Video Article

# Alternative Cultures for Human Pluripotent Stem Cell Production, Maintenance, and Genetic Analysis

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## Abstract

Human pluripotent stem cells (hPSCs) hold great promise for regenerative medicine and biopharmaceutical applications. Currently, optimal culture and efficient expansion of large amounts of clinical-grade hPSCs are critical issues in hPSC-based therapies. Conventionally, hPSCs are propagated as colonies on both feeder and feeder-free culture systems. However, these methods have several major limitations, including low cell yields and generation of heterogeneously differentiated cells. To improve current hPSC culture methods, we have recently developed a new method, which is based on non-colony type monolayer (NCM) culture of dissociated single cells. Here, we present detailed NCM protocols based on the Rho-associated kinase (ROCK) inhibitor Y-27632. We also provide new information regarding NCM culture with different small molecules such as Y-39983 (ROCK I inhibitor), phenylbenzodioxane (ROCK II inhibitor), and thiazovivin (a novel ROCK inhibitor). We further extend our basic protocol to cultivate hPSCs on defined extracellular proteins such as the laminin isoform 521 (LN-521) without the use of ROCK inhibitors. Moreover, based on NCM, we have demonstrated efficient transfection or transduction of plasmid DNAs, lentiviral particles, and oligonucleotide-based microRNAs into hPSCs in order to genetically modify these cells for molecular analyses and drug discovery. The NCM-based methods overcome the major shortcomings of colony-type culture, and thus may be suitable for producing large amounts of homogeneous hPSCs for future clinical therapies, stem cell research, and drug discovery.

## Video Link

The video component of this article can be found at https://www.jove.com/video/51519/

## Introduction

The capacity of hPSCs to differentiate toward multilineage adult tissues has opened new avenues to treating patients who suffer from severe diseases that involve cardiovascular, hepatic, pancreatic, and neurological systems[1-4]. Various cell types derived from hPSCs would also provide robust cellular platforms for disease modeling, genetic engineering, drug screening, and toxicological testing[1,4]. The key issue that ensures their future clinical and pharmacological applications is the generation of large numbers of clinical-grade hPSCs through *in vitro* cell culture. However, current culture systems are either insufficient or inherently variable, involving various feeder and feeder-free cultures of hPSCs as colonies[5,6].

Colony-type growth of hPSCs shares many structural features of the inner cell mass (ICM) of early mammalian embryos. The ICM is prone to differentiate into the three germ-layers in a multicellular environment because of the existence of heterogeneous signaling gradients. Thus, the acquisition of heterogeneity in early embryonic development is considered as a required process for differentiation, but an unwanted feature of hPSC culture. The heterogeneity in hPSC culture is often induced by excessive apoptotic signals and spontaneous differentiation due to suboptimal growth conditions. Thus, in colony-type culture, the heterogeneous cells are often observed in the periphery of the colonies[7,8]. It has been also shown that the cells in human embryonic stem cell (hESC) colonies exhibit differential responses to signaling molecules such as BMP-4 [9]. Moreover, colony culture methods produce low cell yields as well as very low cell recovery rates from cryopreservation due to uncontrollable growth rates and apoptotic signaling pathways[6,9]. In recent years, various suspension cultures have been developed for culturing hPSCs, particularly for expansion of large amounts of hPSCs in feeder- and matrix-free conditions[6,10-13]. Obviously, different culture systems have their own advantages and disadvantages. In general, the heterogeneous nature of hPSCs represents one of the major drawbacks in colony-type and aggregated culture methods, which are suboptimal for delivering DNA and RNA materials into hPSCs for genetic engineering[6].

Clearly, there is an imperative need to develop new systems that circumvent some shortcomings of current culture methods. The discoveries of small molecule inhibitors (such as the ROCK inhibitor Y-27632 and JAK inhibitor 1) that improve single-cell survival pave the way for dissociated-hPSC culture[14,15]. With the use of these small molecules, we have recently developed a culture method based on non-colony type (NCM) growth of dissociated-hPSCs[9]. This novel culture method combines both single-cell passaging and high-density plating methods, allowing us to produce large amounts of homogeneous hPSCs under consistent growth cycles without major chromosomal abnormalities[9]. Alternatively, NCM culture might be implemented with different small molecules and defined matrices (such as laminins) in order to optimize the culture method for wide applications. Here, we present several detailed protocols based on NCM culture and delineate detailed procedures for genetic engineering. To

 

demonstrate the versatility of NCM protocols, we also tested NCM culture with diverse ROCK inhibitors and with the single laminin isoform 521 (*i.e.*, LN-521).

## Protocol

Single-cell based non-colony type monolayer (NCM) culture of hPSCs.

# 1. Preparations

1. Make 500 ml of medium for culture of mouse embryonic fibroblasts (MEFs): DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM non-essential amino acids (NEAA).
2. Isolate mouse embryonic fibroblasts (MEFs) cells derived from the CF1 strain following a routine protocol[16] and culture MEFs on 0.1% gelatin-coated 6-well cell culture plate in DMEM medium. Alternatively, purchase MEF stocks at passage 3 from commercial resources.
3. Prepare Matrigel plates.
    1. Dilute 5 ml of hESC-qualified Matrigel stock with 5 ml of DMEM/F12 medium (chilled at ~4 °C) and store as 50% aliquots in a -20 °C freezer. Thaw the frozen Matrigel stock in a refrigerator (~4 °C) O/N and further dilute the Matrigel in cold DMEM/F12 medium (to give rise to a 2.5% working concentration).
    2. Coat 6-well cell culture plates with 1.5 ml of 2.5% Matrigel per well, store the coated plates in refrigerator O/N (Note: use the Matrigel-coated plate within 2 weeks).
    3. Remove the Matrigel plate from the refrigerator, allow the plate to warm at RT in the cell culture hood for 10 to 30 min (Note: do not warm the plate in a cell culture incubator), and aspirate DMEM medium prior to plating hPSCs.
4. Make 500 ml hESC medium: 80% DMEM/F12 medium, 20% KSR, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml of FGF-2.
5. Prepare 10 ml of 2X hPSC freezing medium: 60% FBS, 20% DMSO, and 20 μM Y-27632 in mTeSR1 medium, sterilize by filtration, and use the medium within 1 week.

