-lot variability by comparing cell morphology and growth rate between old and new media.

6. EB media: KnockOut™ DMEM, 15% fetal bovine serum (FBS), 2 mM Glutamine, 1X Non-essential amino acids and 0.055 mM 2-mercaptoethanol.

7. High quality of ESCs (< 50 passages) routinely tested negative for mycoplasma are highly recommended for all experiments.

| EB Media (Make 40 mL) | Final Concentration | Amount  |
|-----------------------|---------------------|---------|
| KnockOut™ DMEM        | N/A                 | 33.160 mL |
| Fetal bovine serum (FBS) | 15%               | 6 mL |
| Glutamine (200 mM)    | 2 mM                | 400 μL |
| Non-essential amino acids (100X) | 1X             | 400 μL |
| 2-mercaptoethanol (55 mM) | 0.055 mM          | 40 μL |

8. Tetramethylrhodamine Methyl Ester (TMRM) preparation: dissolve 25 mg of TMRM into 5 mL of DMSO to get 10 mM solution. To prepare a stock concentration of 100 μM, add 10 μL of 10 mM TMRM solution into 990 μL of DMSO. All TMRM solutions should be stored at –20°C and used
within 6 months. To avoid detrimental effects of freeze-thaw cycles, TMRM solutions are strongly recommended to aliquot for a single use.

9. Staining buffer preparation: add 10 mM HEPES and 2% FBS into Phenol red-free Hanks’ Balanced Salt Solution (HBSS) 1×.

| Staining Buffer (Make 40 mL) | Final Concentration | Amount |
|-----------------------------|---------------------|--------|
| HEPES 1M                    | 10 mM               | 400 µL |
| FBS                         | 2%                  | 800 µL |
| Phenol red-free Hanks’ Balanced Salt Solution (HBSS) 1× | N/A | 38.8 mL |

**Note:** FBS needs to be heat-inactivated at 56°C for 30 min, mix well and stored at 4°C.

10. FACS running buffer: add 10 mM HEPES into N2B27 medium.

| FACS running buffer (Make 40 mL) | Final Concentration | Amount |
|----------------------------------|---------------------|--------|
| HEPES 1M                         | 10 mM               | 400 µL |
| N2B27 medium                     | N/A                 | 39.6 mL |

11. FACS collection buffer: add 10 mM HEPES and 2% FBS into N2B27 medium.

| FACS Running Buffer (Make 40 mL) | Final Concentration | Amount |
|----------------------------------|---------------------|--------|
| HEPES 1M                         | 10 mM               | 400 µL |
| FBS                              | 2%                  | 800 µL |
| N2B27 medium                     | N/A                 | 38.8 mL |

