

# Derivation of axiolooids, an *in vitro* model of somitogenesis and axial development from human pluripotent stem cells

**Yoshihiro Yamanaka**

Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto 606-8501, Japan

**Sofiane Hamidi**

Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto 606-8501, Japan

<https://orcid.org/0000-0002-1189-843X>

**Rolf Ericsson**

Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto 606-8501, Japan

**Sirajam Munira**

Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto 606-8501, Japan

**Cantas Alev** (✉ [alev.cantas.8m@kyoto-u.ac.jp](mailto:alev.cantas.8m@kyoto-u.ac.jp))

Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto 606-8501, Japan

<https://orcid.org/0000-0002-4879-8782>

---

## Method Article

**Keywords:** mesoderm development, primitive streak, paraxial mesoderm, neuromesodermal progenitor cell, tailbud, segmentation clock, oscillation, symmetry breaking, axial elongation, signaling gradients, somitogenesis, somite, epithelialization, pluripotent stem cell, iPSC, Matrigel, retinoid signaling, disease modeling, in vitro organogenesis

**Posted Date:** February 2nd, 2023

**DOI:** <https://doi.org/10.21203/rs.3.pex-2114/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

Here we describe a step-by-step protocol for the derivation of axioids, a pluripotent stem cell (PSC)-based 3D *in vitro* model of human segmentation and somitogenesis. Mesoderm-based axioids capture reproducibly core features of the segmentation process *in vitro* including axial elongation, sequential formation of properly patterned epithelial somites and coordinated oscillatory activity of the segmentation clock. Axioids recapitulate furthermore key morphogenetic and molecular features of post-gastrulation human embryos, including temporally coordinated emergence and spatial organization of somitogenesis-associated mesodermal cell populations, presence of opposing FGF/WNT and RA signaling gradients and spatiotemporally controlled expression of HOX genes. In addition to increasing our still limited understanding of human axial development, axioids can be used to model and study congenital diseases of the human spine, when utilizing patient-like iPSCs with defined pathogenic mutations in genes such as *HES7* or *MESP2*.

## Introduction

Somitogenesis is a core developmental event during which the metameric body plan is laid out in vertebrates. It is well studied in model organisms such as mouse, zebrafish or chick but remains poorly understood in human and other primates<sup>1</sup>. Despite recent progress with pluripotent stem cell (PSC)-based *in vitro* models of organogenesis and embryonic development<sup>2-4</sup>, an experimental model system that can robustly recapitulate core features of somitogenesis *in vitro* remains largely elusive. Using *in vitro*-derived presomitic mesoderm (PSM), we and others have previously succeeded to reconstitute and quantify oscillatory activity of the segmentation clock, a molecular oscillator believed to control somite formation<sup>5-7</sup>. Interestingly, these *in vitro* models of the segmentation clock did not show any sign of segmentation or somitogenesis despite the presence of oscillatory activity of clock genes such as *HES7*. Extending on these earlier findings we then asked whether we could recapitulate not only the segmentation clock but also the actual process of segmentation and epithelial somite formation *in vitro*. Utilizing again PSCs as starting material we established a 3D *in vitro* model of human somitogenesis, which exhibited periodic formation of properly patterned epithelial somites in synchrony with the segmentation clock. Our self-organizing “axioids” shared morphological and molecular features of the emerging vertebrate embryonic tail and axis including presence of somitogenesis associated major cell populations and opposing morphogen gradients and signaling activities. We further demonstrated a critical albeit unexpected function of retinoic acid (RA) signaling in the stabilization of segments, suggesting a synergistic role for RA and extracellular matrix (ECM) components in the formation and epithelialization of somites. Lastly, we applied our bottom-up model system to study the pathogenesis of human congenital diseases of the spine, using patient-like iPSCs with mutations in *HES7* and *MESP2*, which revealed disease-associated phenotypes including loss of epithelial somite formation and abnormal rostrocaudal patterning. These results suggest that axioids represent a promising novel platform to study early embryonic development and disease in humans.

The here described axioloid derivation protocol encompasses a simple stepwise process that involves the initial derivation of primitive streak cells from human PSCs via simultaneous activation of the WNT and FGF pathways (under 2D culture conditions), followed by the induction of presomitic mesoderm (PSM) via TGF- $\beta$  inhibition in mesodermal aggregates forming under non-attachment conditions. These spontaneously symmetry-breaking mesodermal 3D aggregates are then embedded into Matrigel (MG), which leads to enhanced axial elongation and sequential formation of segments. Presence of retinoid signaling during the MG embedding phase dramatically increases stability of the forming segments and ensures proper epithelialization of somites in human axioloids.

