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🌐 Generation of human Trunk-like Structures (hTLS) from Human Pluripotent Stem Cells

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Abstract

Novel *in vitro* approaches are required to overcome the technical and ethical challenges associated with studying human embryogenesis. 3D stem cell-based embryo models are providing new insights on human biology from cultures of human pluripotent stem cells (hPSCs). This protocol describes the generation of human trunk-like structures (hTLS) as described in Makwana, Tilley *et al.* (2025). The addition of both an extracellular matrix and a Wnt signalling agonist to aggregates of hPSCs enables the formation of structures that co-develop somites and a neural tube. This protocol for generating human trunk-like structures provides an *in vitro* model for the study of both somitogenesis and neural tube development.

Image Attribution

Figure 1: Aggregate morphology at 72h after plating is predictive of hTLS formation. Classification of the morphology of structures at 120h (right) relative to morphology at 72h prior to Matrigel embedding (middle).

Materials

Materials

- Phosphate-buffered saline (PBS) (no CaCl₂ or MgCl₂, (-/-); Sigma-Aldrich D8537)
- Laminin iMatrix-511 silk E8 (AMSBIO, AMS892 021)
- Y-27632 (Tocris Biosciences, 1254)
- mTeSR™1 (STEMCELL Technologies, 85850)
- StemPro™ Accutase™ (Gibco™ Thermo Fisher Scientific, A1110501)
- Neurobasal Medium (Gibco, 21103049)
- Advanced DMEM/F12 Medium (Gibco, 12634028)
- B-27 Supplement (w/o Vitamin A) 50× (Gibco, 12587010)
- N-2 Supplement 100× (Gibco, 17502048)
- GlutaMAX Supplement 100× (Gibco, 35050061)
- CHIR99021 (Tocris Biosciences, 4423)
- SB431542 (Tocris Biosciences, 1614)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich D2438)
- Ultra-low-adherence 96-well plate (Greiner Bio-One, 650970)
- 6-well sterile tissue culture plate (Corning 3516)
- Corning® Matrigel® growth factor-reduced basement membrane matrix, phenol red-free, LDEV-free (Corning, 356231)
- all *trans*-retinal (Merck Sigma-Aldrich, R2500-100MG)
- Paraformaldehyde (PFA) – 16% w/v aq.soln, Methanol Free (ThermoFisher Scientific, 043368.9M)
- 15 mL sterile centrifuge tubes (Corning Falcon 430791)
- 50 mL sterile centrifuge tubes (Corning Falcon 430291)
- 1% (v/v) ChemGene MEDLAB (or other approved disinfectant for work at Containment Level 2) (SKU075A)
- 1L vacuum filtration system (Corning 431098)

Equipment

- NucleoCounter® NC-202™, ChemoMetec – *or equivalent automated cell counter measuring live cell counts*
- Water bath at 37°C
- Class II Microbiological Safety Cabinet (Faster SafeFast Premium)
- Vacuum aspirator with hand operator (SAM Medical Suction SAM14, Integra Vacuboy)
- P1000 micropipette (Gilson FA10006M)
- 30-300 µL multichannel micropipette (Eppendorf ResearchPlus 3125000052)
- Tissue culture microscope with phase contrast optics (Nikon Eclipse Ts2 or similar)
- Fume cupboard

Safety warnings



Safety warnings

Hazard Statements:

- all *trans*-retinal
 - H315: Causes skin irritation
 - H317: May cause an allergic skin reaction
 - H361f: Suspected of damaging fertility
- Paraformaldehyde, 16% w/v aq. soln, Methanol Free
 - H302 + H312 + H332: Harmful if swallowed, in contact with skin or if inhaled
 - H315: Causes skin irritation
 - H317: May cause an allergic skin reaction
 - H319: Causes serious eye irritation
 - H341: Suspected of causing genetic defects
 - H350: May cause cancer

Before start

All human pluripotent cell culture is conducted at Containment Level 2 in Class II Microbiological Safety Cabinet and cells are cultured in humidified incubators at 37 °C and 5% CO₂. All liquid waste is inactivated with 1% (v/v) ChemGene MEDLAB for a contact time of at least 15 minutes prior to disposal.