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-OCT3/4         | Santa Cruz Biotechnology | Cat#sc-5279, RRID:AB_628051 |
| Anti-PECAM-1        | Biolegend | Cat#102402, RRID: AB_312897 |
| APC/FireTM 750 Goat anti-Rat IgG | Biolegend | Cat#405426, RRID:AB_2687098 |
| Rat IgG Isotype Control | Thermo Fisher Scientific | Cat#31933, RRID: AB_10959892 |
| Goat anti-Mouse IgG FITC-conjugated | Thermo Fisher Scientific | Cat#62-6511, RRID:AB_2533946 |
| TruStain FcX™ (anti-mouse CD16/32) | Biolegend | Cat#101320, RRID:AB_1574975 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| ESGRO® leukemia inhibitory factor (LIF) | EMD Millipore | Cat#ESG1107 |
| PD0325901           | SIGMA ALDRICH       | Cat#P20162 |
| CHIR99021           | SIGMA ALDRICH       | Cat#SML1046 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Dimethyl Sulfoxide (DMSO) | SIGMA ALDRICH | Cat#D8418 |
| Geltrex | Thermo Fisher Scientific | Cat#A1413202 |
| 0.1% Gelatin Solution | Millipore | Cat#ES-006-B |
| N2 supplement (100x) | Thermo Fisher Scientific | Cat#17502001 |
| B27 supplement (50x) | Thermo Fisher Scientific | Cat#17504-044 |
| KnockOut™ DMEM medium | Thermo Fisher Scientific | Cat#10829018 |
| 2-mercaptoethanol | Thermo Fisher Scientific | Cat#21985023 |
| Penicillin/Streptomycin | Thermo Fisher Scientific | Cat#15140122 |
| Glutamine | Thermo Fisher Scientific | Cat#25030-024 |
| DMEM/F12 | Thermo Fisher Scientific | Cat#11320-033 |
| Neurobasal | Thermo Fisher Scientific | Cat#21103-049 |
| Fetal Bovine Serum (FBS) | Atlas Biologicals | Cat#F-0500-D |
| HEPES 1M | Thermo Fisher Scientific | Cat#15630106 |
| Hanks’ Balanced Salt Solution (HBSS) 1x | Gibco™ | Cat#14175-095 |
| Dulbecco’s Phosphate-Buffered Saline (DPBS) | Gibco™ | Cat#14190-144 |
| DPBS containing Calcium Chloride and Magnesium Chloride (DPBS+) | Gibco™ | Cat#14040-133 |
| UltraPure™ DNase/RNase-Free distilled H2O | Thermo Fisher Scientific | Cat#10977-015 |
| Accutase | Gibco™ | Cat#A1110501 |
| Cell staining buffer | Biolegend | Cat#420201 |
| Paraformaldehyde | Ted Pella Inc. | Cat#18505 |
| Triton X-100 | SIGMA ALDRICH | Cat#93443 |
| Goat serum | Cell Signaling Technology | Cat#5425 |
| SYTOX™ Blue | Thermo Fisher Scientific | Cat#S34857 |
| Tetramethylrhodamine methyl ester (TMRM) | Thermo Fisher Scientific | Cat#T668 |
| Hoechst 33342 | Thermo Fisher Scientific | Cat#62249 |
| Pyronin Y | SIGMA ALDRICH | Cat#213519 |

**Critical Commercial Assays**

| Assay Name                       | Source                | Identifier |
|----------------------------------|-----------------------|------------|
| VECTOR® Red Alkaline Phosphatase Kit | Vector Laboratories | Cat#SK-5100 |
| LookOut® Mycoplasma PCR Detection Kit | SIGMA ALDRICH | Cat#MP0035 |
| Qubit™ RNA HS assay kit | Thermo Fisher Scientific | Cat#Q32855 |

**Experimental Models: Cell Lines**

| Cell Line                        | Source     | Identifier |
|----------------------------------|------------|------------|
| Mouse ESCs: E14tg2a               | ATCC       | Cat#CRL-1821™ |

**Software and Algorithms**

| Software                        | Source                        | Link |
|---------------------------------|-------------------------------|------|
| ImageJ                          | Schneider et al., 2012         | https://imagej.nih.gov/ij/ |
| GraphPad Prism, v7              | GraphPad                       | https://www.graphpad.com/scientific-software/prismv/ |
| FlowJo, v10.0.7r2               | FlowJo LLC                     | http://docs.flowjo.com |

**Other**

| Equipment                      | Source                       | Identifier |
|--------------------------------|------------------------------|------------|
| Nunclon™ Sphera™ 96U plate dishes | Thermo Fisher Scientific | Cat#174932 |
| Multiwell Tissue Culture Plate, 6 well | Corning                     | Cat#353046 |

(Continued on next page)
STEP-BY-STEP METHOD DETAILS

ESCs are heterogenous with regard to mitochondrial activity (Khoa et al., 2020; Schieke et al., 2008). However, a detailed protocol for determining the biological features of distinct mitochondrial activity is currently lacking. In this section, we list step-by-step procedures, timing as well as critical considerations that ensure success. Following this protocol would result in a successful isolation of quiescent ESCs with intact pluripotent features.

