Corning® Synthegel™ 3D hiPSC Matrix Kit

Guidelines for Use

CORNING

The Corning Synthegel 3D hiPSC Matrix kit is a powerful tool for *in vitro* 3D human induced pluripotent stem cell (hiPSC) culture with more accurate *in vivo* predictions for life science research and development. Corning Synthegel 3D hiPSC Matrix kit consists of a vial of Corning Synthegel 3D hiPSC Matrix peptide nanofiber solution and, a vial of Corning Synthegel X-Link solution along with the required vial of lyophilized Corning Synthegel 3D hiPSC Grow Mix. The Synthegel 3D hiPSC Matrix nanofibrils are formulated into a basal cell culture medium in neutral pH forming a 3D microenvironment for hiPSC spheroid growth. With Synthegel 3D hiPSC Matrix, cells no longer suffer acidic or chilled conditions since all operating procedures can be completed at room temperature or 37°C and in neutral pH. Additionally, cultured cells are easily harvested from the matrix.

FOR FIRST TIME USERS, PLEASE READ AND FOLLOW THESE 3 MESSAGES.

MESSAGE 1: Mixing ratio notice.

The Synthegel 3D hiPSC Matrix solution contains 1% W/V standard peptides. For first time users, experimenting with several mixing ratios, in the range of 0.3% to 0.6% W/V final peptide concentration is recommended for hiPSC encapsulation to identify the optimal mixing ratio.

Table 1 shows examples of two mixing ratios at 0.3% and 0.5% and can be used as reference when mixing Synthegel 3D hiPSC Matrix solutions and cell suspension.

NOTES:

- Strict aseptic techniques must be used when handling solutions and cells. All materials (e.g., pipet tips, centrifuge tubes, well plates, etc.) must be sterile prior to use and all procedures should be performed in a biosafety cabinet (BSC), when appropriate, to minimize the risk of contamination.
- When harvesting it is recommended that all centrifuge steps be performed in a centrifuge equipped with a swinging bucket rotor to allow pelleting at the bottom of conical tubes. If using microcentrifuge tubes, a fixed angle rotor may be used, but caution should be taken during handling as to prevent loss of sample.
- Corning Synthegel X-Link solution should be added to the cell suspension before mixing with the Synthegel 3D hiPSC Matrix solution.

Table 1. Examples of mixing ratios of Corning Synthegel 3D hiPSC Matrix solution, Corning Synthegel X-Link solution* and cell suspension, as well as maximum plating volume per well for different plate formats**.

	0.3% W/V			0.5% W/V			
Plate Size	Cell Suspension (µL)	Corning Synthegel X-Link Solution (µL)	Corning Synthegel 3D hiPSC Matrix Solution (µL)	Cell Suspension (µL)	Corning Synthegel X-Link Solution (µL)	Corning Synthegel 3D hiPSC Matrix Solution (µL)	Maximum Plating Volume for 1 Well (µL)
6-well	1,360	40	600	960	40	1,000	2,000
12-well	680	20	300	480	20	500	1,000
24-well	340	10	150	240	10	250	500
48-well	170	5	75	120	5	125	250
96-well	40.8	1.2	18	28.8	1.2	30	60

*Corning Synthegel X-Link solution is always 2% of the total volume of Synthegel 3D hiPSC Matrix + cell suspension + Synthegel X-Link solution together.
**Nontreated culture plate is recommended for culturing 3D cells in Synthegel 3D hiPSC Matrix.

MESSAGE 2: Add medium on top of the hydrogel to prevent drying and to feed cells for long-term culture.

After hydrogel formation (30 min. at 37°C after mixing), cell medium needs to be added on top of the gel to provide fresh nutrients and prevent drying during long-term culture. Recommended initial volumes of medium overlayed on top of the hydrogel is dependent on different sized well plates (Table 2).

Table 2. The recommended initial volume of medium per well to be added on the top of hydrogel.

	6-well*	12-well	24-well	48-well	96-well**
Plating hydrogel volume (µL) per well	2,000	1,000	500	250	60
Initial medium added on top of gel (µL) per well	6,000	3,000	1,500	750	180

*6-well is not recommended for hiPSC 3D embedded culture; Synthegel hiPSC Suspension Matrix Kit is a better option for large scale hiPSC production with 6-well plates.
**96-well is not usually recommended for hiPSC 3D culture.

