Gastruloids: embryonic organoids from mouse embryonic stem cells to study patterning and development in early mammalian embryos.

Kerim Anlas[1#], Peter Baillie-Johnson[2#], Krisztina Arató[1], David A.Turner*[3], Vikas Trivedi*[1]

*Authors for correspondence: *dat40@liverpool.ac.uk, trivedi@embl.es*

[#]These authors contributed equally to this work

1 – European Molecular Biology Laboratory, Dr. Aiguader, 88, 08003 Barcelona, Spain

2 – Wellcome - MRC Cambridge Stem Cell Institute, University of Cambridge

3 – Institute of Ageing and Chronic Disease, William Henry Duncan Building, University of Liverpool

Abstract

Gastruloids are embryonic organoids made from small, defined numbers of mouse embryonic stem cells (mESCs) aggregated in suspension culture, which, over time form 3D structures that mimic many of the features of early mammalian development. Unlike embryoid bodies, that are usually disorganized when grown over several days, gastruloids display distinct, well organised gene expression domains demarcating the emergence of the three body-axes, anteroposterior axial elongation, and implementation of collinear Hox transcriptional patterns over 5-7 days of culture. As such *gastruloids* represent a useful experimental system that is complementary to *in vivo* approaches in studying early developmental patterning mechanisms regulating the acquisition of cell fates. In this protocol, we describe the most recent method for generating gastruloids with high reproducibility, and provide a comprehensive list of possible challenges as well as steps for protocol optimization.

Key Words

Gastruloids, gastrulation, organoids, mouse embryo, axial development, mouse embryonic stem cells

1. Introduction

The initial body-plan of the embryo is established during early development, where specific regions of gene-expression are defined that serve as a blueprint for coordinating the growth and patterning of the embryo over time. In mammalian embryos, the zygote develops two distinct cell populations, inner cell mass (ICM) and trophectoderm, the former being the source of embryonic stem cells. Around implantation, the ICM segregates into the primitive endoderm and epiblast, with the epiblast giving rise to all tissues of the embryo proper. Prior to gastrulation (which transforms a bilayered embryo into one comprised of three germ layers), a subpopulation in the proximal posterior region of the epiblast initiates the expression of *T*/*Brachyury* (T/Bra) defining the site of gastrulation and the formation of the primitive streak.

Owing to the inaccessibility of mammalian embryo at these early stages of development, studying many of the cellular processes involved in lineage specification *in vivo* has been technically challenging. Embryonic stem cells (ESCs) provide a potential, inexpensive solution to dissect these events as they can generate all tissues of the embryo proper, can be maintained indefinitely in culture, and their directed differentiation can yield different cell fates ***(1-8)***. Studies conducted with mouse ESCs (mESCs) in particular have informed our understanding of the role and requirement of specific signalling molecules and transcription factors in early mouse development. However, these studies have been performed on cells grown in a monolayer (2-dimensional; 2D) which lack the architecture of a 3-dimensional (3D) embryo.

Recently, more attention has been given to developing techniques that allow cells to be cultured in 3D, building on previous work on hanging-drop and mechanically supported cultures ***(9)*** to generate 3D structures termed *organoids*. Organoids, which can be derived from stem cells (embryonic and adult) or fragments of *in vivo* tissues (such as intestinal crypts), mimic the structural architecture and, to some extent the function, of their *in vivo* counterparts. Examples include mesodermal derivatives ***(10,11),*** intestinal ***(12)***, gut ***(13)***, kidney ***(14, 15)***, brain ***(16, 17)***, retinal ***(18, 19)***, and neural ***(20)*** organoids. Together they represent a class of new approaches and model systems to understand embryogenesis *in vitro* ***(21-22)***.

1.1 – Gastruloids and other embryonic organoids

Work by van den Brink *et al.* ***(23)*** showed that a defined number of mESCs when aggregated to form initially spherical 3D structures, mimic morphogenetic events of early mouse embryos, such as polarization of gene expression, primary axis formation, elongation and associated patterning; notably in the absence of extraembryonic tissues and nearly all associated signalling cues. This system, termed *gastruloids,* has been developed since then, demonstrating that mESCs in such aggregates, in a serum- and matrigel-free environment, display gastrulation-like movements and develop gene expression domains associated with all germ layers as well as the three body axes, accompanied by timely implementation of collinear Hox transcriptional patterns over 5-7 days of culture ***(24-27).*** In this article we outline a unified protocol for generating *gastruloids* that has been developed across several labs.