ESC Culture

lâ€“ Timing: 2 days

1. Mouse E14 ESC line is maintained on Geltrex-coated 6-well plates at 37°C and 5% CO2 in 2 mL of N2B27/2i/LIF culture media per well.
2. To keep the cells growing at relatively stable density, we usually seed 1–2 x 10^5 cells per well onto 6-well plates after each split. Medium change is performed daily. Cells are passaged every two days as follows:
   a. Pre-warm N2B27, N2B27/2i/LIF, Accutase and DPBS for 5 min at 37°C. All reagents are in 15 mL or 50 mL conical tubes.
   b. Gently aspirate the old medium and wash the cells once with DPBS.
   c. Incubate the cells with 400 µL of Accutase at 37°C for 4 min.
   d. Add 1.5 mL of N2B27 medium, gently pipette up and down for ten times and collect cells by centrifugation at 300 x g, RT for 3 min.
   e. Resuspend the cells in 1 mL of N2B27/2i/LIF media.
   f. Count the cell numbers by hemocytometer and place cells at a density of 1–2 x 10^5 cells per well of 6-well plate. 2 mL of N2B27/2i/LIF culture media per well are used throughout the culture.
   g. Return the cell plate to the incubator and gently shake the plate to distribute the cells evenly.

â CRITICAL: Cell density plays critical roles in maintaining ESC identity as well as the metabolic status. In our experiences, plating ESCs at a density of 1–2 x 10^5 cells per well of 6-well plate often results in 70%–80% confluence after 2 days in culture (Figure 1A). This can give rise to a consistent cell cycle profile (Figure 1B) as well as fraction of cells with low mitochondrial membrane potential, which are determinant factors in our protocol. Researchers can use our seeding density as a starting point to scale up or down according to their plate formats.

TMRM Staining of ESCs

lâ€“ Timing: 1 h

TMRM is a cell-permeable dye that can label functioning mitochondria. As such, cells with active mitochondrial membrane potential (ΔΨ_m) tend to accumulate more TMRM than those harboring lower ΔΨ_m. Here, we present a step-by-step procedure of TMRM staining at single-cell levels.
3. Preparation of single-cell suspension and reagents for staining.
   a. Inspect the cells under a microscope to confirm the cell density, and make sure the culture is not contaminated at the time of staining.
   b. Gently aspirate the old medium and wash the cells once with DPBS.
   c. Dissociate cells with 400 μL of Accutase at 37°C for 4 min. Tap the plate to facilitate the detachment of colonies.
   d. Cells are gently pipetted up and down ten times, resuspended in 37°C pre-warmed FACS running buffer and centrifuged at 300 x g, RT for 3 min.
   e. Resuspend the cells in 1 mL of 37°C pre-warmed staining buffer.
   f. Take out an aliquot of 100 μM TMRM, and leave at RT.

4. TMRM staining
   a. Count the cells by hemocytometer and adjust the cell numbers at a density of 1 x 10^6 cells/mL.
   b. Add TMRM at a final concentration of 25 nM. Gently pipette up and down for ten times to mix the cells.
   c. Incubate cells at 37°C and 5% CO2 for 15 min.
   d. Wash the cells twice in ice-cold FACS running buffer by centrifugation at 300 x g, 4°C for 3 min.
   e. Resuspend the cells in ice-cold FACS running buffer containing SYTOX™ Blue (1:1,000 dilution). Cells are filtered through 40 μm sterile cell strainer, left on ice, and protected from light by aluminum foil until FACS.

⚠️ CRITICAL: TMRM should be protected from lights. Each aliquot of TMRM is for a single use.

Note: to set up the gating strategy, two additional samples are needed: unstained cells and SYTOX™ Blue-stained cells. Since there is always a small subpopulation of dead cells in culture that can be used for setting up the gating (Figure 2), inducing cell death for a positive control is not necessary.

FACS-Based Isolation of Quiescent ESCs

⏱ Timing: 30 min to 1 h

We recently reported that ground-state ESCs with low ΔΨm (i.e., low TMRM) exhibit defined features of the quiescent state, such as reduced RNA content and extremely slow proliferation.
rate (Khoa et al., 2020). Here we provide a detailed gating strategy for capturing quiescent ESCs after TMRM staining.

Cell sorting based on the intensity of TMRM signals is performed using the MoFlo Astrios Cell Sorter (BECKMAN COULTER life Sciences). TMRM can be detected by the RFP filter set with absorbance peak at 548 nm and emission peak at 574 nm. Excitation and emission of SYTOX™ Blue are at 444 nm and 480 nm, respectively, which are detectable with the 405 nm violet laser light.