Message 3. Examples of cell density and gel concentration.

Table 3*. Recommended Cell densities and gel concentrations for 3D hiPSC cultures in Corning® Synthegel™ 3D hiPSC Matrix products.

Cells	Corning Synthegel 3D hiPSC Matrix	Gel Concentration (%)*	Gelation Time (min.)	Cell Seeding Density (cell/mL)	Cell Medium
hiPSC derived from Fibroblast	Corning Synthegel 3D hiPSC Matrix	0.5	30	(2-3) x 10^5	mTeSR™1 complete medium + Corning Synthegel 3D hiPSC Grow Mix
Episomal hiPSC	Corning Synthegel 3D hiPSC Matrix	0.5	30	(2.5-3.5) x 10^5	mTeSR1 complete medium + Corning Synthegel 3D hiPSC Grow Mix

*For E8 medium, 0.3% gel concentration is recommended

3D hiPSC Culture Protocols

Cell Encapsulation and Culture

1.0 Encapsulate cells from subculture (2D or 3D) or cryopreserved hiPSC.

1.1 Synthegel 3D hiPSC Matrix solution and Synthegel X-Link solution should be at room temperature (15°C to 25°C) or 37°C (37°C water bath) prior to starting.

1.2 Synthegel 3D hiPSC Grow Mix reconstitution and medium stock solution preparation.

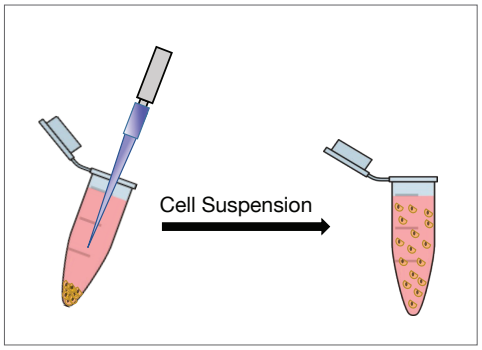
- Add 300 µL sterilized DPBS (without Mg^{2+} and Ca^{2+}) to one (1 mg) vial of lyophilized Synthegel 3D hiPSC Grow Mix, pipet gently and invert the vial 4 to 5 times to obtain a homogeneous Synthegel 3D hiPSC Grow Mix solution*.
- The reconstituted Synthegel 3D hiPSC Grow Mix solution is stable for up to 6 months at -20°C. It is recommended that aliquots of working volumes be made and stored at -20°C to avoid repeated freeze-thaw cycles.
- Add the reconstituted Synthegel 3D hiPSC Grow Mix solution into culture medium (i.e., mTeSR1 complete medium) at ratio 1:1000 v/v (Synthegel 3D hiPSC Grow Mix: mTeSR1 complete medium) to prepare hiPSC culture complete medium stock solution.

*Synthegel 3D hiPSC Grow Mix is used as a cell culture supplement. Synthegel 3D hiPSC Grow Mix solution should be diluted into mTeSR1 complete medium immediately before use and used within two weeks after dilution. Media used for cell culture in this protocol are all supplemented with Synthegel 3D hiPSC Grow Mix.

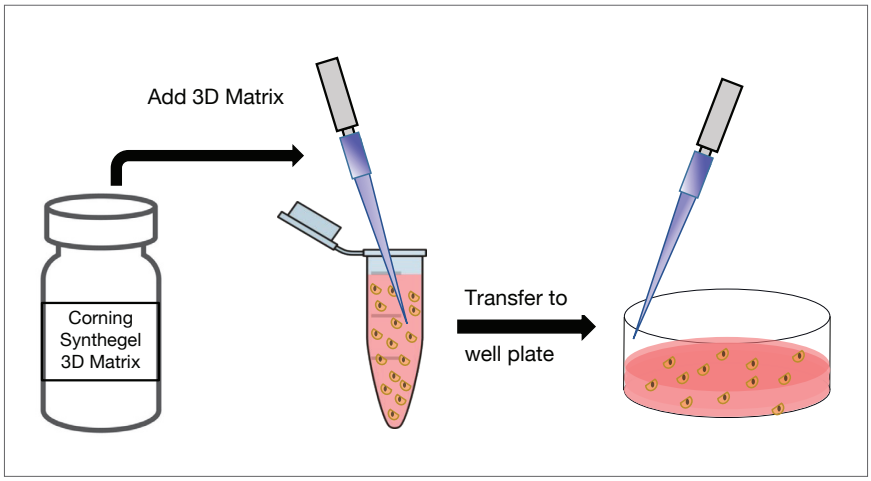
NOTE: The addition of ROCK Inhibitor is not necessary for 3D hiPSC culture using Synthegel 3D hiPSC Matrix kit as it is included in the Synthegel 3D hiPSC Grow Mix.