## Reagents

### Basic reagents

- 0.5%-Trypan blue stain solution (Nacalai, Cat#: 29853-34)
- Phosphate buffered saline (PBS) tablets (Takara, Cat#: T900)
- 0.5 mmol/l-EDTA/PBS solution (Nacalai, Cat#: 13567-84)
- Albumin, Bovine Serum, F- , pH5.2 (Nacalai, Cat#: 01863-77)
- Polyvinyl Alcohol (PVA) (Nacalai, Cat#: 11738-62)
- StemPro™ Accutase™ cell dissociation reagent (Thermo Fisher Scientific, Cat#: A11105-01)
- TrypLE™ Select enzyme (Thermo Fisher Scientific, Cat#: 12563-011)
- D-Luciferin Potassium Salt (Bioworld, Cat#: 40400076-1)

### Culture medium and extra-cellular matrix components

- Penicillin-Streptomycin (Thermo Fisher Scientific, Cat#: 15140122)
- StemFit® AK02N (Reprocell, Cat#: AJ100) medium is constituted of three different components termed A, B and C that need to be mixed prior to use. In the following protocol:
- AK02N refers to the medium used for hiPSC maintenance; it is obtained by mixing the three components A+B+C and Penicillin-Streptomycin (100U/mL).
- AK02N-C is used for differentiation of hiPSCs and is obtained by omitting the component C and mixing the components A with B only, in addition to Penicillin-Streptomycin (100U/mL).
- Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, Cat#: 356231)

- iMatrix-511 (Nippi, Cat#: 892021 or Cat# 892012)

## **Cytokines and small molecules**

- Y-27632 (Rock-Inhibitor) (Nacalai, Cat#: 1267954)
- Recombinant human bFGF protein (rh-bFGF) (Peprotech, Cat#: 100-18B)
- SB431542 (TGF- $\beta$  inhibitor) (Selleck Chemicals, Cat#: S1067)
- CHIR99021 (GSK3 $\alpha/\beta$  inhibitor) (Nacalai, Cat#: 1876444)
- Retinol (Sigma-Aldrich, Cat#: R7632)
- All trans-Retinal (Sigma-Aldrich, Cat#: R2500)
- Retinoic acid (Sigma-Aldrich, Cat#: R2625)

## **Cell lines**

- hiPSC 409B2 (RIKEN BRC, HPS0076)
- hiPSC 201B7 (RIKEN BRC, HPS0063) and *HES7*-luciferase reporter based on 201B7 as reported in our earlier manuscript on the *in vitro* human segmentation clock<sup>5</sup>.

## **Equipment**

- Electronic pipette (Eppendorf, Cat#: 4861000040)
- Mechanical 12-channel pipette (Eppendorf, Cat#: 3122000060)
- Pipette controller (Drummond, Cat#: 4-040-101-J)
- Pipettes (Gilson, Cat#: FA10001P, FA10002P, FA10003P, FA10005P, FA10006P)
- Pipette tips (Watson, Cat#: 123R-254CS, 123R-755CS, 123R-757CS)
- Serological pipettes (Corning, Cat#: 4487, 4488, 4489, 4490)
- 6 well sterile cell culture plate (WATSON, Cat#: 197-06CPS)
- 96 well flat-bottom sterile suspension culture plate (WATSON, Cat#: 197-96CS or 197-96CIS)

- 96 well glass-bottom EZVIEW plate (Iwaki, Cat#: 5899-096)
- 24 well cell imaging plates (Eppendorf, Cat#: 0030741005)
- PrimeSurface 96U plate (Sumitomo Bakelite, Cat#: MS-9096U)
- Cell scraper (Iwaki, Cat#: 9000-220)
- Cell counting chamber slides (OLYMPUS)
- Automated Cell Counter (OLYMPUS, Cat#: model R1)
- 1.5 ml microtube (Watson, Cat#: 131-715C)
- 15 ml tube (Corning, Cat#: 430791)
- 50 ml tube (Corning, Cat#: 430829)
- CO2 incubator (Thermo Fisher Scientific, Cat#: 51030301)
- Centrifuge (Tomy, Cat#: AX-521)
- Clean bench (Showa Kagaku, Cat#: S-1301PRV)
- Suction pump (Wakenbtech, Cat#: SP40)
- Vortex mixer (Scientific Industries, Cat#: SI-0286)

## Procedure

- The here described induction and differentiation conditions are based on the feeder-free 2D culture of human induced pluripotent stem cell (iPSC) lines 201B7<sup>8</sup> and 409B2<sup>9</sup> on laminin-511 E8 fragment coated cell culture plates with AK02N being the iPSC-culture and maintenance medium of choice.
- Media are always used after being equilibrated 30 minutes to 1 hour at RT except if stated otherwise.
- Stable and high-quality culture of established human iPSC lines is critical for subsequent successful differentiation of mesodermal cell types or induction of axioids described in this protocol (Figure 1).

### A. Human iPSC culture in feeder-free condition

#### a) Coating of cell culture dishes & plates for feeder-free iPSC culture

Conditions described hereafter have been adjusted for a single well of a 6 well culture plate and should be adjusted accordingly (see Table 1).