5. Gating strategy is shown in Figure 2.
6. Collect cells in 15 mL tubes containing FACS collection buffer.
7. After sorting, gently invert the collection tubes in order to resuspend cells attached on the tube’s wall.
8. Centrifuge the sorted cells at 300 \( \times \) g for 5 min.
9. Carefully remove the supernatant and resuspend the cells in 1 mL of N2B27/2i/LIF or EB differentiation media.

△ CRITICAL: To lessen potential cell death due to the time-consuming process of FACS sorting, the cell concentration needs to be optimized appropriately. We recommend quickly performing FACS within 1 h after TMRM staining and use SYTOX™ Blue dye for staining to exclude the dead cells.

Note: TMRM and SYTOX™ Blue are light sensitive. All samples and FACS collection tubes should be protected from light and kept on ice during sorting operation.

Validating Pluripotent Features of Quiescent ESCs: Measure Total RNA Amounts per Cell

△ Timing: variable

Diapaused embryos can resume their normal development in response to favorable environments (He et al., 2019; Renfree and Fenelon, 2017). To determine whether quiescent ESCs are capable
of responding to distinct environmental cues, we analyze their behaviors under self-renewal and differentiation conditions.

A canonical characteristic of quiescent stem cells is the drastic reduction in RNA content as a result of minimal metabolic activity, low DNA replication, and slow cell cycle progression.

10. Total amounts of RNAs in each cell are determined as previously described (Khoa et al., 2020). Equal cell numbers of top and bottom <5% of TMRM fluorescence intensity are sorted as above (Figure 2). We use the RNeasy mini kit (QIAGEN) to extract total RNAs. Extracted RNAs are stored at −80°C, and thawed on ice prior to further analyses. Quantifications of RNA amounts are performed using Qubit™ RNA HS assay kit. Data are normalized against the cell numbers (Figure 3).

Validating Pluripotent Features of Quiescent ESCs: Colony Formation

- Timing: 5 days

To examine the self-renewal capacity of low $\Delta \Psi_m$ cells (i.e., quiescent ESCs) as compared to high $\Delta \Psi_m$ cells, we seed sorted cells with distinct TMRM signals at clonal density for colony formation assay.

11. Seed 500 sorted cells per well of Geltrex-coated 6-well plates in 2 mL of N2B27/2i/LIF culture media.
12. Cells are cultured for 5 days in N2B27/2i/LIF media. Medium change starts 2 days after initial seeding and is subsequently performed every other day.

13. At day 5, the old media are discarded. Cells are washed once with DPBS containing Calcium Chloride and Magnesium Chloride (i.e., DPBS (+)) and fixed with 1 mL of 4% paraformaldehyde (PFA)/DPBS (+) at RT for 10 min. Fixed cells are then washed once with UltraPure™ DNase/RNase-Free distilled H2O and subjected for alkaline phosphatase (AP) staining in accordance with manufacturer’s instructions (Figure 4).

14. Images were captured by the color scanner (Epson) and the IX73 microscope system (Olympus).

⚠ CRITICAL: Medium change should be carefully implemented during the course of the experiment. Tilt the dish at a 45°C angle, wait 5 s, gently remove the old media and replenish with the fresh media. Because the colony is easy to detach from the dish, we recommend using the pipettetips to change the media.

Note: At the step 13, we recommend washing the cells with DPBS (+) to help avoid the detachment of the colonies.

Validating Pluripotent Features of Quiescent ESCs: Immunofluorescence for OCT3/4

© Timing: 2 days

OCT3/4 is a canonical factor that plays an indispensable role in maintenance of ESC pluripotency. To investigate the expression levels of OCT3/4 in high and low $\Delta \Psi_m$ cells, we perform immunofluorescence as follows:

15. Seed sorted high and low TMRM cells onto Geltrex-coated Lab-Tek II Chamber cover glass. Cells are grown for 24 h prior to OCT3/4 immunofluorescence analysis using the standard protocol.
   a. Fix cells in 4% PFA/DPBS (+) for 30 min at 4°C. Wash cells twice with DPBS (+).
   b. Permeabilize cells in 0.25% Triton X-100/DPBS (+) (PBST) for 30 min at 4°C.
   c. Block cells in blocking solution containing 10% Goat serum in DPBS (+) for 1 h at 4°C.
   d. Incubate cells with anti-OCT3/4 antibody (1:50) diluted in blocking solution for 14 h at 4°C.
   e. Wash cells with 0.25% PBST three times with gentle shaking for 5 min each, and incubate cells with Goat anti-Mouse IgG FITC-conjugated secondary antibody for 1 h at RT.