1.3 Suspend cells in mTeSR1 complete medium supplemented with Synthegel 3D hiPSC Grow Mix and add Synthegel X-Link solution to the cell suspension according to the Mixing Ratio (Table 1). Pipet well without introducing air bubbles.

TIP: Fully expel air from the pipet before immersing the pipet tip in the cell solution and keep the pipet tip in the cell solution during pipetting.



1.4 Mix the Synthegel 3D hiPSC Matrix solution carefully into the cell suspension of Step 3 at the desired mixing ratio (Table 1) and pipet well. Transfer the mixture into the center of each well, then swirl the plate to uniformly cover the entire well bottom surface. For larger area plate (i.e., 6-well plate), gently hand-shake the plate front to back and side to side to uniformly cover the entire surface. Examples of cell seeding densities and gel concentrations are given in Table 3.



1.5 Incubate the plate at 37°C (5% CO_2) for 30 min. (but no more than 60 min.) to complete gelation.

1.6 After gelation, overlay the hydrogel with mTeSR1 complete medium supplemented with Synthegel 3D hiPSC Grow Mix to prevent drying and provide nutrients for the cells (Table 2).

TIP: Gently add cell culture medium along the wall of each well without disturbing the hydrogel.

1.7 Feeding cells, medium feeding volume vs. well plate size (Table 4):

- Day 0: See Step 1.6 above.
- Days 1 and 2: No medium change is needed; initial medium feeding is enough to support cell growth if Table 4 is followed.
- Days 3 and 4: Gently remove about 2/3 medium above gel and add fresh medium along the wall of the well plate slowly. (Table 4)
- Day 5: hiPSCs are usually harvested.
- Synthegel Grow Mix is added to mTeSR1 complete medium at ratio 1:1000 v/v. See Section 1.2 for reconstitution procedure.

Table 4. Recommend medium feeding volume per well for various plate formats.

	Day 0	Day 1, 2	Day 3		Day 4	
Plate Size	Initial Medium Added on Top of Gel (µL)		Medium Removal Above Gel (µL)	Fresh Medium (µL)	Medium Removal Above Gel (µL)	Fresh Medium (µL)
6-well	6,000	No medium change	4,000	4,000	4,000	6,000
12-well	3,000		2,000	2,000	2,000	3,000
24-well	1,500		1,000	1,000	1,000	1,500
48-well	750		600	600	600	700
96-well	180		100	100	100	150

NOTES:

- hiPSCs are usually harvested on day 5 when cells exhibit 10-15 fold increase in numbers. If harvesting on day 4 (96 hr. of cell culture) it is recommended that a larger volume of medium is added on day 3. For day 6 harvesting, feed the cells twice on day 5 due to the fast cell growth.
- The feeding volumes are for reference only. Several factors including cell lines, cell seeding density, culture passages and culture duration impact hiPSC growth. Frequency of medium exchanges will depend on cell feeding strategy for each hiPSC and will need to be determined empirically. Color change of the medium can also be used as an indicator for timing of medium exchanges.

Thawing hiPSC Directly In 3D*

1.8 Bring the Synthegel 3D hiPSC Matrix solution and Synthegel X-Link solution to room temperature (15°C to 25°C) or 37°C (37°C water bath) prior to use.

1.9 To prepare cell medium stock solution following the procedure stated in Section 1.2.

1.10 Thaw the vial of cryopreserved hiPSC by gently agitating in a 37°C water bath. To reduce the risk of contamination, avoid exposure of the O-ring and cap to water in the water bath.

1.11 Remove the vial from the water bath as soon as the contents start to thaw. Spray the outside of the vial with 70% ethanol to decontaminate prior to placing it into a biosafety cabinet for further manipulations. Add 1 mL of prewarmed mTeSR1 complete medium to the vial and gently pipette until the cell suspension is completely thawed.

1.12 Transfer the cell suspension to a 15 mL conical tube, add 1 mL of mTeSR1 complete medium to rinse the vial for the remaining cells, and combine into the 15 mL conical tube.