# 2. Protocol 1 (Basic): Grow hPSC Colonies on Feeders

1. Use MEF passage numbers at 5 or 6 (designated as p5 and p6) for hPSC culture in order to get consistent results. Mitotically inactivate MEFs by treating the cells with 10 μg/ml mitomycin C for 3 hr at 37 °C, wash the cells 3x with 1X Dulbecco's Phosphate-Buffered Saline (D-PBS), and then dissociate MEFs with 0.05% Trypsin in 0.53 mM EDTA. Alternatively, irradiate MEFs at a dose of 8,000 rads with an X-ray irradiator.
2. Count cell numbers using the Trypan Blue stain exclusion method under microscope. Alternatively, use an automatic cell counter.
3. Plate irradiated MEFs on 6-well polystyrene plates coated with 0.1% gelatin at a density of 1.88 x $10^5$ cells per well (*i.e.*, 1.96 x $10^4$ cells/$cm^2$). Incubate the cells at 37 °C and 5% $CO_2$ for 24 hr.
4. Remove the MEF culture medium by aspiration with a Pasteur pipette (Note: no wash steps needed at this time).
5. Triturate hPSC colonies from previous culture into small clumps, examine the clumps under microscope to ensure their sizes ranging from 50 to 100 μm in diameters, and plate hPSC clumps on the top of MEF feeder layers in one well containing 2 ml of hPSC medium.
6. Change hESC medium daily for 3 to 5 days, record colony growth by photograph, mark any morphologically altered or differentiated colonies (~5%), and manually remove the marked colonies by gently aspiration with a Pasteur pipette.
7. Rinse the remaining colonies on MEFs twice (2 min each with D-PBS), and incubate the colonies with 2 ml of 1 mg/ml collagenase IV in hPSC medium for 10 to 30 min), and examine the detachment of hPSC colonies from the MEF-coated surface (Note: use a scraper to help the detachment process only if the colonies are tightly attached to the plate).
8. Add 5 ml of hPSC medium to each well to minimize enzymatic reactions, transfer colonies to a 15 ml tube, allow hPSC colonies to sediment for 3 to 5 min at RT, and ensure the sedimentation of colonies by direct visualization of the cells at the bottom of the tube (Note: do not centrifuge the tube at this step).
9. Remove the supernatants containing residual MEFs and dissociated single cells, resuspend the colonies with 5 ml of hESC medium, and repeat the sedimentation step twice.
10. Remove the medium, triturate hPSC colonies into small clumps, store in 1X hPSC freezing medium (or CryoStore CS10 freezing medium) for cryopreservation (1 confluent well per frozen vial) or plate on a 6-well plate for cell passaging.

# 3. Protocol 2: Convert hPSC Colonies from Feeders to NCM

1. Rinse hPSC pellets in D-PBS once, incubate the pellets with 1 ml of 1X Accutase for 10 min, and examine the enzymatic reaction under a microscope to ensure single-cell dissociation.
2. Terminate the enzymatic reactions by gently resuspending the cells in 5 ml of mTeSR1 medium containing 10 μM Y-27632 followed by centrifugation at 200 x g at RT for 5 min (Note, do not use excessive centrifugation forces which cause cell damage).
3. Add 5 ml of mTeSR1 medium to the pellet, resuspend the pellet as single cells, and filter dissociated cells through a 40 μm cell strainer (to remove any residual cell aggregates).
4. Seed 1.3-2 x $10^6$ hPSCs into one well (1.4-2.1 x $10^5$ cells/$cm^2$) of a 6-well plate coated with 2.5% hESC-qualified Matrigel, add mTeSR1 medium up to 2.5 ml, and include 10 μM Y-27632 in the medium (to facilitate the initial 24 hr single-cell plating). Alternatively, utilize the following small molecules to replace 10 μM Y-27632 for enhancing single-cell plating: 1 μM Y-39983 (ROCK I inhibitor), 1 μM phenylbenzodioxane (ROCK II inhibitor), 1 μM thiazovivin (a novel ROCK inhibitor), and 2 μM JAK inhibitor 1.
5. Replace the medium with drug-free mTeSR1 medium on the next day (within 24 hr), dissociate cells from one extra well, and count cell number to determine single-cell plating efficiency (Note: approximately, 50 to 90% of single-cell plating efficiency that can be achieved at this stage).

6. Allow the cells to grow as a single-cell-formed monolayer for a few days with 3 ml of mTeSR1 medium. Change medium daily.
7. Empirically determine the schedule for passaging adapted NCM depending on the cell density. Passage when cell growth reaches confluence at day 3 or day 4, or at the 4 hr time point after the disappearance of cell-to-cell borders. Dissociate the cells in 1 ml of Accutase, use a 1 to 3 splitting ratio (*i.e.*, 1 confluent well of cells plated in 3 wells of 6-well plate) for cell passaging, and stabilize adapted NCM by 5 passages.
8. Cell freezing
    1. Utilize one confluent well (~ 5-6 x$10^6$ cells) for one frozen vial: dissociate cells from one confluent well with 1 ml of Accutase for 10 min.
    2. Dilute with 5 ml of mTeSR1 medium and centrifuge cells as described above.
    3. Resuspend the pellet in 500 µl of mTeSR1 medium and then slowly add 500 µl of 2X hPSC freezing medium (containing 20 µM Y-27632) in a cryopreservation vial. Alternatively, resuspend the cells in 1X hPSC freezing medium containing 2 µM JAK inhibitor 1 or 1X CryoStor CS10 medium in the presence of either 10 µM Y-27632 or 2 µM JAK inhibitor 1.
    4. Place frozen vials into an ice-chilled cryocontainer (bottom-filled with isopranol) and transfer the cryocontainer to a -80 °C freezer immediately.
    5. Transfer cells from the -80 °C freezer to a liquid nitrogen tank (on the next day) for long-term cryopreservation.
9. Cell thawing
    1. Utilize one cryopreservation vial for plating 1 well of a 6-well plate.
    2. Pre-warm mTeSR1 medium in a 37 °C water bath for 10 min, thaw cells in the same water bath for 2 min, drop-wise add cells to 5 ml of pre-warmed mTeSR1 medium containing 10 µM Y-27632, and centrifuge as described above.
    3. Gently resuspend the cell pellets with 2 ml of mTeSR1 medium containing 10 µM Y-27632 and slowly transfer cells to a well pre-coated with Matrigel (Note: avoid producing air bubbles).
    4. Change medium daily and expect hPSC growth to reach confluence at day 3 or 4.