As an alternative to *in vivo* studies of early embryogenesis and pattern formation, several embryonic organoid model systems have been developed, each with its own unique strengths and it is important to recognise key differences between them and *gastruloids* discussed in this protocol. Embryoid bodies, first developed by ten Berge *et al* ***(10)***, differ in both the protocol (in terms of number of cells and media composition) as well as the final outcome. Unlike *gastruloids*, that are grown in serum free media and start with 300 mESCs, embryoid bodies are typically grown in media with serum and start with ~1000-2000 cells in suspension. Two other remarkable systems use both embryonic and extraembryonic stem cells to mimic embryogenesis *in vitro* at two distinct stages: (i) *blastoids*, which are aggregates of trophoblast and embryonic stem cells that resemble embryonic

day 3.5 blastocysts ***(28,29)*** (ii) ETX embryos that consist of embryonic, trophoblast and extra-embryonic endoderm stem cells and mimic an epiblast with embryonic and extra-embryonic compartments ***(30,31)***. These differ from gastruloids that consist of only embryonic stem cells and thereby provide an opportunity to study the self-organizing potential of embryonic cells in the absence of any extra-embryonic tissue. Altogether, gastruloids represent a highly tractable, medium-throughput *in vitro* system that is suitable for live-imaging, and for dissecting and elucidating the underlying events involved in early mouse development such as symmetry breaking, cell fate decisions and tissue patterning dynamics.

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2. Materials

2.1 Cell lines tested with this protocol

We have tested a number of cell lines from various backgrounds, assessed their ability to generate gastruloids and modified their culture conditions to ensure they are in a similar responsive state just prior to *gastruloid* formation. We usually consider the frequency and extent of elongation observed in *gastruloids* at around 96-120h as a proxy for assessing how 'competent' the cells are in responding to differentiating signals when taken from culture prior to the gastruloid protocol. While the number of cells that usually yields elongating gastruloids starting from 72hrs in culture is typically 300, certain cells lines require an optimization of the number of cells. Certain cell lines might also require a day long exposure to 2i+LIF medium (termed '2iL-Pulse', [ESL-2iL]) prior to the *gastruloid* protocol.

2.2 Routine Culture Medium

For the following medium, ensure the maximum volume remains 500ml, removing sufficient volume of the base medium to allow for the total volume of medium supplements. Always avoid using 'old' medium where the pH is too high (i.e. purple medium when phenol red is the pH indicator). Store all complete medium at 4°C and warm to 37°C before use.

2.2.1 ESL Medium:

This is the standard medium for culturing most mESC lines.

a. Base medium:

   i) 500ml Glasgow's Minimal Essential Medium (GMEM, Gibco 11710-035)

   Or

   ii) 500ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco 11960044)

b. 550µl mouse Leukaemia Inhibitory Factor (mLIF; 1000U, either inhouse or commercial)

c. 50ml foetal bovine serum (FBS; 10% final concentration, requires batch testing for specific applications)

d. 5ml Non-Essential Amino Acids (NEAA; 100x, Thermo Fisher Scientific 11140050)

e. 5ml Sodium Pyruvate (100x, Thermo Fisher Scientific 11360070)

f. 5ml Glutamax (100x, Thermo Fisher Scientific 35050038)

g. 1ml 2-mercaptoethanol (0.1 mM final concentration, Thermo Fisher Scientific 31350010)

Note 1: Other base media can be used depending on the mESC line in question. For most mESC lines, either GMEM or DMEM is sufficient. Also different labs might use slightly different concentrations of the components b-g for making ESL medium that works for lab-specific culture conditions.

Note 2: Although we do not routinely use antibiotics in mESC culture for making gastruloids, their use is optional. It is important to monitor the cell line over time as low-level infection suppressed by antibiotics may be misrepresented as non-contamination, and the down-stream effects of this have not been examined regarding gastruloid culture.