The expected duration of the protocol is 10-11 days from plating out single cells to mature hTLS structures, occurring at a frequency of 80-90%.

Human Pluripotent Stem Cell Culture

1 Thawing ESCs/iPSCs:

- 1.1 Retrieve cryovial of single iPSC/ESCs from vapour phase liquid nitrogen storage. Keep on dry ice. 

S.N.: To transfer iPSC/ESCs from other culture conditions, cells can be brought up immediately with iMatrix-511 but begin with original culture medium and then switch to mTeSR™1 in next passage.

- 1.2 Label 6-well plate with cell line, date, name and passage.

- 1.3 Prepare enough medium (dependent on medium cells were grown in prior to freezing) supplemented with 10 μ M Y-27632 (reconstituted in PBS (-/-)) for plating (2 mL for each well) plus 4 mL for centrifugation of cell pellet and 1 mL for resuspension of cell pellet.

- 1.4 Take medium for plating and add to 15 mL Falcon tube. Add iMatrix-511 to plating medium at a concentration of 1.2 μ g/mL (4.8 μ L of 0.5 mg/mL stock (0.25 μ g/cm²) per well).

S.N.: Plates can also be precoated with iMatrix-511 using PBS(-/-) with a final coating density of 0.5 μ g/cm².

- 1.5 Add 2 mL of plating medium to each well and place in incubator to pre-equilibrate. 

- 1.6 To thaw frozen cells, suspend the cryovial in a 37°C water bath, until almost all the cell suspension is in a liquid state (a small block of ice should remain).

- 1.7 Transfer all liquid from the cryovial to the resuspension medium. Use a small amount of the cell suspension to thaw any remaining ice and transfer to the resuspension medium, leaving the cryovial empty.

- 1.8 Centrifuge cells in resuspension medium at 170 x g for 4 minutes. 

- 1.9 Aspirate supernatant and resuspend in resuspension medium using a P1000 micropipette.

- 1.10 Determine cell concentration using an automated cell counter or similar. 



S.N.: A reliable cell counter is very important for ensuring reproducible results for this protocol.

- 1.11 Seed 3-4 different seeding densities of cells/well onto iMatrix-511 coated 6-well plate that already contains 2 mL mTeSR™1 supplemented with 10 µM Y-27632 per well.

S.N.: Seeding densities need to be adjusted for each cell line. Cells may also grow at different rates between passages.

- 1.12 Distribute cells evenly in wells by gentle rocking.

- 1.13 Incubate at 37°C, 5% CO₂ for 24 hours.



- 1.14 Warm mTeSR™1 (or medium) to room temperature (RT).

- 1.15 Change medium to mTeSR™1 without Y-27632 by aspirating the whole culture volume and replacing it with fresh medium. Return the plate to the incubator.



- 1.16 Change medium every day. If using a different medium, change over to mTeSR™1 once colonies have formed or on the next passage. Passage after 4-5 days, dependent on confluency.



2 **Passaging ESCs/iPSCs:**

- 2.1 Passage ESC/iPSCs when they reach 80% confluency (as estimated by eye).

- 2.2 Thaw Accutase at RT (do not thaw at 37 °C) and prepare 4 mL of PBS in a 15 mL Falcon tube. Pre-warm in the 37°C water bath.

- 2.3 Warm mTeSR™1 and PBS to RT.

- 2.4 Prepare 2 mL mTeSR™1 with 10 µM Y-27632 and 4.8 µL iMatrix-511 for each well of a 6-well plate in 15 mL Falcon tube.

- 2.5 Label 6-well plate and add 2 mL of pre-warmed media (with Y-27632 and iMatrix-511) per well. Incubate plate at 37 °C.



- 2.6 Prepare 1 mL mTeSR™1 with 10 µM Y-27632. This will be used to resuspend the cell pellet.
- 2.7 Take 6-well plate with cells out of the incubator and aspirate spent culture medium.
- 2.8 Wash cells with 2 mL PBS and aspirate PBS.
- 2.9 Add 1 mL of RT Accutase.
- 2.10 Incubate at 37 °C for 8 minutes. Check cells under microscope to see if cells are detaching.