1.13 Add mTeSR1 complete medium to bring the final volume up to 10 mL and centrifuge at 200 g for 5 min.

1.14 Prior to enumeration, remove the supernatant and resuspend the cell pellet gently in mTeSR1 complete medium.

1.15 Follow Steps 1.1-1.7 (Encapsulate cells from subculture (2D or 3D) or cryopreserved hiPSC) for the hiPSC encapsulation and culture in Synthegel 3D hiPSC Matrix.

***NOTE:** The recommended hiPSC seeding density from thawing in hiPSC-qualified Synthegel 3D hiPSC Matrix is higher (i.e., 3 to 4 x 10^5 cell/mL) than when encapsulating cells from subculture. This increase in cell density is necessary to offset loss of cell viability and proliferation that often occurs upon thaw.

2.0 hiPSC Spheroid Recovery from Hyrdogel for Passaging, Enumerating, and Downstream Applications

(Example is given in Section 3.2 for new users).

2.1 **Gel disruption:** First transfer about 1/3 of the culture medium from the well to a conical centrifuge tube A, then mechanically disrupt the gel GENTLY and THOROUGHLY by pipetting the remaining medium and hydrogel several times (6 to 8 times). Transfer this mixture to conical centrifuge tube A (recommended conical centrifuge tube size vs. well-plate size are listed in Table 5)

NOTES:

- Thorough disruption of the gel is very important for cell isolation from the gel. Follow the steps below for better gel disruption results:
  - Slightly tilt the well plate.
  - Expel air from the pipet prior to submerging in liquid.
  - Immerse the pipet tip into the gel without touching the bottom of the well plate.
  - Aspirate the mixture of gel and medium slowly, and dispense slowly along the top edge of the wall in the well plate.
  - Repeat Steps 3 and 4 (6 to 8 times).
- Avoid air bubbles during pipetting.

2.2 **Rinse:** Use PBS or DPBS (without Mg^{2+} and Ca^{2+}) to rinse the well and combine into centrifuge tube A, mix thoroughly (pipet 3 to 4 times). Recommended rinse volume is double the maximum plating volume for each well (Table 1) (i.e., 120 µL for 96-well microplate or 1000 µL for 24-well plate).

2.3 **Gel dilution:** Add additional PBS or DPBS to centrifuge tube A to further dilute the mixture by 20-fold of the original plating volume (Table 5) and mix well (pipet 3 to 4 times).

2.4 **Centrifuge:** Centrifuge at 200 to 400 g for 5 min. Discard supernatant, and collect the cell pellet (**TIP:** When approaching the bottom of tube A, use a 1 mL pipet to gently remove and discard the supernatant without disturbing the spheroid cell pellet).

Table 5. Recommended conical centrifuge tube size vs. well-plate size for gel dilution.

	6-well	12-well	24-well	48-well	96-well
Total plating volume per well	2,000 µL	1,000 µL	500 µL	250 µL	60 µL
Final volume of diluted gel and cell mixture from one well (20 folds)	40 mL	20 mL	10 mL	5 mL	1.2 mL
Suggested conical tube size	50 mL	50 mL	50 mL**	15 mL	5 mL

NOTES:

- The conical tube size suggested here is only good for one well cultured cell harvesting. If more than one well cultured cells are harvested at the same time, the tube size will need to be increased accordingly. For example, 50 mL tube can be used for harvesting no more than 3 wells of 24-well plate at the same time (3 x 10 mL = 30 mL).

**For better results, it is recommended that a 50 mL tube be used for pipetting and the mixture transfered to a 15 mL conical tube for centrifugation and easy collection of the hiPSC pellet.

2.5 **hiPSC spheroid dissociation**

1. To dissociate hiPSC spheroids, add TrypLE™ Express Enzyme (1X) to the spheroid pellet. Volume of TrypLE depends upon the quantity and size of the spheroids. Start by adding twice the plating hydrogel volume/well (Table 2) into the conical tube.