## 4. Protocol 3: Convert hPSC Colonies on Matrigel to NCM Culture

1. Remove a Matrigel-coated plate from refrigerator, place the plate in the tissue culture hood for 10 to 30 min, remove the medium from each well of the plate, and add 2 ml of pre-warmed mTeSR1 medium to each well.
2. Transfer triturated hPSC clumps from the feeder culture (Step 2.10 of the basic protocol) to the above Matrigel plate. Add mTeSR1 medium up to 2.5 ml final volume in each well.
3. Change medium daily for 3 to 4 days until colonies reach 80 to 90% confluence.
4. For cell passaging: rinse the colonies with D-PBS twice, treat the cells with 1 ml of 2 mg/ml dispase at 37 °C for 15 to 20 min, and sediment and passage cells as clumps.
5. For NCM culture: repeat Steps 3.1 to 3.9 described in Protocol 2.

## 5. Protocol 4: NCM Culture of hPSCs on LN-521

1. Thaw the recombinant LN-521 solution at 4 °C before the day of use and make the laminin coating solution (LCS) by diluting the thawed laminin with 1X D-PBS (containing $Ca^{2+}$/$Mg^{2+}$) to a final concentration of 10 µg/ml.
2. Add 1 ml of the LCS to one well in 6-well plate (note: avoid dry-out during the coating process).
3. Seal the coated plate with Parafilm to prevent evaporation and store the plate in refrigerator (4 °C) O/N (note: use the plate within 1 week).
4. Gently remove the LCS with a Pasteur pipette without touching the coated surface and add 2 ml of mTeSR1 medium to one well.
5. Warm all culture solutions (including D-PBS) in a 37 °C water bath for 25 min.
6. Convert hPSC colonies to NCM
    1. Dissociate cell aggregates to single cells with Accutase as described in Protocol 2.
    2. Seed 1.3-2 x $10^6$ hPSCs into one LN-521-coated well (1.4-2.1 x $10^5$ cells/$cm^2$) without the presence of ROCK inhibitors.
    3. Change medium daily for 3 to 4 days.
    4. Dissociate the cells in 1 ml of Accutase at day 3 or 4 for the next cell passaging using a 1 to 3 splitting ratio.

## 6. Protocol 5: NCM Culture for Plasmid DNA Transfection

1. Adapt hPSC colonies to NCM culture as described in Protocols 2 to 4.
2. Plate dissociated hPSCs in 12-well plate with a cell density of 7.5 x $10^5$ cells/well in mTeSR1 medium in the presence of 10 µM Y-27632.
3. Replace mTeSR1 medium with drug-free mTeSR1 medium between 4-8 hr after plating the cells.
4. For each transfection, dilute 2.5 µg of expression plasmids (*e.g.*, pmaxGFP) and 5 µl of Lipofectamine 2000 in separate Eppendorf tubes in 125 µl each of Opti-MEM Reduced Serum Medium.
5. After 5 min, mix the diluted reagents and incubate for 20 min at RT (to form transfection complexes).
6. Add the 250 µl transfection complexes to each well containing hPSCs in 1 ml mTeSR1 medium and incubate the cells at 37 °C in a $CO_2$ incubator for 24 hr.
7. Examine the transfection efficiency under a fluorescence microscope and photograph randomly for calculation of the actual transfection efficiency.

## 7. Protocol 6: NCM Culture for Transfection of MicroRNAs

1. Dissociate semi-confluent hPSCs at day 2 under NCM conditions by adding 1 ml of Accutase per well in 6-well plate.
2. Add 10 ml of mTeSR1 medium to dilute enzymatic reactions and centrifuge at 200 x g for 5 min.
3. Resuspend the cell pellet with mTeSR1 medium, seed the cells at a density of 7.5 x $10^5$ cells per well in a 12-well plate in mTeSR1 medium containing 10 µM Y-27632, and let the cells attach for 4 hr.

4. Replace the medium with Y27632-free mTeSR1 medium.
5. Use commercially available microRNAs (*e.g.*, a non-targeting miRIDIAN miRNA Transfection Control labeled with Dy547). Titrate concentrations ranging from 0-160 nM using the provided reagents and follow the manufacturers' instructions.
6. Optimize transfection efficiency for each concentration in hPSC lines by imaging the Dy547 fluorescence of the live cells under a microscope at 24 hr after the transfection.
7. Calculate the transfection efficiencies based on the percentage of Dy547 positive cells over all imaged cells.