1. Add 1.5 ml of PBS containing iMatrix-511 (0.5  $\mu\text{g}/\text{cm}^2$ ) into each well of a 6 well culture plate.
2. Incubate the plate for at least 1 hour at 37°C, 3 hours at room temperature or overnight at 4°C. Usually coating is performed overnight at 4°C.
3. Aspirate\* iMatrix-511 containing PBS and add 1.5 ml of StemFit® AK02N containing Y-27632 (10  $\mu\text{M}$ ).
4. Place the plate into a humidified 5% CO<sub>2</sub> incubator at 37°C until further use.

\*Do not let the plate dry out when changing solutions.

*b) Passaging of human iPSCs in feeder-free conditions*

Passage should be done when iPSCs cultures are between 50% and 80% cell confluency.

1. Aspirate the culture medium.
2. Wash the cells with 2 ml of PBS.
3. Aspirate the PBS and add 300  $\mu\text{l}$  of 0.5X TrypLE™ Select solution (1:1 dilution with 0.5 mmol/l-EDTA/PBS).
4. Gently shake the plate to evenly distribute the solution in the well, and place into a humidified CO<sub>2</sub> incubator set to 37°C.
5. After 2 minutes, take out the plate and check the cells under the microscope; if cells have already started to detach move to the next step, otherwise gently shake the plate again to re-distribute the 0.5X TrypLE™ Select solution and place into the incubator for another 2 minutes.
6. Aspirate the TrypLE™ Select solution and gently wash with 2 ml of PBS.
7. Aspirate the PBS and add 1 ml of StemFit® AK02N with Y-27632 (10  $\mu\text{M}$ ).
8. Harvest cells with cell scraper or pipetting into a 1.5 ml microtube.

9. Dissociate the cells by gently pipetting the cell suspension using P1000 pipette (about 15 times) and measure cell density.
10. Seed  $1.3 \times 10^4$  cells/well\* onto the previously prepared iMatrix-511 coated 6 well culture plate containing 1.5 ml StemFit® AK02N with Y-27632 (10  $\mu$ M) (for other plates or dishes see Table 1).
11. Gently shake the plate to distribute cells evenly and place in a humidified 5% CO<sub>2</sub> incubator at 37°C.
12. On the next day, change the medium to AK02N without Y-27632.
13. Change medium every other day until day 4, then every day until axioloid induction at day 5 or passage\*\*.

\* Initial cell seeding density should be adjusted in order for hiPSCs to be at ~60% confluency at day 5 of culture.

\*\* Cells should not be cultured for more than 7 days before being passaged.

## **B. Induction of axiolooids from human iPSCs**

### *a) Initiation of differentiation of hiPSCs under 2D culture conditions*

At day 5 of culture, check the quality of cultured iPSCs\* and confirm that they reached ~60% confluency (good initial cell density is a critical factor!).

1. Prepare 1.5 ml per well (when using a 6 well plate) induction medium consisting of AK02N-C supplemented with CHIR 5  $\mu$ M\*\* and bFGF 20 ng/ml.
2. Expose the cells to the induction medium and culture for 24 hours.

\* If hiPSC conditions are not good (e.g. confluency too high or too low, many spontaneously differentiated cells are present or colony sizes/shapes are unusual) do not use cells for induction.

\*\* We found that CHIR concentrations need to be slightly adjusted depending on the cell line which is used. In our hands, for most human iPSC lines the optimal CHIR concentration is usually in the range of 4-6  $\mu$ M.

b) Aggregation step and 3D differentiation of cells

24 hours after initial primitive streak/mesoderm induction with WNT agonist and FGF recombinant protein, treated cells are detached and aggregated under 3D conditions in non-attachment plates.

1. Prepare aggregation medium by supplementing AK02N-C with CHIR (use a concentration identical to the one used for the initial 2D induction phase), bFGF 20 ng/mL, SB431542 10  $\mu$ M, and Y-27632 10  $\mu$ M.
2. Wash the cells with 2 ml of PBS.
3. Add 0.3 ml of Accutase<sup>TM</sup> and distribute it evenly in the well by gently shaking the plate.
4. Incubate for an initial 2 minutes in a humidified CO<sub>2</sub> incubator set to 37°C .
5. Observe the cells under the microscope whether they are starting to detach or not; if not, re-distribute the Accutase<sup>TM</sup> and incubate it for another 2 minutes.
6. Neutralize by adding 0.7 ml of aggregation medium.
7. Harvest the cells using a cell scraper or pipetting into a 1.5 ml tube and centrifuge (800 rpm, 22°C, 4 minutes).
8. Aspirate the supernatant and add 1 ml of aggregation medium.
9. Mechanically dissociate the cells by gently pipetting (about 15 times) with P1000 and measure the cell concentration.
10. Prepare a cell suspension of  $1.0 \times 10^4$  cells/ml (in aggregation medium).
11. Add 50  $\mu$ l of this cell suspension per well into a 96 well U-bottom low attachment plate (500 cells/well)\* using an electronic pipette or mechanical 12-channel pipette.
12. Centrifuge the plate (800 rpm, 22°C, 2 minutes).
13. Place the plate in the cell incubator at 37°C for 24 hours.
14. The next day (day 1), add 150  $\mu$ l per well of AK02N-C medium.
15. The following day (day 2) aspirate delicately 150  $\mu$ l of the culture medium per well (using a 12-channel pipettes) and replace with 150  $\mu$ l of fresh AK02N-C medium.