2.2.2 N2B27/NDiff227:

This medium can either be commercially bought (NDiff227; Takara Y40002) or made in-house (N2B27) as described previously ***(4, 32)*** and below:

a. 250ml DMEM/F12 (50:50; Thermo Fisher Scientific 11320074)

b. 250ml Neurobasal (Thermo Fisher Scientific 21103049)

c. 2.5ml N2 (100x; Thermo Fisher Scientific 17502048)

d. 5ml B27 (50x; Thermo Fisher Scientific 17504044)

e. 5ml glutamax (100x; Thermo Fisher Scientific 35050038)

f. 1ml 2-mercaptoethanol (Thermo Fisher Scientific 31350010)

g. BSA fraction V (Gibco™ 15260037)

Note 1: N2 and B27 show batch variability and each batch must be tested prior to use. Note that NDiff227 must be defrosted carefully as described below (section 3.2). BSA fraction V is optional. Also note that the concentration of N2 used is 0.5x final concentration.

*Note 2: N2B27 can be modified depending on the downstream application, as indicated in a recent publication **(4)**, where three versions of N2B27 are provided.*

2.2.3 2iL:

a. 50ml N2B27 (or NDiff227)

b. 3µM CHIR99021 (Chi; 10mM stock dissolved in DMSO, Tocris Bioscience 4423)

c. 1µM PD0325901 (PD03; 10mM stock dissolved in DMSO, Tocris Bioscience 4192)

d. 55µl mLIF

2.3 Tissue-culture Reagents

a. 1x $PBS^{+/+}$ (with Ca^{2+} and Mg^{2+})

b. Gelatin; a 1% (w/v) stock solution prepared in sterile water and autoclaved. Further diluted to 0.1% (v/v) in PBS (with Ca^{2+} and Mg^{2+}). *Alternatively acquire ready-to-use 0.1% gelatin in H_2O suitable for ESC culture applications.*

c. Trypsin-EDTA (0.05%, Thermo Fisher Scientific 25300054)

2.4 Tissue-culture plastics and equipment

a. Tissue culture treated flasks: 25cm^2 flasks routinely used; coated in 0.1% gelatin before use
b. 50ml or 15ml sterile centrifuge tubes
c. Sterile reservoir
d. U-Bottomed 96-well plate (Greiner Bio-One 650185)
e. U-Bottomed 96-well plate with low-adherence (for extended culture to 144h; Greiner Bio-One 650970 or Corning® CLS7007)
f. Low-adherence 24-well plate for extended culture (from 120h-168h)
g. Cell counter/haemocytometer
h. BSL-2 biosafety cabinet
i. Benchtop centrifuge with capacity for 15ml or 50ml centrifuge tubes
j. Water Bath set to 37°C
k. An optional requirement for an incubator-compatible horizontal shaker (Infors Celltron 69222) for extended culture up to 168h after aggregation
l. Inverted benchtop tissue-culture microscope
m. Multichannel pipette(s) for 40µl and 150µl volumes

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3. Methods

3.1 Routine cell culture

In order to generate consistent gastruloids, it is essential that the cells are well maintained and that they are competent to respond to differentiating signals. Ensure cells are *Mycoplasma* free and have not been maintained in culture for excessive passage numbers which, while naturally dependent on the "age" of the respective cell line, generally amounts to a maximum of around 25-30 passages from thawing a fresh aliquot. Cells must be in culture for 2-3 passages after defrosting before making gastruloids. Typically, cells are cultured in 25cm^2 tissue culture-treated flasks that have been coated with 0.1% gelatin/PBS or 0.1.% gelatin/H_2O, in GMEM or DMEM containing serum and LIF (see above for formulation; ESL). Ensure medium and Trypsin is warmed up to 37°C prior to use.

3.1.1 Prepare a fresh 25cm^2 tissue-culture flask and coat with 3ml 0.1% gelatin/PBS for at least 30 min at RT. Coating can continue overnight at room temperature (RT) if required. Aspirate gelatin solution just prior to use.

3.1.2 When the flask containing mESCs is approximately 60% confluent, aspirate the culture medium and wash twice with PBS to remove traces of serum. Aspirate PBS and incubate at 37°C until cells have detached (<5 min) in the humidified tissue-culture incubator with 2ml Trypsin. Gentle mechanical agitation aids cell detachment.