## 8. Protocol 7: NCM Culture for Transduction of Lentiviral Vector

1. Repeat steps 7.1 to 7.4 of Protocol 6.
2. Calculate the amounts of viral particles to be used for transduction: Use the following formula: TU = (MOI x CN) / VT, whereas TU denotes the total numbers of transforming units, MOI equals the desired multiplicity of infection in the well (MOI or TU/cell), CN specifies the number of cells in each well, and VT indicates stock viral titers (TU/ml). For example, given that stock viral titers for SMART-shRNA vector equals 1 x $10^9$ TU/ml (transforming units per ml), 7.5 x $10^5$ cells per well at the time of transduction, and an expected MOI of 20: use 15 µl of the stock viral particles for each well (note: this condition is recommended for transient lentiviral induction).
3. Pre-warm 300 µl of mTeSR1 medium with 10 mg/ml of polybrene for 30 min.
4. Add 15 µl of stock viral particles into the pre-warmed mTeSR1 medium containing polybrene and gently mix the solution.
5. Replace the cell culture medium with 300 µl of prewarmed medium containing the viral particles.
6. After 4 hr incubation, examine turboGFP fluorescence (a maker for shRNA expression) to determine the transduction efficiency.
7. Add 300 µl of additional mTeSR1 medium to the transducing well when the cells begin to express turboGFP.
8. After 12-16 hr incubation, reexamine turbo-GFP expression.
9. Perform desired follow-up experiments with these transduced cells within 72 hr.

## Representative Results

**A general schema of NCM culture**

**Figure 1** represents a typical NCM culture schema showing the dynamic changes of hPSCs after high-density single-cell plating in the presence of the ROCK inhibitor Y-27632. These morphological changes include intercellular connections after plating, cellular clusters formation, and exponential cell growth followed by cell condensation (**Figure 1A**). A representative experiment indicates WA01 (H1) hESCs, plated as single-cells at a density of 1.9 x $10^5$ cells/$cm^2$ in the presence of 10 µM Y-27632 at day 1 (**Figure 1B**, left panel), further propagated without the formation of colonies (**Figure 1B**, middle panel) at day 2, and condensed as a homogeneous monolayer that is suitable for desired experiments or for cell passaging at day 3 (**Figure 1B**, right panel).

**Various ROCK inhibitors support NCM culture**

A 96-well plate assay was used for proof-of-concept of high-throughput drug screening. It was also designed to optimize the use of various ROCK inhibitors to support NCM culture. Approximately, 31,000 dissociated SCU-i10 cells, human induced pluripotent cells (hiPSCs)[17], were plated on one Matrigel-coated well in the presence of different concentrations of ROCK inhibitors. After 24 hr, the cells were subjected to the CCK-8 based survival assay to determine cell survival under these conditions. We have previously shown that the NCM method requires the use of the ROCK inhibitor, Y-27632, at 10 µM to enhance single-cell plating. In this report, we confirmed that 10 µM Y-27632 significantly increase 24 hr single-cell plating efficiency of hiPSCs ($P < 0.05$) (**Figure 2**). We also found that Y-39983 (ROCK I inhibitor), phenylbenzodioxane (ROCK II inhibitor), and thiazovivin (a novel ROCK inhibitor) significantly modulate single-cell plating efficiency and promote NCM growth at 1 µM when compared with their controls ($P < 0.05$) (**Figure 2**). Moreover, the effects of the three ROCK inhibitors (at 1 µM) on single-cell plating efficiency were comparable to that of Y-27632 at 10 µM ($P > 0.05$) (**Figure 2**). Notably, the ROCK I inhibitor (at 5 µM) appears to show pronounced cytotoxicity compared with the drug at 1 µM ($P < 0.05$), implicating a more specific interaction than other molecules. Thus, various ROCK inhibitors may be used for supporting NCM culture in the future. However, a complete characterization of both hESCs and hiPSCs under NCM with these new inhibitors would be required for future use.

**LN-521 supports NCM culture without the use of ROCK inhibitors**

To determine the role of a specific laminin isoform in supporting hESC growth, we cultured SCU-i30 hiPSCs on LN-521-coated plates in the xeno-free medium TeSR2. Interestingly, LN-521 alone, without the presence of ROCK inhibitors, supports single-cell plating and subsequent NCM growth for 15 passages under this condition (**Figure 3**). Immunostaining of SCU-i30 cells with an anti-NANOG polyclonal antibody indicated that the cells under this condition had high NANOG expression in the nuclei (**Figure 3A**). Flow cytometric analysis showed that the hESC marker expression profile was similar to the cells grown as NCM using the ROCK inhibitor Y-27632 (**Figure 3B**).

**High efficiency of microRNA delivery without the use of lentiviral particles**

Transfection with Dy547-labeled oligonucleotide microRNAs was carried out in WA01 (H1) cells under NCM conditions. WA01 hESCs were grown as NCM on 2.5% Matrigel in mTeSR1 for 2 (**Figure 4A**) and 18 (**Figure 4B**) passages respectively. These cells showed high transfection efficiency 24 hr post-transfection (**Figures 4A** and **4B**). Generally, we can obtain transfection efficiency up to 91% in hESCs at 24 hr after the transfection.