S.N.: Incubation time depends on confluency of cells - shorter incubation for less confluent cells, and whether the cells have been pre-treated – longer incubation is required after 24-hour pre-treatment with Chiron.
- 2.11 Detach cells immediately by tilting plate and gently pipetting up and down the Accutase (avoid bubbles or foaming) using a P1000 micropipette to ensure cells detach. Turn plate 180° and repeat. Check for detachment under microscope. Take the 1 mL cell suspension and pipette once into the middle of the well to promote dissociation to single cells.
- 2.12 Transfer cells into the 15 mL Falcon tube with the prewarmed 4 mL of PBS. Take 1 mL of cell suspension and wash over to collect and remaining cells.
- 2.13 Centrifuge for 4 minutes at 170 x g.
- 2.14 Aspirate supernatant and resuspend pellet in 1 mL mTeSR™1 + 10 µM Y-27632 by pipetting up and down 3 times.
- 2.15 Determine cell concentration using an automated cell counter or similar.
- 2.16 Remove 6-well plate with 2 mL of mTeSR™1 medium supplemented with 10 µM Y-27632 and iMatrix-511 from incubator.
- 2.17 Seed $4-6 \times 10^4$ cells/well onto iMatrix-511 coated 6-well plate that already contains 2 mL mTeSR™1 supplemented with 10 µM Y-27632 (4.2×10^3 to 6.3×10^3 cells/cm²).





S.N.: Seeding 3-4 different wells at different densities is advised, to accommodate any variation in growth rate.

2.18 Distribute cells evenly in wells by gentle rocking.

2.19 Incubate at 37 °C, 5% CO₂ for 24 hours.



2.20 Warm mTeSR™1 to RT.

2.21 Change medium to mTeSR™1 without Y-27632 by aspirating the whole culture volume and replacing it with fresh medium. Return the plate to the incubator.



2.22 Change medium every day.



2.23 Passage the cells every 4-5 days.

Generation of hTLSs from human ESC/iPSC

3 N2B27 Medium Preparation (1L)

N2B27 is used for pre-treatment and for the generation of hTLSs. There are many protocols for generating N2B27, alternatives may also be suitable.

3.1 Thaw B-27 Supplement (w/o Vitamin A) (50×, 10 mL) and N-2 Supplement (100×, 5 mL).

3.2 Mix 500 mL Advanced DMEM/F12 Medium, 500 mL Neurobasal Medium, 10 mL B-27 Supplement (w/o Vitamin A) (50×) and 5 mL N-2 Supplement (100×).

3.3 Sterile filter the combined medium and aliquot into 40 mL aliquots in 50 mL Falcon centrifuge tubes.

3.4 Refreeze at -20 °C if not using immediately.

S.N.: Freezing N2B27 medium has worked using Corning 50 mL Centrifuge Tubes (430291) and closed tightly. Medium when frozen should have an orange ring at top of



medium. If it is pink/magenta – do not use.

- 3.5 When needed, thaw N2B27 aliquot and add 1% GlutaMAX Supplement 100×. Mix thoroughly.

- 3.6 Do not re-freeze thawed aliquots, and store thawed aliquots at 4 °C for up to one week in the dark.



4 Pre-Treatment of Human ESC/iPSC (PT)

- 4.1 Culture ESC/iPSCs for 5-6 days after seeding as single cells in a 6-well plate. ESC/iPSC need to be approximately 70% confluency (as estimated by eye).

S.N: The seeding density required to reach the right confluency for pre-treatment is heavily cell line dependent and on how well the cells are growing. Cell confluency is important as it will determine the day of pre-treatment. If the cells are growing at a good rate, cells are usually pretreated on day 5 of culture. Typically, this is using a seeding density of $2-2.5 \times 10^4$ cells/well. However, slower growing cells can be pretreated on day 6 at 70% confluency.

- 4.2 Do not pretreat cultures with differentiation or that have too sparse or small colonies.