\* The initial number of aggregated cells is critical to obtain proper axial elongation and subsequent segmentation and somite formation; carefully homogenize your cell suspension and check for high survival rate of dissociated cells prior to aggregation. Initial cell number to be used might vary and needs to be optimized depending on the used cell line. We usually use 500 cells/well but slightly higher or lower cell numbers will work depending on utilized cell line.

c) Matrigel embedding of hiPSC-derived 3D mesodermal aggregates

72 hours after the initial WNT and FGF pulse, axioids which have undergone initial symmetry breaking and have started to slightly elongate are embedded into AK02N-C supplemented with 10% Matrigel (MG). To stabilize forming segments and induce epithelialization of somites, retinoid signaling molecules have to be added, *i.e.* add retinol (10  $\mu$ M), all trans-retinal (1  $\mu$ M) or retinoic acid (100 nM) to the MG containing embedding medium\*.

1. On ice, prepare embedding medium by mixing cold (4°C) AK02N-C with 10% cold (4°C) Matrigel\*\*.
2. Add 80  $\mu$ l of 1% BSA in PBS into each well of a 96 well flat-bottom suspension culture plate in order to quench possible adhesion of axioids to the walls/bottom of the well.
3. Aspirate the BSA solution, place the plate on ice and add 80  $\mu$ l of embedding medium into each well with an electronic pipette.
4. Carefully transfer\*\*\* each axioid into a well of a 96 well flat-bottom suspension culture plate containing embedding medium. Using a P2 pipette aspirate each axioid one by one without damaging them and in the smallest volume possible, e.g. 1-1.5  $\mu$ l\*\*\*\*.
5. After 24 or 48 hours, embedded axioids can be fixed and processed for further staining or differentiation.

\* Other molecules, *e.g.* cytokines, small molecule agonists or inhibitors can also be added directly into the embedding medium.

\*\* Matrigel concentration can be lowered to 5% without any apparent effect on axioid induction efficiency or morphogenesis of axioids. Other Matrigel-equivalent ECM compounds or mixtures can be also used but need to be tested and titrated accordingly.

\*\*\* Mesodermal aggregates/axioids are transferred manually; to be able to see small aggregates place your 96 well in front of a white background and tilt it slightly or place an LED light (avoid heat) behind the

plate and tilt it to see the aggregates.

\*\*\*\*Axioloids to be embedded into Matrigel should be individually selected based on their general morphology. Axioloids with cylindrical elongation features are ideal, nevertheless the more asymmetrical ones can be also chosen if their NMP containing tailbud region ( $BRA^+/SOX2^+/MEOX1^-$ ) is still large. In general, the tailbud end should always be the same size or bigger than the anterior ( $MEOX1^+$ ) “head part” (see Figures 2 and 3). If you embed inappropriate or suboptimal “starting points” into Matrigel +/- retinoids, axioloids will not elongate or epithelialize properly (Figure 4). Optimal “starting shapes” might differ between utilized PSC lines and need to be determined for optimal outcomes.

### C. Analysis of axioloids

#### a) Live imaging and luminometric analysis of axioloids

Using *HES7*-Luciferase reporter iPSC line (201B7 Luc)-derived axioloids we can visualize the oscillatory expression of the segmentation clock associated gene *HES7*. To this end axioloids are embedded into MG-containing medium (+/- retinoids) supplemented with 100  $\mu$ M of luciferin in specific culture vessels. For Kronos HT luminometer measurements 24 well cell imaging plates are used and for IX-83-based bioluminescent imaging 96 well EZVIEW glass bottom plates coated with 1.5% of PVA are used. Other live imaging experiments are performed in standard cell suspension culture plates used for standard axioloid embedding. Further details on various molecular and functional assessment methods and analytical tools applied for characterization of axioloids can be found in our accompanying manuscript.

#### b) Collection and fixation of axioloids

- Prepare a wash buffer (BSA 0.1%, EDTA 0.25 mM in PBS).
- Coat any tips or tubes with the wash buffer prior to use.
- Collect axioloids in a pretreated tube.
- Wash 3 times with wash buffer; between each washing step let the aggregates sink to the bottom of the tube.
- Aspirate carefully the supernatant and proceed with fixation process.