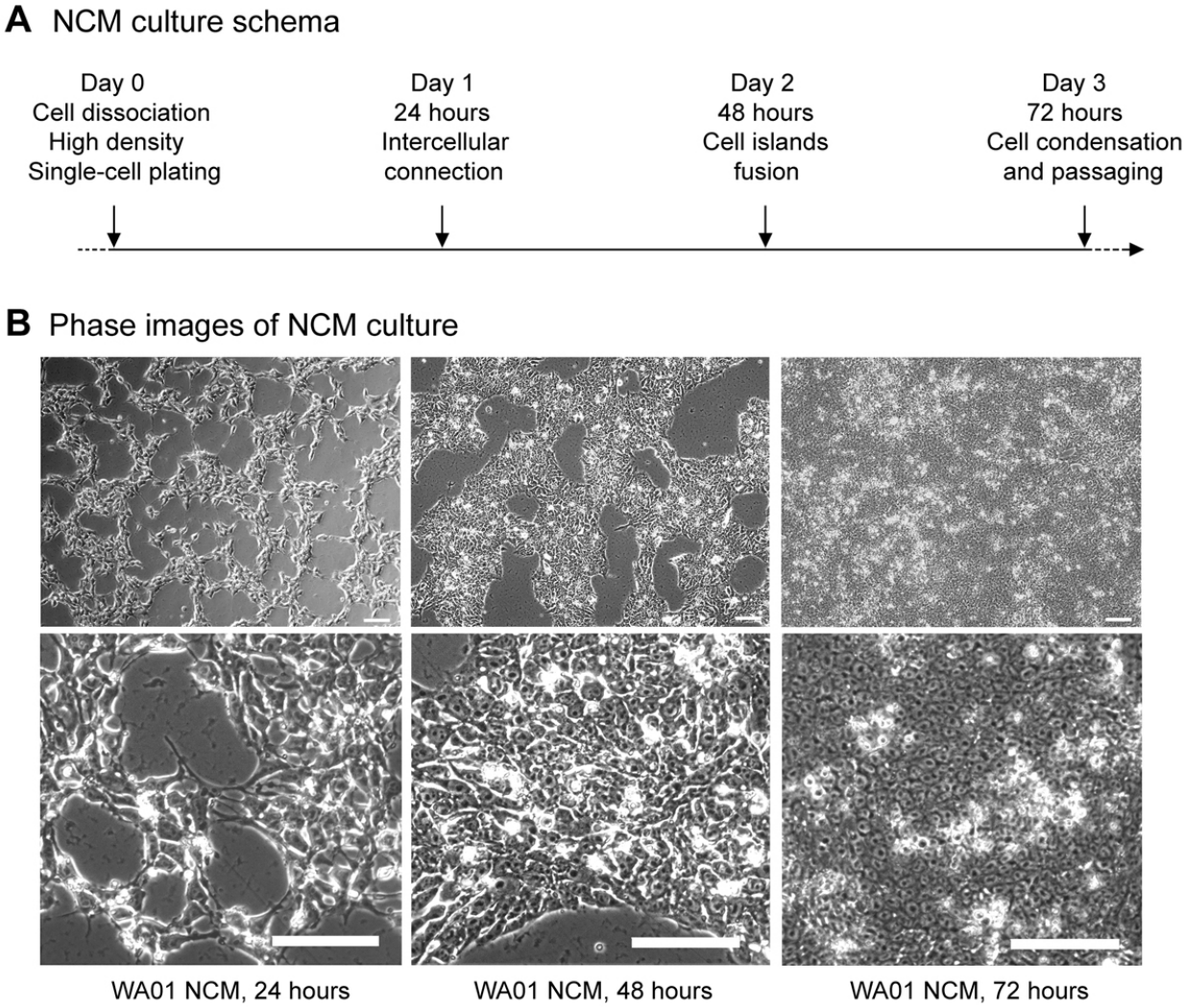


**Figure 1. (A)** Schema of NCM culture. The line graph delineates the dynamic changes of multicellular association in a typical 3-day culture under NCM conditions. **(B)** Representative phase images of WA01 (H1) hESCs propagated under an NCM condition on 2.5% Matrigel in mTeSR1 medium for 16 passages (designated as WA01, mcp16). The lower panel is the enlarged view of the upper panel. Scale bars indicate 100 μm. Please click here to view a larger version of this figure.