- 4.3 Prepare pre-treatment medium: 3 mL N2B27 (w/ 1% Glutamax) supplemented with 3-5 μ M CHIR99021 (reconstituted in DMSO).

S.N: The concentration of CHIR99021 needed for pretreatment is highly cell line dependent. Every cell line will need to be optimised and therefore it is recommended to try out a few concentrations for each new cell line. A good range of concentrations to test is 3-5 μ M.

- 4.4 Aspirate medium from cells and add fresh pre-treatment medium.

- 4.5 Return to incubator and culture for 24 hours.



5 Aggregation of Human ESC/iPSC

- 5.1 Prewarm N2B27 and Accutase to RT. Thaw aliquots of small molecules.

- 5.2 Label 96-well U-bottom low attachment plate.
- 5.3 Prepare a 15 mL Falcon tube with 4 mL PBS for each pretreatment well and incubate in 37 °C water bath.
- 5.4 Prepare base medium for aggregation formation and resuspension (Total volume should include medium for cell suspension plus 10% extra as dead volume and 1 mL for each PT well): N2B27 (w/ 1% Glutamax), 10 µM SB431542 (TGFβ inhibitor, reconstituted in DMSO), 10 µM Y-27632.
- 5.5 Aliquot base medium for volume required for each aggregation treatment and supplement with 3-6 µM CHIR99021.

S.N: The concentration of CHIR99021 needed for aggregation is highly cell line dependent. Every cell line will need to be optimised and therefore it is recommended to try out a few concentrations for each new cell line. E.g. for each PT condition, 3-4 aggregation concentrations are tested – typically 3-6 µM.

- 5.6 Add aggregation media into labelled tubes.
- 5.7 Aspirate media and wash pre-treated hPSCs with 2 mL of PBS.
- 5.8 Add 1 mL of Accutase and distribute across well by gentle rocking.
- 5.9 Incubate at 37 °C for 11 minutes. Check cells under microscope to see if cells are detaching. 
- 5.10 Detach cells immediately by tilting plate and gently pipetting up and down the Accutase (avoid bubbles or foaming) using a P1000 (about 5 times) to ensure cells detach. Turn plate 180° and repeat. Look under microscope. Take the 1 mL cell suspension and pipette against the middle of the well to promote dissociation to a single cell suspension. 
- 5.11 Transfer cells into the 15 mL falcon with the prewarmed 4 mL of PBS. Take 1 mL of cell suspension and wash over the growth surface to collect any remaining cells.
- 5.12 Centrifuge in a 15 mL Falcon centrifuge tube at 170 x g for 4 minutes. 



5.13 Aspirate supernatant and add 1 mL of (resuspension) aggregation medium.

5.14 Dissociate the cells gently by pipetting up and down with a P1000 pipette.

5.15 Count the cells using an automated cell counter. Repeat count and take an average.



5.16 Prepare a cell suspension at 1×10^4 cells/mL.

5.17 Add 50 μ L of the cell suspension into each well of a 96-well U-bottom low attachment plate, equivalent to 500 cells/well by reverse pipetting using a multichannel pipette.



5.18 Centrifuge the plate at 160 x g for 2 minutes. Repeat centrifugation. Cells will appear grouped together under the microscope.



S.N: Be careful not to nudge plate during transport or cells will disperse. If this occurs, the plate will need to be centrifuged again.

5.19 Incubate the plate in a humidified incubator at 5% CO₂ and 37 °C for 24 hours.



6 Culturing of Human ESC/iPSC Aggregates

6.1 24 hours after plating, add 150 μ L of prewarmed (RT) basal N2B27 (w/ 1% Glutamax) per well using a multichannel micropipette.



6.2 At 48 hours after plating, collect 150 μ L of spent culture medium with a multichannel pipette and then add fresh 150 μ L basal N2B27 (w/ 1% Glutamax).