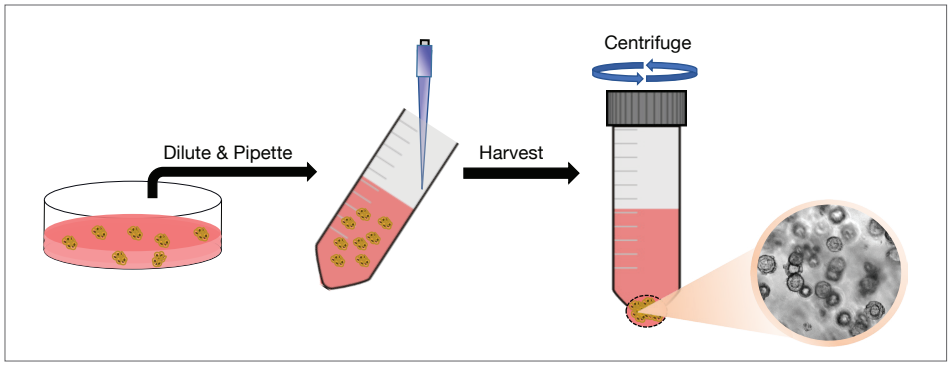
Example: hiPSC cell pellet harvested from 1 well of 24-well plate on Day 5 with an initial seeding density 2 x 10^5 cell/mL and a final concentration of single hiPSC of 1 to 1.5 x 10^6 cells (10-fold increase) will need 1 mL TrypLE Express Enzyme (1X) solution to dissociate the spheroids.

2. Pipet mix gently to resuspend the spheroid pellet in TrypLE and incubate at 37°C for 10 to 20 min. The incubation will depend on the size and quantity of the hiPSC spheroids.

TIP: At approx. 10 min. of incubation, pipet the spheroids and TrypLE Express Enzyme (1X) solution mixture gently to help break-up the spheroids. Observe the cell cluster size under a microscope to determine if an extension of incubation time is needed. If most of the cells (≥ 90%) have become single cells, then proceed to the next step. If larger cell clusters are preferred for passage, incubation time can be shortened as needed.

3. Add DPBS equal to volume of TrypLE Express Enzyme (1X) solution used to each tube (i.e., add 1 mL DPBS of TrypLE Express) to dilute dissociation reagent.
4. Centrifuge at 200 g for 5 min.
5. Discard supernatant and resuspend cell pellet in mTeSR1 complete medium for enumeration or passage. (i.e., one well of 24-well plate harvested at 5 days usually produces about 1 to 1.5 x 10^6 cells, which can be resuspended in 400 to 500 µL medium).

NOTE: The use of TrypLE Express Enzyme (1X) is highly recommended for hiPSC spheroids dissociation, as cell viability is higher than using EDTA for dissociation. The dissociated hiPSC (single and small clusters) display viability above 95%, compared to 80% to 85% for 0.05 mM EDTA.



3.0 Growth Performance and Recovery of hiPSC in Corning Synthegel 3D hiPSC Matrix (24-well plate cultured for 5 days)

3.1. Culture condition

- **Culture medium:** Completed mTeSR medium supplemented with Synthegel 3D hiPSC Grow Mix
- **Seeding density:** 1 x 10^5 cells per well for 24-well plate (500 µL of 2 x 10^5 cells/mL solution)
- **Gel concentration:** 0.5% Synthegel 3D hiPSC Matrix (Table 1)
- **Gel plating volume:** 500 µL per well for 24-well plate (2 mm gel thickness)
- **Cell feeding:** Add 1.5 mL medium above gel at Day 0
  - Exchange 1 mL of medium with fresh medium at Day 3
  - Exchange 1 mL of medium with fresh medium at Day 4.

TIP: DO NOT disturb the gel. Slightly tilt the culture plate and remove the conditioned medium along the wall of the well, keeping the tip close to the medium surface. Add fresh medium, dropwise, along the well wall.