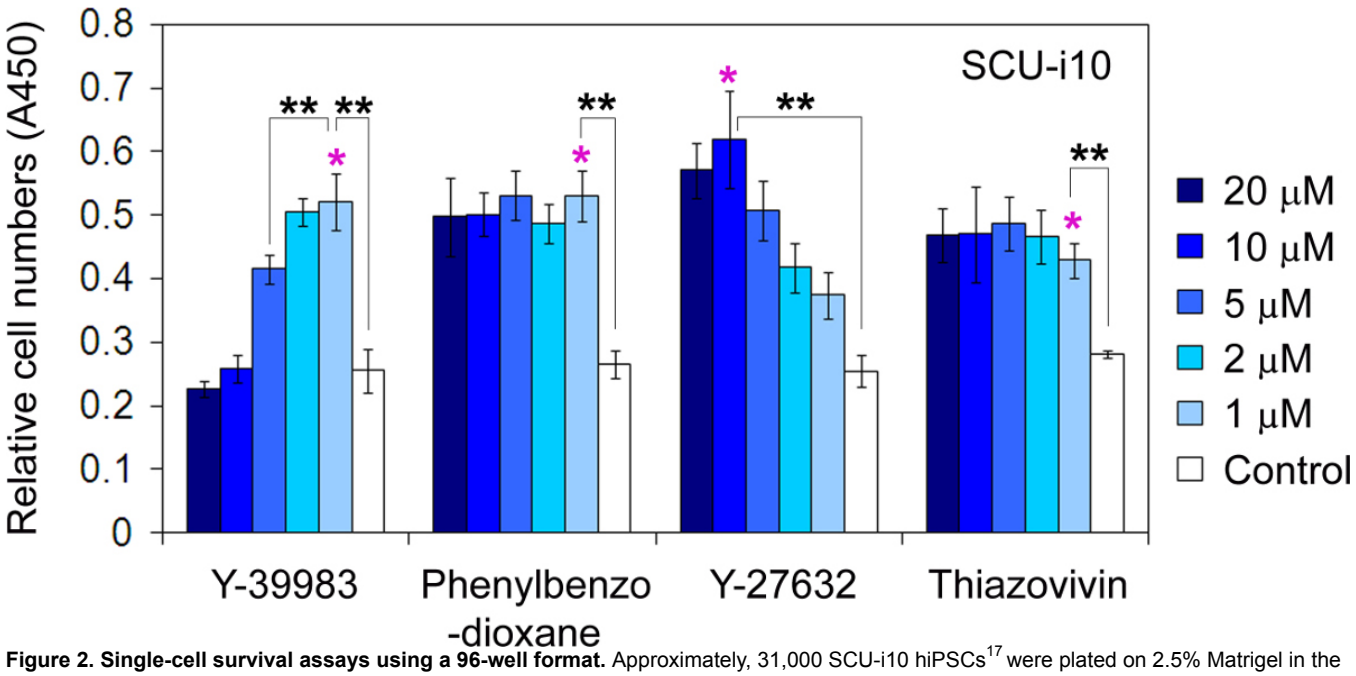


**Figure 2. Single-cell survival assays using a 96-well format.** Approximately, 31,000 SCU-i10 hiPSCs[17] were plated on 2.5% Matrigel in the presence of various small molecule inhibitors related to Rho-associated kinase (ROCK) pathways at indicated concentrations. After 24 hr, the cells were subjected to the CCK-8 survival assay by measuring the absorbance at 450 nm (A450). The student t-test was used to determine whether the differences in single-cell plating efficiency between various ROCK inhibitors are of statistical significance. The singular asterisk sign (*) indicates no significant differences between the inhibitors ($P > 0.05$), whereas the double asterisk signs (**) denotes the observed difference is of statistical significance ($P < 0.05$). Columns in the histograms designate the mean values of the quadruplicate determinants and bars represent standard deviations.

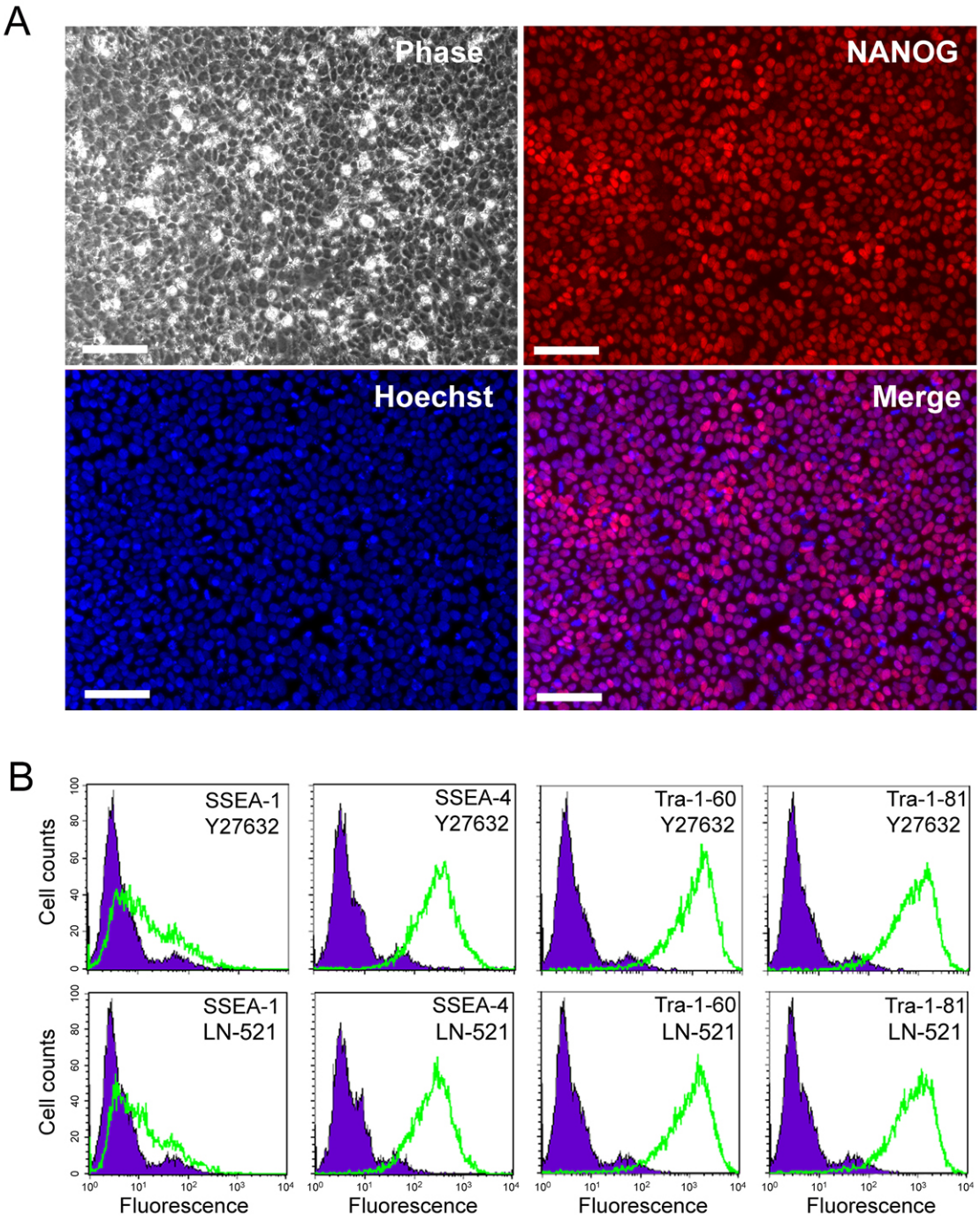


**Figure 3. NCM culture of hiPSCs on laminin-521 (LN-521) without the presence of ROCK inhibitors.** The SCU-i30 hiPSC line was established at the NIH Stem Cell Unit. SCU-i30 cells were grown on LN-521-coated 6-well plates in the xeno-free medium TeSR2 for 15 passages without the use of small molecule inhibitors such as ROCK inhibitors. **(A)** Immunostaining of SCU-i30 cells with an anti-NANOG polyclonal antibody and counterstained with Hoechst 33342 (Hoechst). Notably, some cells that have a negative NANOG staining are the cells under mitosis. **(B)** Flow cytometric analysis of hPSC marker expression in SCU-i30 cells grown as NCM on 2.5% Matrigel with the use of 10 μM Y-27632 (Upper panel) or NCM on LN-521 without Y-27632 (Lower panel). The procedures for both immunostaining and flow cytometric analysis were described previously[5]. Scale bars depict 100 μm. Please click here to view a larger version of this figure.