#### Fixation for immunofluorescence (IF):

- Fix with 4% PFA for 30 minutes at RT.

- Remove PFA and wash 3 times with the wash buffer for 5 minutes each.
- Store in PBS at 4°C until further processing.

### Fixation for in situ hybridization chain reaction (HCR):

- Fix in 4% PFA 1 hour at RT.
- Wash 3 times with PBST for 5 minutes each.
- Add 1 mL of 100% MeOH per tube.
- Store at -30°C until further processing.

## Troubleshooting

### 1. PSCs start detaching before passage

- iMatrix-511 coating was not done properly or dried during passage.

### 2. Initial PSC culture seems not optimal at day 5

- Initial cell seeding density should be adjusted in order for hiPSCs to be at ~60% confluency at day 5 of culture.
- Cells should not be cultured for more than 7 days before being passaged or it will result in the presence of differentiated cells or unusual colony sizes and/or shapes.

### 3. Cells do not aggregate or high cell death rate during the aggregation step

- Aspirate carefully the supernatant after initial centrifugation to remove any traces of active Accutase.
- Reduce the time between cell detachment and aggregation; as a general rule we suggest to make aggregates as fast as possible and in general ~30 minutes per cell line/conditions.
- Avoid detaching/aggregating too many cell lines and/or conditions in parallel.

### 4. Aggregates at 24 hours have variation in size

- Make sure to regularly and carefully homogenize your cell solutions and do not let cell solutions sit for extended periods of time before transferring them into the 96 well U-bottom plate.
- Make sure you centrifuge the plate before placing it into the incubator.

#### 5. Aggregates at 72 hours have abnormal shapes compared to recommended ones

- Optimize the initial CHIR concentration.
- The initial number of aggregated cells is too big or too small.
- Passage number of used iPSC line is too high; use early passage numbers.

#### 6. Axioloids do not elongate or segments don't form properly upon Matrigel embedding

- The initial cell culture quality was not appropriate and/or contained differentiated cells.
- The cell line has been in culture for a long time (high passage number), please unfreeze a low passage number of the iPSC line in question; if possible, unfreeze and restart culture of cells from early passages every 2 months. Freshly unfrozen cells should be passaged two or three times prior to be used for axioloid induction.
- Medium was old; do not use AK02N medium (induction or maintenance) for more than two weeks.
- Prepare fresh induction and aggregation mediums every time you use.
- Use new growth factors/small molecule aliquots and avoid repeated freeze and thaw cycles.
- Mix Matrigel and AK02N-C medium well on ice.
- Make sure to not damage the aggregates with the tips when transferring them into the new 96 well plate.
- Axioloids are too close to the edge of the wells. When transferring individual aggregates to AK02N-C + MG (+/- retinoid) containing medium be sure to position them closest to the center of each well. Position can be adjusted by gently tapping the edges of the plate.
- Matrigel in the embedding medium reticulated before aggregates were placed; keep the plate on ice until you finish transferring all aggregates into the 96 well plate.
- The aggregates picked for embedding were not optimal. Ensure that optimal "embedding starting points/shapes" are used; do not use 72h axioloids with unusual or abnormal sizes or shapes.

- Embedding medium does not contain retinoids; in the absence of retinoids in the culture, forming segments do not stabilize and segments within axiolooids become disorganized and/or disappear over time. Make sure to have proper concentrations of retinoids (retinol, retinal or retinoic acid) present in the embedding medium. Recommended concentrations and timing of retinoid addition usually works for 409B2 and 201B7-Luc but may need to be optimized depending on utilized iPSC lines.

#### 7. Axiolooids attach to the bottom of the well

- The 1% BSA in PBS solution used for coating of wells is too old.
- The 1% BSA in PBS solution has not been spread correctly.

#### 8. Axiolooids attach to the surface of 1.5 ml tubes or tips

- Use BSA 0.1%, EDTA 0.25mM in PBS for washing and make sure to coat the tips and tube surfaces with this solution.
- Gently mechanically flush and detach attached axiolooids using a P1000 pipette.

## Time Taken

The total duration of this protocol is six (6) days starting from initial induction under 2D conditions considered as  $t = -24$  hours until termination of the experiment (e.g. final fixation) at  $t = 120$  hours. The cell detachment and aggregation step is considered to be  $t = 0$  hour (Figure 1).

Times described hereafter include the time for medium preparation:

- **Initial induction ( $t = -24$  hours):** approximately 15 minutes per condition.
- **Making aggregates ( $t = 0$  hours):** approximately 30 minutes per condition.
- **Changing medium ( $t = 24$  hours and 48 hours):** approximately 5 minutes per plate.
- **Embedding axiolooids into Matrigel ( $t = 72$  hours):** approximately 45 minutes per plate.
- **Fixation of axiolooids:** approximately 45-60 minutes per plate.