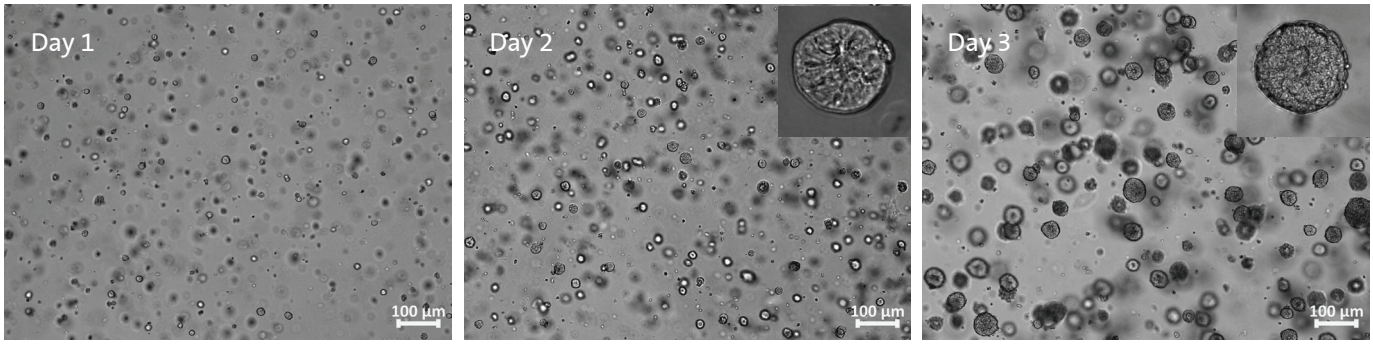


Figure 1. Physiological morphologies of hiPSC in Corning Synthegel 3D hiPSC Matrix.

Table 6. hiPSC growth performance within 0.5% Synthegel hiPSC matrix (24-well plate).

Cell source	Viability	Cell amount harvested per well at Day 5	Doublings	Starting seeding density
hiPSC derived from fibroblast	95% to 98%	1 to 1.5 x 10^6	10 to 15 folds	1 x 10^5

NOTE: Results are for reference only. Performance will vary depending upon selected cell line, culture medium, and seeding density for 3D hiPSC growth.

3.2. Spheroid recovery

Gel disruption: Transfer 1/3 (500 to 700 µL) of medium above the hydrogel from the culture well to a 50 mL conical centrifuge tube A. Mechanically disrupt the gel GENTLY and THOROUGHLY in the well by pipetting the gel and the remaining medium a several times (6 to 8 times) and transfer to conical centrifuge tube. (Recommended conical centrifuge tube size vs. well-plate size is listed in Table 5)

NOTE: Thorough disruption of the gel is critical for cell isolation. Follow the steps below:

- Slightly tilt the well plate
- Expel air from the pipet prior to submerging in liquid
- Immerse the pipet tip into the gel without touching the bottom of the well plate
- Aspirate the mixture of gel and medium slowly, and dispense slowly along the top edge of the wall in the well plate
- Repeat Steps 3 and 4 (6 to 8 times).

Rinse: Use 1,000 µL PBS/DPBS (without Mg^{2+} and Ca^{2+}) to rinse the well, combine the solution into centrifuge tube A, and pipette mix thoroughly (3 to 4 times).

Gel dilution: Add 7 to 8 mL PBS/DPBS to centrifuge tube A to further dilute the mixture and pipette mix well (3 to 4 times).

Centrifuge: Transfer the mixture from the 50 ml tube A into a 15 mL conical centrifuge tube B and centrifuge at 200 to 400 g for 5 min. Discard supernatant and collect the spheroid pellet for further handling.

TIP: When approaching the bottom of tube, use 1000 µL pipet to gently remove and discard the remaining supernatant without disturbing the cell pellet. The spheroids can now be broken-up into smaller clusters or single cells as needed following the procedure in Section 2.5.

4.0 hiPSC Cryopreservation

1. After enumeration, centrifuge solution at 100 to 200 g for 5 min. (depending on cell type).
2. Carefully remove supernatant and discard. Resuspend hiPSC pellet (single cell or small clusters) in ESC-Sure™ Human ESC Freezing Medium or complete growth medium with 5% to 10% DMSO to a final concentration of 1 x 10^6 to 5 x 10^6 cells/mL depending on cell type. Aliquot into cryopreservation vials and incubate at room temperature 15 min. to allow diffusion of cryoprotectant into the cells.

   NOTE: Prepare solution of complete cell growth medium with 5% to 10% DMSO prior to resuspending cell pellet. Do not add DMSO directly to cells in complete growth medium.
3. Slowly freeze cells at 1°C/min. using a Corning CoolCell® module (Corning 432000) placed in a -80°C freezer for at least 24 hours.
4. Quickly transfer the frozen vials to liquid nitrogen or -130°C freezer for long-term storage. Do not store cells at -80°C for extended periods of time as it negatively impacts cell viability.