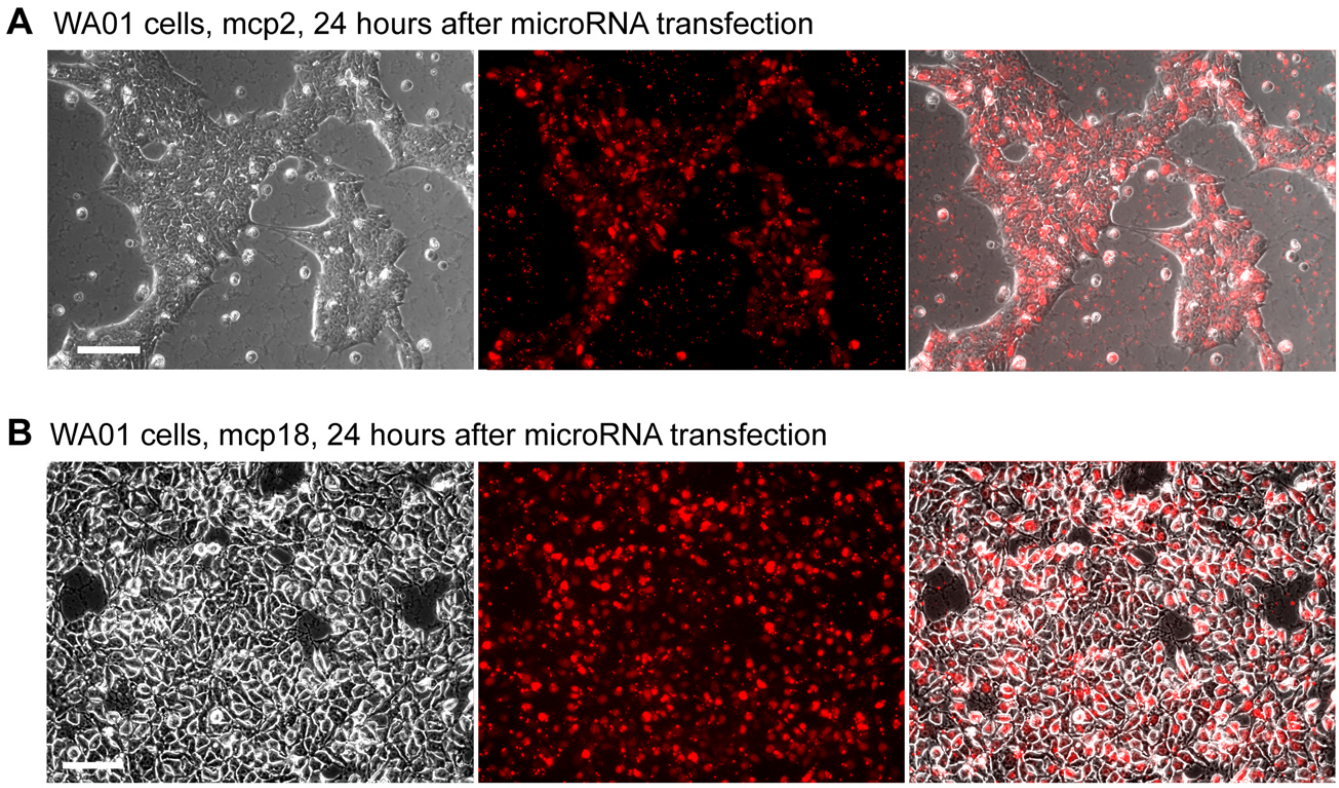


**Figure 4. NCM culture of hPSCs for microRNA transfection.** WA01 hESCs were grown as NCM on 2.5% Matrigel in mTeSR1 for 2 **(A)** and 18 **(B)** passages, respectively. Representative phase and fluorescence images of WA01 cells transfected with Dy547-labeled control microRNAs for monitoring transfection efficiency at 24 hr after the transfection. Scale bars represent 100 µm. Please click here to view a larger version of this figure.

## Discussion

There are two major ways to culture hPSCs *in vitro*: conventional colony-type culture (of cells on feeders or extracellular matrices) and suspension culture of hPSCs as aggregates without feeders[6]. The limitations of both colony-type and suspension culture methods include accumulated heterogeneity and inheritable epigenetic changes. NCM culture, based on both single-cell passaging and high-density cell plating, represents a new culture method for hPSC growth[6,18]. Although various single-cell passaging methods have been documented in the literature, but none of them are used for routine propagation. For example, Wu and colleagues employed a single-cell passaging method to study the effects of various small molecules cocktails on hPSC growth[19]. However, their final culture products are colonies. So, low-density-based single-cell passaging methods still belong to colony-type culture. With regard to NCM culture, the high-density plating transforms this culture to a new method, since the final cell product is a homogenous monolayer. Recently, Dvorak and colleagues used similar method to comprehensively analyze hPSC properties under this growth condition[18]. Their results also support our major conclusions. It is clear now that NCM culture is an emerging method that possesses several new properties and can be further modified for many potential applications in pluripotent stem cell biology.