3.1.3 Once the cells have detached, inactivate the Trypsin with 8ml ESL, gently pipetting up and down with a 10ml pipette over the growth surface ~3-4 times to dislodge any cells still adherent and to break up any remaining clumps of cells.

3.1.4 Transfer cells to a centrifuge tube (50ml or 15ml) and centrifuge for 3 min at 170*g* (~1000rpm). Following centrifugation, aspirate the supernatant carefully to prevent the pellet being dislodged, and resuspend in an appropriate volume of fresh ESL (i.e. 1ml). Ensure

generation of a homogeneously dispersed single cell suspension by gentle repeated pipetting with a P1000 pipette, avoiding the generation of bubbles.

3.1.5 Determine the cell density by counting with an automated cell counter or a haemocytometer. Remove gelatin from the fresh tissue-culture flask, and plate an appropriate number of cells in 6ml ESL. *Note: an appropriate number of cells plated is the number which is required to produce a flask after 2 days that is 60-70% confluent. This is specific for each cell line. Depending on an experimentalist's experience, cell counting can be omitted and cells can be split into a new gelatin-coated flask at a ratio between 1:5 to 1:20, provided that appropriate confluency is reached after 2 days.*

3.1.6 Place the flask carefully in the humidified 37°C incubator (5% CO_2), and ensure the cells are evenly spread along the tissue-culture flask.

3.1.7 The next day, check the cells to ensure they are growing well (estimated confluency at this stage is ~20%). Aspirate medium and replace with fresh ESL.

3.2 Preparation of N2B27

If using commercial N2B27 (NDiff227), extreme care is required when defrosting the stock bottle to ensure there is no precipitation in the medium which would interfere with gastruloid aggregation and their on-going culture. It has been found that NDiff227 readily precipitates during defrosting, and the following method (adapted from the manufacturer's instructions) prevents this from occurring.

3.2.1 Place NDiff227 in the water-bath set to 37°C and immediately protect from light via covering with aluminium-foil or using a darkened incubator lid.

3.2.2 After 15 min, remove the stock bottle from water-bath and gently invert it repeatedly (~3-4 times) to gradually equilibrate the temperature around the bottle. Place the bottle back in the water-bath (protected from light).

3.2.3 Continue to return to and invert the bottle every 5 min. Remove the bottle from the water-bath at the point where the ice has dissolved to the size of a ball of ~4-5cm, invert the bottle once more and place in the fridge at 4°C overnight.

3.2.4 The next day, check for precipitate and if clear, invert the bottle 3-4 times (to fully mix and avoid any concentration gradients that have formed) and aliquot. Store aliquots at 4°C protected from light for a month.

3.3 Preparing cells for Gastruloid plating

We have developed two protocols for culturing cells in preparation for gastruloid formation (Fig. 1): Protocols [ESL-ESL] and [ESL-2iL]. The first protocol [ESL-ESL] is the 'standard protocol' *(24-28)*, where cells are exposed to their normal culture medium for the duration of the passage, whereas the second protocol [ESL-2iL] requires a pulse of 2iL medium on the second day in culture. It must be stressed that this second protocol is not necessary for all cell lines, and is only necessary if the cells produce variable gastruloid morphology by 120h (i.e. <70% consistent elongation). Further, the end results in terms of gastruloid morphology and gene expression may be different for the two protocols. See Table 1 for the cell lines we have tested and their requirement for 2iL pre-treatment [ESL-2iL].

3.3.1 For the passage before plating gastruloids, passage the cells as described above (section 3.1), and plate the required number of cells into two 25cm^2 tissue-culture flasks in 6ml routine cell culture medium. Incubate overnight in a humidified incubator (37°C; 5% CO_2).

The first flask is to continue the culture of cells ('passage flask' Fig. 1; section 3.1) whereas the other is solely for gastruloid culture ('gastruloid flask'; Fig. 1).

3.3.2 The next day, aspirate the medium in the 'gastruloid flask', and replace with either 6ml of routine tissue-culture [ESL-ESL] or wash cells with 6ml $PBS^{+/+}$ and then replace with 2iL [ESL-2iL], depending on the cell line. Incubate cells overnight in a humidified incubator (37°C; 5% CO_2).