5.0 Immunostaining

All reagents and procedures are done at room temperature unless otherwise noted. Two recommended staining methods, either in Synthegel 3D matrix or in solution are described below:

5.1. Staining in Corning Synthegel 3D Matrix.

1. Carefully remove medium on top of the hydrogel in cultured wells.
2. Carefully rinse each well of hydrogel surface once with DPBS (without Ca^{2+} and Mg^{2+}). Rinse volume should be equal to initial media volume (Table 2) (i.e., 300 µL/well for 96-well microplate or 1.5 mL/well for 24-well plate).
3. Carefully remove DPBS overlay.
4. Fix cells by slowly adding 10% neutral buffered formalin above the hydrogel and incubating for 30 to 40 min. at room temperature (or 100% methanol, or 4% paraformaldehyde). Volume of buffered formalin should be equal to initial media volume (Table 2) (i.e., 300 µL/well for 96-well microplate or 1.5 mL/well for 24-well plate).
5. Carefully remove buffered formalin overlay.
6. Wash hydrogel formalin twice with DPBS (without Ca^{2+} and Mg^{2+}) for 15 min. each. The fixed sample can be used directly or sliced into 80 µm slices by using cryo-sectioning protocol prior to staining.
7. Prepare washing buffer and blocking solutions.

   a. Washing buffer: DPBS (with Ca^{2+} and Mg^{2+}) + 0.2% V/V Triton X-100 + 0.1% V/V cold water fish gelatin. Mix by pipetting thoroughly or by vortexing gently.

   b. Blocking solution: washing buffer + 10% V/V serum from the same source animal as secondary antibody. Mix by pipetting thoroughly or by vortexing gently.

   NOTE: Blocking solution should be prepared just before applying to the sample.
8. Carefully remove DPBS overlay.
9. Wash twice with washing buffer for 10 min. each. Volume of washing buffer should be equal to initial media volume (Table 2) (i.e., 300 µL/well for 96-well microplate or 1.5 mL/well for 24-well plate).

   NOTE: If the sample is not blocked and stained immediately, it can be stored after the addition of washing buffer. Stored sample plate should be sealed with parafilm, to minimize evaporation and stored at 4°C until ready for immunostaining. Remove plate from 4°C and allow to reach room temperature before proceeding to the Blocking step.
10. Blocking (Day 1)
    - Carefully remove washing buffer overlay and add blocking solution to the fixed samples. Volumes of blocking solution should be 100% to 150% of maximum plating volume/well (Table 2) (i.e., 500 to 750 µL/well for 24-well plates or 100-150 µL/well for 96-well microplates).
    - Incubate overnight or >12 hrs.
11. Primary Antibody (1Ab) (Day 2)
    - Prepare primary antibody solution by diluting the primary antibody into washing buffer. Primary antibody concentrations for 3D staining should be the same or higher than what is used for 2D culture staining (i.e., Goat Oct 3/4 antibody (N-19), with final concentration of 3 µg/mL, was used for hiPSC staining directly in 3D Synthegel 3D hiPSC Matrix)[1].
    - Carefully remove blocking buffer
    - Add primary antibody solution to the gel. Volumes of blocking solution should be twice the maximum plating volume/well (Table 2) (i.e., 1000 µL/well for 24-well plates or 200 µL/well for 96-well microplates).
    - Incubate overnight or >12 hrs.

NOTES:

- Volume of primary antibody solution should be enough to completely soak the gel.
- Blocking buffer should be added to one well as a negative control to assess background staining.

12. Rinse (Day 3)
    - Carefully remove primary antibody solution or blocking buffer from wells.
    - Wash four times with washing buffer for 2 hours each. Volume of washing buffer should be equal to initial media volume (Table 2) (i.e., 300µL/well for 96-well microplate or 1.5 mL/well for 24-well plate).
13. Secondary antibody (2Ab) (Day 3)
    - Prepare secondary antibody solution by diluting the secondary antibody into washing buffer. Secondary antibody concentration for 3D staining should be the same or higher than that used for 2D culture staining. Use the concentration recommended by vendor or determined empirically[8]. (i.e., Rabbit anti-goat lgG (H+L) secondary antibody Alexa Fluor™ 488 with a final concentration of 5 µg/mL was used for hiPSC staining directly in Synthegel 3D hiPSC Matrix.
    - Wrap the plate with aluminum foil to prevent bleaching of fluorescent dye
    - Incubate overnight or >12 hrs.
14. Rinse and Image (Day 4)
    - Carefully remove secondary antibody solution from wells.
    - Wash a minimum of 6 times with washing buffer for 1 hr. each. Volume of washing buffer should be equal to initial media volume (Table 2) (i.e., 300 µL/well for 96-well microplate or 1.5 mL/well for 24-well plate).
    - After final wash, replace washing buffer with Glycerin for imaging.
    - Proceed with imaging.