## Anticipated Results

When the initial steps of axiolooid induction are performed under optimal conditions with

- correct/ideal cell density during initial FGF/WNT pulse
- correct/ideal CHIR concentrations
- correct/ideal cell number used for initial aggregates
- correct/ideal timing of aggregate embedding into MG
- correct/ideal iPSC line used for axioid induction

the efficiency of axioid induction after Matrigel embedding at 96 or 120 hours is extremely high (usually more than 80%). Obtained axioids can be classified into 4 categories based on their overall morphological features (please see Extended Data Fig. 1e and Supplementary Figs. 1a, 2b and 2c in corresponding publication) with at least ~50% of axioids usually showing nice elongation and clear segment/somite formation.

2D PSC culture condition at the time of initial induction, characterized by the overall cell number, colony diameter and shape of colonies, is critical for the success of axioid derivation possibly due to variations in mesoderm induction efficiency based on e.g. differences in epithelial status of the cells in treated PSC colonies<sup>10,11</sup>.

A similar phenomenon might be responsible for variations in both efficiency and observed phenotypes when modulating the number of cells which are aggregated. This may be due to differences in the effect of cytokines and small molecules on cells located either in the center or periphery of the forming aggregates.

Properly induced axioids will spontaneously break symmetry and form a pole high in “progenitor cells” positive for TBXT (BRA) and SOX2. This initial polarization process will in turn initiate an elongation process resulting in the transition from a spherical aggregate at 24 hours to a “bean shaped” one at 72 hours. From this point onwards and upon Matrigel embedding, aggregates will undergo enhanced axial elongation and segmentation along their antero-posterior axis, forming individual segments that epithelialize upon MG embedding and retinoid stimulation.

Embedded axioids can be imaged directly in the 96 well plate in which they were derived or transferred into designated plates or dishes during the embedding stage for more specific analytical purposes, such as studying the oscillatory expression of the segmentation clock gene *HES7*. Axiooids can furthermore

be e.g. fixed and stained for confocal imaging or dissociated and processed for single cell RNA-seq analysis.

## References

- 1 Maroto, M., Bone, R. A. & Dale, J. K. Somitogenesis. *Development* **139**, 2453-2456, (2012).
- 2 Moris, N. *et al.* An in vitro model of early anteroposterior organization during human development. *Nature* **582**, 410-415, (2020).
- 3 Rossi, G. *et al.* Capturing Cardiogenesis in Gastruloids. *Cell Stem Cell* **28**, 230-240 e236, (2021).
- 4 Olmsted, Z. T. & Paluh, J. L. Co-development of central and peripheral neurons with trunk mesendoderm in human elongating multi-lineage organized gastruloids. *Nat Commun* **12**, 3020, (2021).
- 5 Matsuda, M. *et al.* Recapitulating the human segmentation clock with pluripotent stem cells. *Nature* **580**, 124-129, (2020).
- 6 Diaz-Cuadros, M. *et al.* In vitro characterization of the human segmentation clock. *Nature* **580**, 113-118, (2020).
- 7 Chu, L. F. *et al.* An In Vitro Human Segmentation Clock Model Derived from Embryonic Stem Cells. *Cell Rep* **28**, 2247-2255 e2245, (2019).
- 8 Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872, (2007).
- 9 Okita, K. *et al.* A more efficient method to generate integration-free human iPS cells. *Nat Methods* **8**, 409-412, (2011).
- 10 Ismagulov, G., Hamidi, S. & Sheng, G. Epithelial-Mesenchymal Transition Drives Three-Dimensional Morphogenesis in Mammalian Early Development. *Front Cell Dev Biol* **9**, 639244, (2021).
- 11 Hamidi, S. *et al.* Mesenchymal-epithelial transition regulates initiation of pluripotency exit before gastrulation. *Development* **147**, (2020).

## Acknowledgements

The authors thank all members of ASHBi at Kyoto University; this work was supported by a Naito Foundation Scientific Research Grant to C.A.; Takeda Science Foundation Grant to C.A.; Japan Agency for Medical Research and Development (AMED) Grant Number JP20bm0704050 to C.A.; the ISHIZUE 2020 of Kyoto University Research Development Program to C.A.; the ASHBi Fusion Research Program to Y.Y., C.A.; ASHBi is supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan.