Figure 4. Colony Formation Assay
Left, AP staining in sorted high and low TMRM cells. Scale bar, 100 µm. Right, qualifications of AP-positive colonies from left. Data are shown as means ± SEM from three biological replicates. ***p < 0.001.
f. Wash cells with 0.25% PBST three times with gentle shaking for 5 min each and stain cells with DAPI diluted in 0.25% PBST for 10 min at RT.
g. Wash cells with DPBS (+) three times. Cells are ready for imaging (Figure 5).

16. Images are captured using the Leica SP5 upright 2-photon confocal microscope (Leica Microsystems). Relative fluorescence intensities are quantified in each cell using ImageJ (Figure 5).

Validating Pluripotent Features of Quiescent ESCs: TMRM and PECAM-1 Co-staining

© Timing: 2.5 h

The purpose of this experiment is to provide evidence that low TMRM cells (i.e., quiescent ESCs) in bulk culture are still pluripotent using cell-surface marker PECAM-1 staining.

To additionally characterize the pluripotent features of quiescent ESCs in bulk culture, cells are stained for platelet endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31). PECAM-1 protein is highly expressed in the inner cell mass of blastocysts and in ESCs (Robson et al., 2001; Rugg-Gunn et al., 2012). We develop a co-staining protocol for TMRM and PECAM-1 to determine the expression levels of PECAM-1 in distinct fractions of high and low TMRM cells.

17. Repeat from step 3 to step 4d.
18. Resuspend the cells in 5 mL of the cell staining buffer, centrifuge at 300 × g, 4°C for 5 min and discard the supernatant.
19. In 1.5 mL Eppendorf tube, add 1 μg of TruStain FoX™ in 100 μL staining buffer (1:50) for blocking reaction, gently mix and incubate for 10 min on ice.
20. Add 1 μg of PECAM-1 antibody (1:50) and incubate for 20 min on ice.
21. Wash cells twice with 1 mL of the cell staining buffer and spin at 300 × g, 4°C for 5 min.
22. Add 0.25 μg of APC/Fire™ 750 Goat anti-rat IgG diluted in 100 μL of the staining buffer (1:80) and incubate 20 min on ice in dark.
23. Wash cells twice with 1 mL of the cell staining buffer and spin at 300 × g, 4°C for 5 min.
24. Resuspend cells with 0.5 mL of the cell staining buffer containing SYTOX™ Blue (1:1,000) to exclude dead cells.
25. Samples are ready for flow cytometry (Figure 6).

△ CRITICAL: We do not recommend staining PECAM-1 after TMRM sorting, because the time-consuming FACS sorting per se coupled with PECAM-1 staining afterward would cause profound cell death as well as drastic changes in the metabolic state of the cells. Instead, using the protocol and gating strategy described above would allow for integrative analyses of the pluripotent marker and ΔΨm in bulk ESCs.
In addition to single stained samples, including IgG sample is important for compensation and elimination of unspecific signals.

Validating Pluripotent Features of Quiescent ESCs: Embryoid Body (EB) Differentiation

© Timing: 13 days

To explore whether quiescent ESCs (i.e., low TMRM cells) undergo normal differentiation in vitro, we perform EB formation using all three TMRM-sorted populations (see step 5 and Figure 2).

26. 2000 FACS-sorted cells of high, low and whole TMRM signals are seeded onto each well of the Nunclon™ Sphera™ 96U plate format consisting of 200 μL EB media.

27. EBs are cultured in suspension at 37°C and 5% CO₂. Half of medium is replaced every other day.
   a. EB formation is recorded at day 3 and 5 (Figure 7A), whereas beating EBs are counted at day 9 (Figure 7B).
   b. At day 5, EBs are transferred to gelatin-coated plates for additional 8-day differentiation (Figure 7C).

△ CRITICAL: For EB formation of FACS-sorted cells, we recommend using the Nunclon™ Sphera™ 96U plate format to reproducibly generate single EBs with uniform sizes.