    NOTES:
    - To achieve a strong and specific signal, it is critical to thoroughly block non-specific binding of antibodies and allow the diffusion of antibodies through the hydrogel matrix (Synthegel 3D hiPSC Matrix. To facilitate these requirements, extended blocking and incubation times as well as multiple washes are required.
    - The background signal, in 3D systems, is influenced by cells located in different plane positions. Therefore, post-processing of images may be needed to reduce background brightness.

5.2. Staining in solution.

1. Isolate hiPSC spheroids from Synthegel 3D matrix culture following the Spheroids Recovery Section 3.2.
2. Fix the hiPSC spheroids by suspending them in 10% neutral buffered formalin (or 100% methanol, or 4% paraformaldehyde) from 60 min. to overnight at room temperature).
3. Perform staining process following regular staining procedures recommended by manufacturer for 2D but longer staining time is recommended for reagents fully to penetrate into the spheroids. Centrifuge process is used for washing steps to save cell spheroids.
4. For imaging, the stained hiPSC spheroids can be suspended in 100% Glycerin and dropped on a glass panel for imaging.

5.3. Imaging and Staining Tips

1. For cell spheroids stained in solution (Section 5.2) regular microscope imaging systems or high quality microscope (i.e., Tender imager 3D) or regular confocal imaging system (i.e., ImageXpress Confocal by Molecular Devices) can be used for imaging.
2. For cell spheroids stained in Synthegel 3D matrix (Section 5.1) the stained samples can be imaged directly using confocal image system (i.e., ImageXpress Confocal by Molecular Devices) or cryo-sectioning protocol (below) is recommended to prepare 80 µm slices for high quality images using regular confocal or microscope imaging systems.
3. Staining in solution is the simplest method for high quality images. However, if staining in 3D Synthegel 3D matrix is preferred, cryo-sectioning protocol (below) is recommended to slice the fixed sample into 80 µm slices for easy staining and high quality imaging.
4. **Cryo-sectioning Protocol:** The fixed or printed samples in Synthegel 3D matrix is first embedded in Cryostat embedding medium and put in the slicing chamber to freeze (at -20°C). After fully frozen, the sample is sectioned into 80 µm slices and collected onto glass slides. The sample slides are then warmed in a slide warmer for 2 hours before washing and staining or imaging.

References

1. Nethercott HE, Brick DJ, Schwartz PH. Immunocytochemical analysis of human pluripotent stem cells. Human Pluripotent Stem Cells: Methods and Protocols. 2011:201-20.

Appendix A

Reagents used for 3D hiPSC culture in Synthegel 3D hiPSC Matrix and immunostaining (reference only).

Reagents	Cat. No.	Supplier
Human induced pluripotent stem cell (hiPSC)	ASE-9203	Applied Stem Cell
	A18945	ThermoFisher
mTeSR™1/mTeSR™1 Plus medium	05850 / 05825	Stem Cell Technology
E8	A1517001	Thermofisher
Dulbecco's Phosphate-Buffered Saline (DPBS without Ca^{2+} and Mg^{2+})	21-031-CM	Corning
Dulbecco's Phosphate-Buffered Saline (DPBS with Ca^{2+} and Mg^{2+})	21-030-CM	Corning
Phosphate Buffered Saline (PBS)	46-013-CM	Corning
TrypLE™ Express Enzyme (1X)	12604021	Thermofisher
EDTA	IB70185	MidSci
10% neutral buffered formalin	23-305510	Fisher Scientific
Triton™ X-100	T8787	Sigma-Aldrich
Cold water fish gelatin (Teleostean Gelatin)	G7765	Sigma-Aldrich
Normal rabbit serum control	31883	ThermoFisher
Goat Oct 3/4 antibody (N-19) 100 µg/mL	sc-8628	Santa Cruz Biotechnology
Rabbit anti-goat IgG(H+L) 2nd antibody Alexa Fluor 488	A-11078	ThermoFisher
ESC-Sure™ Human ESC Freezing Medium	ASM-5004	Applied Stem Cell
Corning® CoolCell® module	432003	Corning

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