3.3.3 Aspirate the medium in the 'passage flask', and replace with normal culture medium. This flask will be used to continue the culture of the cell line in parallel to gastruloid culture (section 3.1).

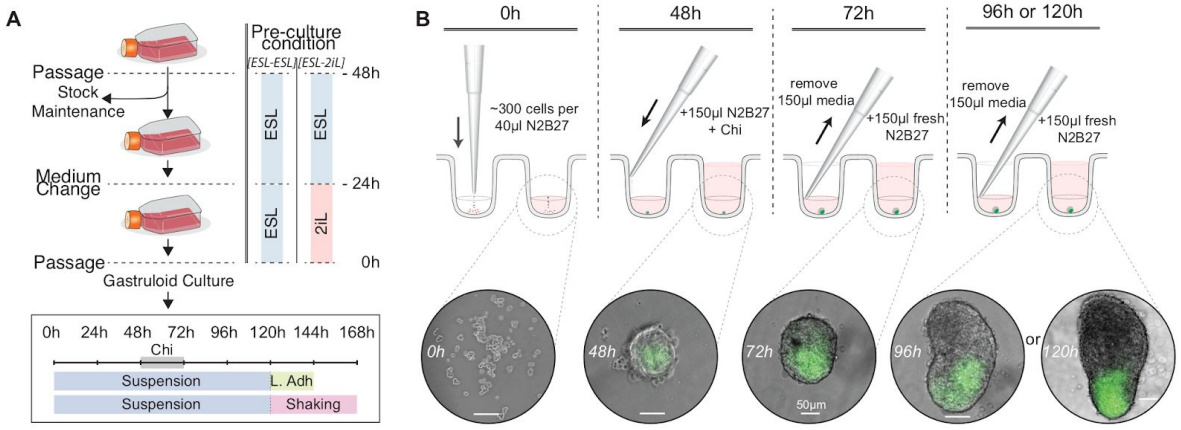

Figure 1: The generic work-flow for gastruloid generation. (**A**) General mouse embryonic stem cell (mESC) maintenance. For making gastruloids, flasks (25cm^2) with ~60% confluency are passaged and cells are plated for stock maintenance or for gastruloid culture in normal growth medium (ESL), and changed into either ESL or 2iL depending on their requirement (*see Sections 3.1-3.3*). Cells are then passaged the next day to generate gastruloids. The time-line of gastruloid development is shown below. (**B**) A 40µl droplet containing the required number of mESCs (*See Sections 3.4*) is plated directly into the centre of the wells of a 96-well plate (0h; left panel) with a multichannel micropipette. At 48h, 150µl fresh N2B27/NDiff227 containing 3µM Chi is added carefully to the sides of the well (second panel from left). From 72h onwards cell culture (150µl) medium is replaced every 24h with fresh N2B27/NDiff227 by carefully removing the medium from the base of the vertical sides of the well so as not to disturb the developing gastruloid, holding the pipette at an angle to the well. 40µl is always left in the well to prevent gastruloids from drying out. Image panels below show representative gastruloids, made with $Bra^{+/GFP}$ cell line ***(35),*** at the indicated time points after aggregation. All scale bars denote 50µm.

3.4 Gastruloid generation and culture

This section describes the method of producing a single 96-well plate of gastruloids from the above mentioned 'gastruloid flask'. Once the experimenter is familiar with the protocol, it is possible to scale up the quantities for multiple 96-well plates. However, we do not recommend more than 4 plates set up at a time (as this will increase the time the cells are out of the incubator, possibly leading to an increase in variability). Prior to starting this section of the protocol, ensure the flask is

~60% confluent and the cells appear healthy by examining the flask of cells on an inverted tissue culture microscope.

0h after aggregation (AA): cell plating

3.4.1 Pre-warm all media (ESL and N2B27/NDiff227), Trypsin, and $PBS^{+/+}$ in a water-bath set to 37°C.

3.4.2 Aspirate medium from the mESC culture flask ('gastruloid flask'), wash once with 5ml PBS+/+, and incubate cells with 2ml Trypsin/EDTA for <5 min in a humidified incubator (37°C; 5% CO_2). Excessive incubation with Trypsin has a detrimental effect on gastruloid formation, so it is imperative that the cells are checked every ~2 min. Rock the cells to aid detachment of the cells from the flask and remove the flask from the incubator once cells are no longer adhering to the flask growth surface.