EXPECTED OUTCOMES

We show here a straightforward protocol for in vitro derivation of quiescent ESCs. In our protocol, ESCs are cultured under the ground-state condition (i.e., 2i/LIF) with consistent density and cell cycle profiles (Figure 1). By following our cell culture conditions, one can not only obtain typical cell cycle profile of ground-state ESCs (Ter Huurne et al., 2017), but also robustly identify a fraction of cells that reside in G0 phase (<5%) (Figure 1).

Using our gating strategy, cells with distinct levels of ΔΨᵢ can be FACS-isolated and subsequently analyzed (Figure 2). We demonstrate that the low ΔΨᵢ cells exhibit features of quiescent state. They
display low RNA contents and reduced self-renewal in colony formation assay, yet maintain high expression levels of pluripotency markers such as AP, OTC3/4 and PECAM-1 (Figures 3, 4, 5, and 6). Importantly, these quiescent ESCs can undergo normal differentiation through three-dimensional EB formation in a suspension culture, similar to that of bulk ESCs (Figure 7). It is important to note that at day 3 of EB differentiation, the low $\Delta \Psi_m$ cells form EBs with smaller sizes than the whole and high $\Delta \Psi_m$ cells (Figure 7A). However, EB sizes become comparable in all levels of $\Delta \Psi_m$ cells at day 5 (Figure 7A).

**LIMITATIONS**

Cell culture condition is crucial for obtaining the reproducible data, yet affected by multiple factors such as splitting ratio, status of starting cell lines and reagents. A strict adherence to our cell culture protocol is highly recommended. It is important to note that we have not tested our protocol in ESCs cultured in serum/LIF medium, which have higher degree of heterogeneity than the ground-state ESCs (Chambers et al., 2007; Marks et al., 2012). We anticipate that our protocol can be applied to other pluripotent stem cell types (e.g., epiblast stem cells and human ESCs), which show distinct levels of metabolic and transcriptional heterogeneity (Khoa le et al., 2016; Messmer et al., 2019; Zhang et al., 2016; Zhou et al., 2012).

Our protocol enables isolation of a small population of cells (<5%) that has low $\Delta \Psi_m$. These cells display biological characteristics of quiescence. However, we have not yet determined whether the low $\Delta \Psi_m$ cells are equivalent to those in G0 phase (Figure 1B). To clarify this intriguing point, scientists may consider using knockin reporter cell lines that facilitate the simultaneous evaluation of those two parameters.

Finally, although our protocol reveals biological features of low $\Delta \Psi_m$ quiescent ESCs that may represent embryonic diapause in vivo, in-depth comparisons of transcriptome and epigenome between
low $\Delta \Psi_m$ quiescent ESCs and diapaused epiblasts are needed. In addition, advantages of low $\Delta \Psi_m$ quiescent ESCs with respect to directed differentiation as compared to self-renewing ESCs warrant further investigations.

**TROUBLESHOOTING**

**Problem 1**
In ESC culture step: cells exhibit signs of differentiation and flattened colony morphology (steps 1 and 2).

**Potential Solution**
Researchers should double check the expiration date of basal N2B27 medium and Geltrex. N2B27 medium should be used within one week. Suboptimal N2B27 may introduce unexpected biases in cell culture, including flattened colony morphology. It is worth paying attention to each component of N2B27 medium when a new lot is used. For preparation of ready-to-use Geltrex, researchers are highly recommended to follow our protocol (see “Geltrex preparation” step). Finally, overgrowth may lead to indiscernible signs of differentiation, which results from seeding inappropriate cell numbers. High cell density can lead to unhealthy colonies and spontaneous differentiation. Whenever those problems arise, the cell culture should be discarded and replaced with new cell stock.

**Problem 2**
There are no significant differences between the high and low $\Delta \Psi_m$ cells with regard to self-renewal capacity, total RNA amounts per cell and EB differentiation (steps 3–9).

**Potential Solution**
Researchers should pay attention to changes in the gating during the course of FACS sorting to ensure that cells of <5% of low TMRM intensity are sorted. This can be accomplished by keyboard-mediated manual adjustment. In our experiences, sorting cells of <5% of the low TMRM signals always leads to reproducible derivation of quiescent ESCs. We strongly recommend testing stabilization of the gating for at least 30 s before performing the actual FACS sorting. In addition, TMRM, when used at high concentrations, can result in unspecific signals by labeling other cellular compartments. Therefore, TMRM concentrations need to be optimized.