# Figures

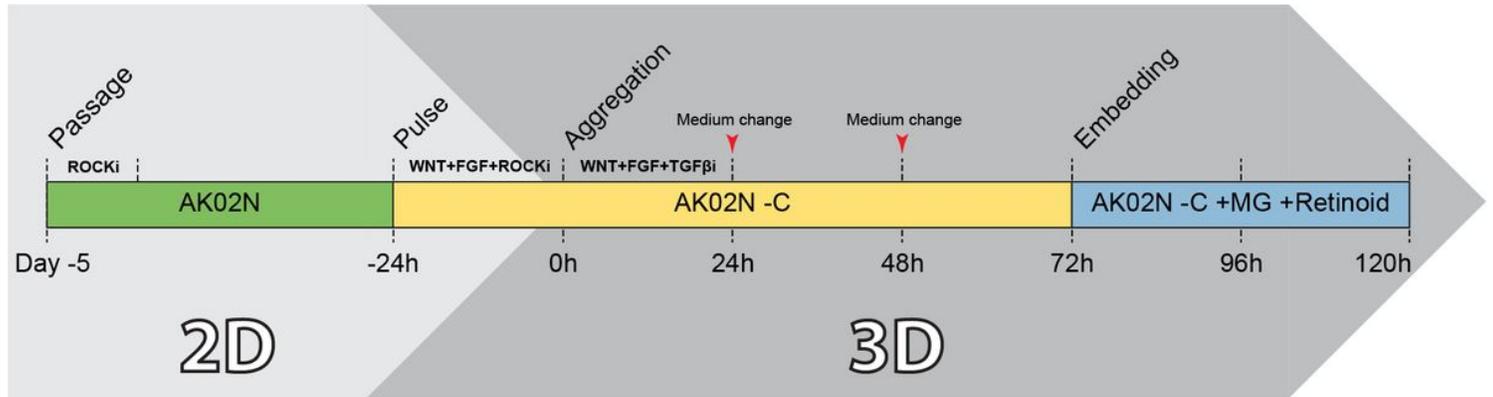


Figure 1

Overview of the axioid induction protocol.

72h

Good



Bad



Figure 2

Images of human axioids at 72 hours.

Examples of good (top) and bad (bottom) axioids at 72 hours prior to embedding into MG. Scale bar is 200  $\mu$ m.