6.3 At 72 hours after plating, aggregates will be ready to embed in Matrigel.

6.4 Whether or not a hTLS will form after embedding is highly dependent on the structure of 72-hour aggregate. See Figure below:

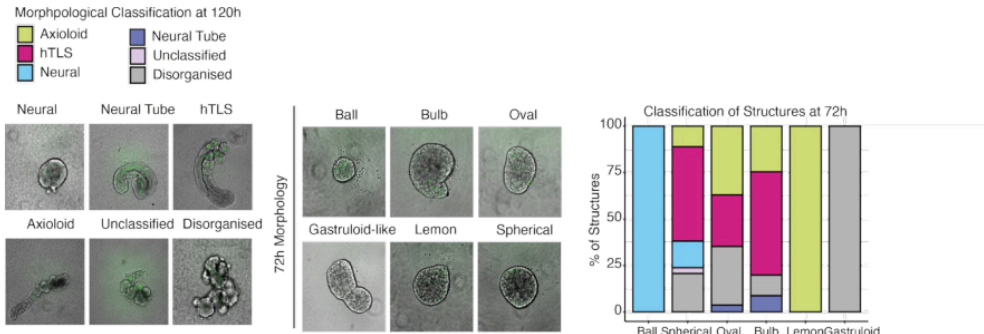


Figure 1: Aggregate morphology at 72h after plating is predictive of hTLS formation. Classification of the morphology of structures at 120h (right) relative to morphology at 72h prior to Matrigel embedding (middle).

7 Matrigel Embedding

- 7.1 Thaw aliquots of Matrigel in ice at 4 °C.

S.N: Aliquot stock Matrigel before use.

- 7.2 Dilute thawed Matrigel with ice-cold N2B27 (w/ 1% Glutamax) medium, to a final concentration of 16% Matrigel (v/v). Keep on ice.

- 7.3 Supplement the 16% Matrigel (v/v) solution with 1.6 μM Retinal (reconstituted in DMSO). Keep on ice.

- 7.4 Remove 150 μL of medium from each well of the aggregate plate using a multichannel micropipette.

- 7.5 Add 50 μL of 16% Matrigel solution into each well of the plate. Do not pipette up and down; the Matrigel will mix with medium and any pipetting will create bubbles/disturbance.

S.N: After medium removal, we assume that 30 μL of medium is left, hence the 16% Matrigel supplemented with 1.6 μM Retinal will be diluted to final concentration of 10% Matrigel with 1 μM Retinal.

- 7.6 Return the plate to the incubator (37 °C, 5% CO_2).

- 7.7 Embedded aggregates are cultured for 24-72 hours without medium changes.

8 Fixation of hTLS

8.1 In a fume cupboard, prepare a working stock of 4% (v/v) paraformaldehyde (PFA) in PBS (-/-) by dilution of the 16% (v/v) stock.



8.2 Aspirate media and hTLS from each well using a P1000 micropipette (single release of plunger with tip at bottom of well), transferring into a 15 mL Falcon centrifuge tube.

8.3 Check 96-well plate under microscope at low magnification to ensure all hTLS have been collected.

8.4 Remove as much excess medium as possible from the collection tube, without aspirating the hTLS.

S.N: hTLS will be floating due to Matrigel so be extra careful when removing excessive liquid. A bright light can be helpful to visualise the hTLS in suspension during aspiration.

8.5 Wash hTLS twice using PBS-/-, aspirating as much excess medium as possible.

S.N: Each wash will dilute the Matrigel and allow the hTLS to settle to the bottom of the tube.

Do not use Corning Cell Recovery Solution to remove Matrigel – this will adversely affect the structure of the hTLS.

8.6 In a fume cupboard, add approximately 300 μ L 4% PFA in PBS (-/-) to fix hTLS.



8.7 Incubate at 4 °C for 2hrs on an orbital shaker, upright in a tube rack.

S.N: Samples can be incubated in 4% PFA for up to 24 hours.

8.8 Once fixed, aspirate PFA in the fume cupboard and add 0.5 mL PBS (-/-). Discard PFA and PFA wash volumes as hazardous liquid waste.



8.9 Repeat PBS wash a further two times. Remove final wash and store hTLS in PBS (-/-) at 4 °C in the collection tube.





Protocol references

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