**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yali Dou ([yalidou@usc.edu](mailto:yalidou@usc.edu)).

**Materials Availability**
This study did not generate new unique reagents.

**Data and Code Availability**
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
L.T.P.K. designed and performed experiments and wrote the protocol. Y.D. supervised the overall study.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vanra, J., Jones, K., Grotewold, L., and Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. Nature 450, 1230–1234.

He, B., Zhang, H., Wang, J., Liu, M., Sun, Y., Guo, C., Lu, J., Wang, H., and Kong, S. (2019). Blastocyst activation engenders transcriptome reprogram affecting X-chromosome reactivation and inflammatory trigger of implantation. Proc. Natl. Acad. Sci. U S A 116, 16621–16630.

Khoa Le, T.P., Azami, T., Tsukiyama, T., Matsushita, J., Tsukiyama-Fujii, S., Takahashi, S., and Ema, M. (2016). Visualization of the epiblast and visceral endodermal cells using Fgf5-P2A-venus BAC transgenic mice and epiblast stem cells. PLoS One 11, e0159246.

Khoa, L.T.P., Tsan, Y.C., Mao, F., Kremer, D.M., Sajjakulnukit, P., Zhang, L., Zhou, B., Tong, X., Bhanu, N.V., Choudhary, C., et al. (2020). Histone acetyltransferase mof blocks acquisition of quiescence in ground-state ESCs through activating fatty acid oxidation. Cell Stem Cell 27, 441–458 e410.

Marks, H., Kalkan, T., Menafra, R., Denissov, S., Jones, K., Hofermeister, H., Nichols, J., Kranz, A., Stewart, A.F., Smith, A., et al. (2012). The transcriptional and epigenomic foundations of ground state pluripotency. Cell 149, 590–604.

Messmer, T., von Meyenn, F., Savino, A., Santos, F., Mohammed, H., Lun, A.T.L., Marioni, J.C., and Reik, W. (2019). Transcriptional heterogeneity in naïve and primed human pluripotent stem cells at single-cell resolution. Cell Rep. 26, 815–824.e4.

Renfree, M.B., and Fenelon, J.C. (2017). The enigma of embryonic diapause. Development 144, 3199–3210.

Robson, P., Stein, P., Zhou, B., Schultz, R.M., and Baldwin, H.S. (2001). Inner cell mass-specific expression of a cell adhesion molecule (PECAM-1/CD31) in the mouse blastocyst. Dev. Biol. 234, 317–329.

Rugg-Gunn, P.J., Cox, B.J., Lanner, F., Sharma, P., Ignatchenko, V., McDonald, A.C., Garner, J., Gramolini, A.O., Rossant, J., and Kislinger, T. (2012). Cell-surface proteomics identifies lineage-specific markers of embryo-derived stem cells. Dev. Cell 22, 887–901.

Schieke, S.M., Ma, M., Cao, L., McCoy, J.P., Jr., Liu, C., Hensel, N.F., Barrett, A.J., Boehm, M., and Finkel, T. (2008). Mitochondrial metabolism modulates differentiation and teratoma formation capacity in mouse embryonic stem cells. J. Biol. Chem. 283, 28506–28512.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9, 671–675.

Ter Huurne, M., Chappell, J., Dalton, S., and Stunnenberg, H.G. (2017). Distinct cell-cycle control in two different states of mouse pluripotency. Cell Stem Cell 21, 449–455.e4.

Zhang, H., Badur, M.G., Divakaruni, A.S., Parker, S.J., Jager, C., Hiller, K., Murphy, A.N., and Metallo, C.M. (2016). Distinct metabolic states can support self-renewal and lipogenesis in human pluripotent stem cells under different culture conditions. Cell Rep. 16, 1536–1547.

Zhou, W., Choi, M., Margineantu, D., Margaretha, L., Hesson, J., Cavanaugh, C., Blau, C.A., Horwitz, M.S., Hockenberg, D., Ware, C., et al. (2012). HIF1alpha induced switch from bivalent to exclusively glycolytic metabolic during ESC-to-EpiSC/ESC transition. EMBO J. 31, 2103–2116.