### Basic properties of hPSCs from NCM culture

It is conceivable that hPSCs under NCM culture share many characteristics with the same types of cells grown as colonies[9,18]. The expression levels of hESC markers in hPSCs, which include Oct-4, NANOG, SSEA-3/4, Tra-1-60, Tra-1-81, and SSEA-1, are similar in both NCM and colony-type culture. NCM-adapted cells also retain similar global mRNA expression patterns to hPSC colonies grown on MEF feeder layers[9]. Clearly, NCM-adapted cells sustain the pluripotent state as determined by teratoma assay[9,18]. However, unlike hPSC colonies, cells under NCM conditions have a predictable growth rate that is characterized by consistent growth curves, cell cycles, and cell numbers[9]. Due to the high-density single-cell plating, hPSCs under NCM conditions exhibit exponential growth between days 2 and 4 (**Figure 1B**), thereby increasing cell number by 4-fold compared with hPSC colonies grown on MEFs. Prolonged culture does not increase cell production, but rather increases apoptotic and differentiation stress[9]. NCM also enables optimal cryopreservation, thus allowing rapid cell recovery upon plating thawed cells (Protocol 1). Moreover, NCM facilitates the adaptation of hPSC culture to various xeno-free protocols[9]. In general, NCM supports the growth of hPSCs, maintains the pluripotent state, and sustains the potential of hPSCs to differentiate into adult tissues of the three germ layers.

### Chromosomal stability in hPSCs under NCM

In terms of chromosomal stability, there is no direct comparison between the cells from different culture methods. The majority of hPSCs under our NCM culture conditions have normal karyotypes and gene copy numbers as determined by G-banding, array-based comparative genomic hybridization (aCGH), and fluorescence *in situ* hybridization (FISH)[9]. Two lines (~13%) showed abnormal karyotypes, in which one line (*i.e.*, WA09) exhibited elevated polyploidy and another one (ES01) had 14% of cells with trisomy 20 [9]. It is unclear whether these abnormal karyotypes

were derived from the selection of preexisting mutated cells prior to NCM culture or induced during NCM adaption. It is important that the starting hPSC colonies or subset of colonies used for NCM culture should be well-characterized in terms of their homogeneity and chromosomal stability at the time for NCM adaptation. Notably, the rate of abnormal karyotypes under NCM conditions is much lower than that reported in a recent cohort analysis, in which the authors reveal 34% abnormal karyotypes under predominant colony-type culture conditions[20]. Hence, our study indicates that we can grow dissociated single-cells and maintain their chromosomal stability under NCM conditions. Nevertheless, we also need to use more sensitive methods to examine chromosomal abnormalities of hPSCs in the coming years. Particularly, we need to scan chromosomes 1, 12, 17, and 20 using higher resolution probes (*i.e.*, < 50 kb), as some minor lesions (*e.g.*, the 20q11.21 amplicon) at these frequently altered chromosomes cannot be detected by conventional karyotyping and FISH[20-22]. Furthermore, the development of diverse NCM protocols would enable us to identify robust and safe methods for future applications.

**NCM culture under diverse modifications**

The use of the ROCK inhibitor, Y-27632, has opened the door for single-cell based assays. The availability of diverse ROCK inhibitors would provide additional choices for NCM-based expansion and cryopreservation of hPSCs (**Figure 2**). In theory, any small molecule, that can significantly increase single-cell plating efficiency, can be used to facilitate NCM culture. JAK inhibitor 1, which functions differently from those ROCK inhibitors, represents such an example[9]. The use of single molecules or combinatorial approaches may provide us optimal cell growth, cellular assays, and cryopreservation of hPSCs. However, alternative NCM culture of hPSCs on defined extracellular matrix proteins, such as the laminin isoform LN-521, normally expressed in hESCs[23-25], may eliminate the interference of small molecules (**Figure 3**). LN-521 might enhance dissociated-hPSC survival and sustains pluripotency through the activation of the α6β1-PI3K/AKT pathway[24]. The simplicity of passaging hPSCs with LN-521 reduces the heterogeneity of the cells, compatible with various feeder-free and xeno-free cell culture systems using completely defined medium (Protocol 4). It also provides an additional module for high-throughput assays without the influence of small molecules. Although the iPSCs under NCM culture on LN-521 retained the expression of a panel of hPSC markers, further characterization of these cells using teratoma assay and embryoid body-mediated multilineage differentiation may be necessary to confirm the pluripotent states in these cells. Of note, hiPSCs cultured on the laminin isoform LN-521 are reportedly cytogenetically stable (http://biolamina.com/). However, we also need to apply higher-resolution and sensitive methods (as discussed above) to examine the chromosomal stability in these cells.

**Versatility of NCM culture for genetic engineering**

NCM-based methods represent a simple, robust, and economical system that may be particularly useful for genetic manipulation of hPSCs (Protocols 5-7). It is known that hESC colonies are difficult to transfect or transduce, with great variability in transfection/transduction efficiencies among different laboratories[26-28]. For example, transfection efficiency in hESCs ranges from 3 to 35% under colony culture conditions[26]. Lentiviral transduction signals in BG01 cells under colony conditions were found to be extremely low[9]. However, the transfection efficiency mediated by NCM was greater than 75%[9] and modification of transduction methods can increase lentivirus-mediated transduction efficiency up to 90%[28]. A tight comparative study has revealed that it is the multicellular associations in hESC colonies that contribute to the low transfection efficiency[9]. Furthermore, we have recently modified a microRNA transfection protocol and are able to achieve high transfection efficiency (~91%) without the use of lentiviruses (Protocol 6 and **Figure 4**). This protocol is easy to use and particularly useful for transient transfection experiments within a 3- to 5-day time frame. As we delineated in above protocols, multiple factors should be taken into considerations when we optimize transfection/transduction efficiency in hPSCs. These factors include cell density, plasmid concentrations, lentiviral titers, multiplicity of infection (MOI), duration of transfection/transduction, cytotoxicity of the reagents, and methods used for monitoring transfection/transduction efficiency.

We elaborate and extend NCM methods to culture hPSCs on Matrigel and on defined extracellular matrices. This culture method is an efficient way to eliminate the heterogeneity commonly found in hPSC colony and aggregated cultures. Human pluripotent stem cells under NCM growth conditions are pluripotent and chromosomally stable. This novel culture system is simple and versatile for hPSC maintenance, large-scale expansion, and genetic manipulations.

## Disclosures

The authors declare that they have no competing financial interests.

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