409B2\_72h

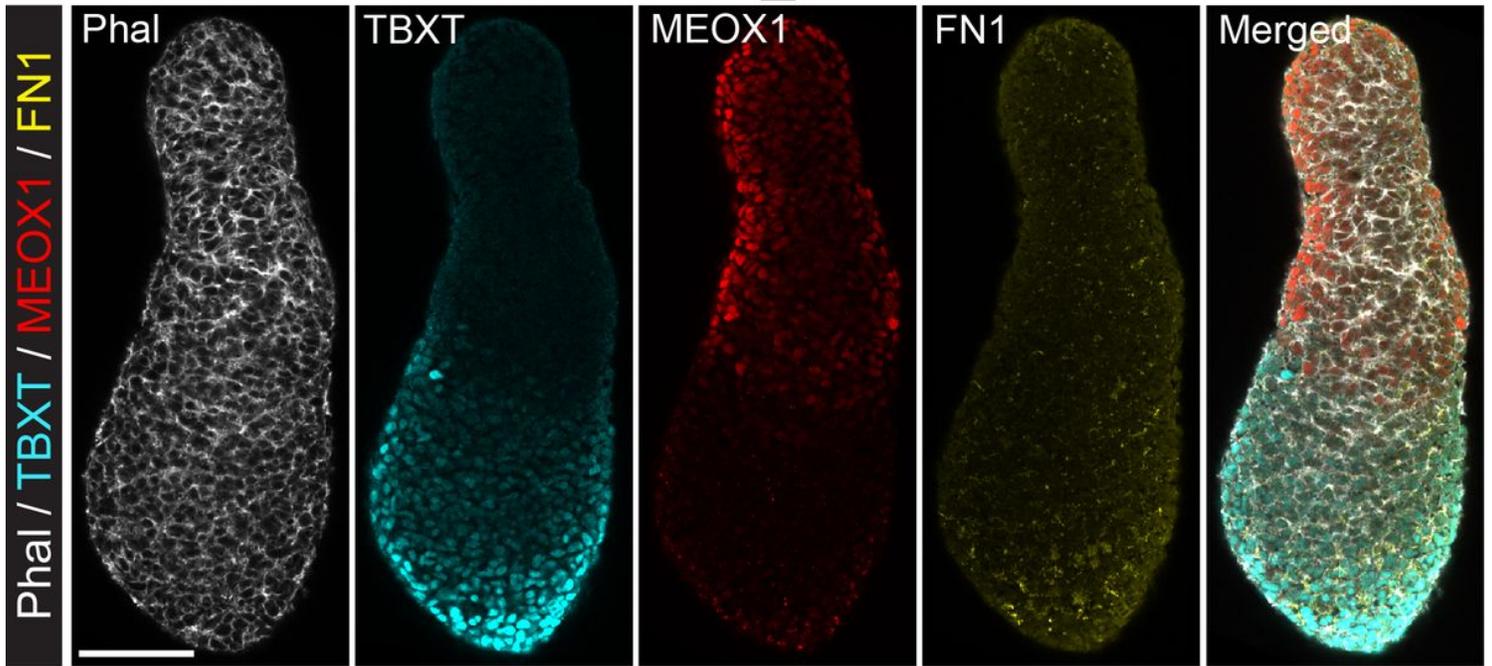
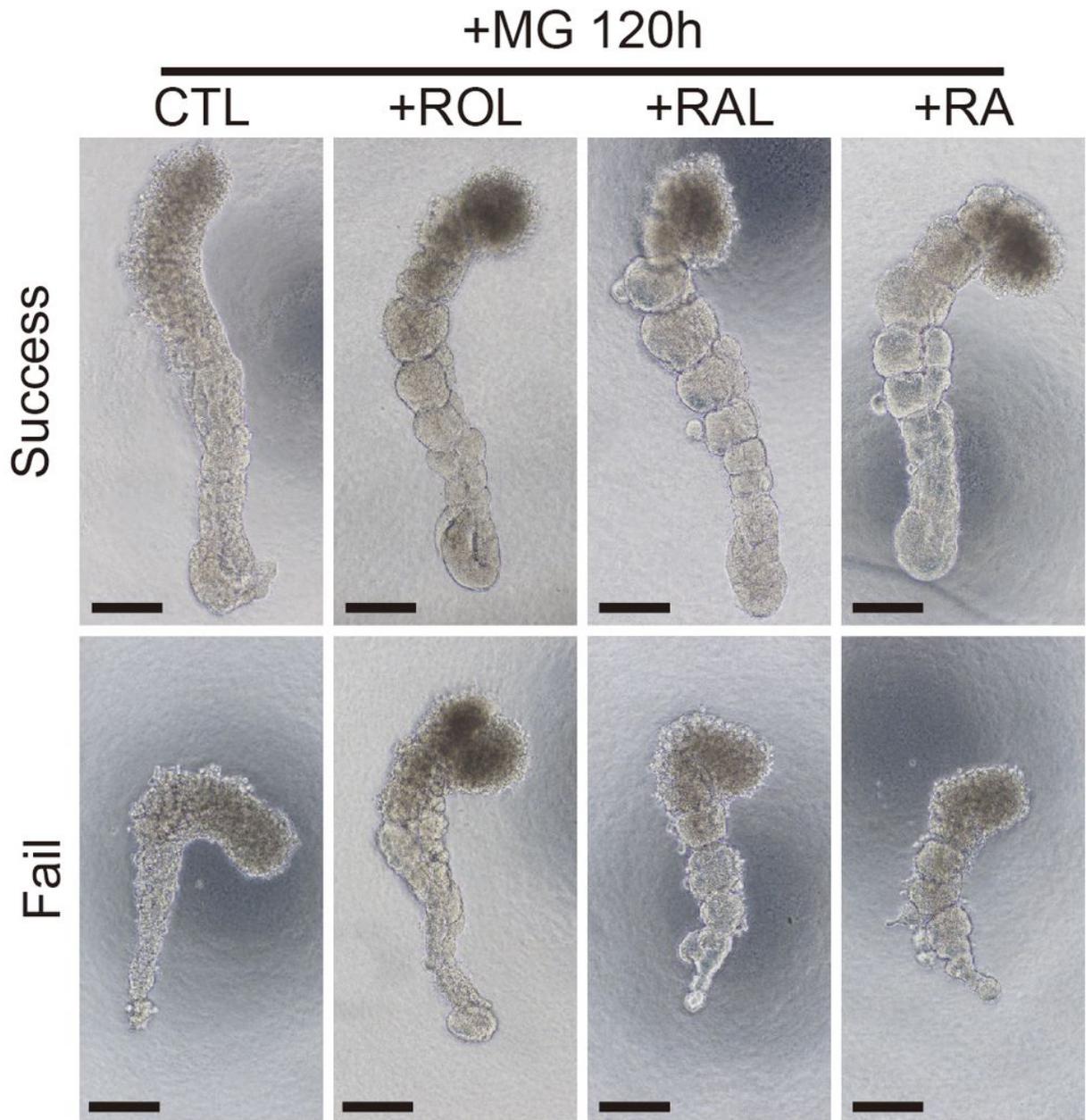


Figure 3

Confocal image of human axioid at 72 hours.

Example of a good axioid at 72 hours, stained for Phalloidin in gray, Brachyury (TBXT) in cyan, MEOX1 in red and Fibronectin in yellow. Scale bar is 100  $\mu$ m.



**Figure 4**

**Images of human axioids at 120 hours.**

Examples of successful (top) and failed (bottom) axioids after Matrigel embedding supplemented with different retinoids (retinol (ROL), retinal (RAL) or retinoic acid (RA)). Scale bar is 200  $\mu$ m.

## Supplementary Files

This is a list of supplementary files associated with this preprint. [Click to download.](#)

- [Table1.xlsx](#)