3.4.3 Inactivate the Trypsin by the addition of 8ml ESL, and dissociate the colonies by pipetting up and down ~3 times across the growth surface. Transfer the 10ml cell suspension to a 15ml or 50ml centrifuge tube and centrifuge the cell suspension at approximately 170*g* for 3 min.

3.4.4 Aspirate the supernatant and dissociate the cell pellet by adding 10ml warm $PBS^{+/+}$. Centrifuge the cell suspension at 170*g* for 3 min.

3.4.5 Aspirate the supernatant and dissociate the cell pellet for a second time by adding 10ml warm $PBS^{+/+}$ and centrifuge at 170*g* for 3 min.

3.4.6 Aspirate the supernatant, ensuring minimal carryover of PBS whilst maintaining integrity of pellet by tilting the tube to ~45° and removing the liquid from the side of the tube.

3.4.7 Cover the pellet in 1ml warm N2B27/NDiff227 and fully resuspend the pellet using a p1000 pipette set to 1ml. Pipette up and down (~3 times for most cell lines) to ensure a single-cell suspension is obtained, minimising bubbling. This solution can be further diluted with a suitable volume of N2B27/NDiff227 (e.g. 3ml) if required.

3.4.8 Accurately determine the concentration of the cell suspension with either an automated cell counter or a haemocytometer, and calculate the required volume of cell suspension for one 96-well plate e.g. if one is plating 300 cells per well, transfer 37,500 cells to 5ml medium such that the final concentration is 300cells/40µl. The 5ml volume is sufficient for 40µl to be transferred to each well and includes adequate dead-volume following later pipetting.

3.4.9 Transfer the correct volume of cell suspension to fresh, warm N2B27 to give a final volume of 5ml, and mix the tube gently by hand, or carefully pipette the solution up and down with a 5ml pipette (avoiding bubbles). Transfer this cell suspension to a reservoir.

3.4.10 Using a multichannel micropipette, transfer 40µl from the reservoir to each well of a sterile U-bottomed, non-tissue-culture-treated 96-well plate, pipetting the droplet into the centre of each well (Fig. 1B; 0h, top panel). If needed, gently tap each of the four sides of the plate to force any droplets that were pipetted to the sides on to the bottom of their wells. If gastruloid culture to 144h or 168h after aggregation is desired, it is recommended to use low adherence 96-well plates (see 2.4.5).

3.4.11 Confirm that cells have been transferred by sampling a region of the plate under an inverted tissue-culture microscope (Fig. 1B; 0h, bottom panel).

3.4.12 Transfer the 96-well plate to the humidified incubator (37°C; 5% CO_2) and incubate for 48h.

3.4.13 At this stage, if the cell stock is being maintained, follow the steps in section 3.1 for the 'passage flask'.

3.4.14 Following aggregation gastruloid culture can be halted at this or any following stage for imaging, fixation or other downstream techniques (see section 3.5). *Note: By observing the gastruloids at 24 hours one can get a good sense of whether they are forming correctly or whether too many/too few cells have been plated which will affect their development over the next days.*

48h after aggregation: Addition of secondary medium with Chiron

3.4.15 Pre-warm the required volume of N2B27/NDiff227 in a water-bath set to 37°C.

3.4.16 Prepare a 3μM solution of Chi in 16ml N2B27/NDiff227; this volume is sufficient for a single 96-well plate including 'dead-volume' following pipetting. Mix well by hand, avoiding excessive medium bubbling. *Note: other combinations of factors can be added to the medium* ***(23,25)****, and this will yield different results in morphology and gene expression.*

3.4.17 Assess the quality of the gastruloids by observing them on an inverted benchtop microscope. They should have formed a single, spherical aggregate of smooth appearance approximately 150μm in diameter (Fig. 1B; 48h). Only slight deviations from this morphology can be expected depending on the cell line (e.g. slightly ovoid). *See Section 4, notes 4.1-4.5 for trouble-shooting.*

3.4.18 Pipette the Chi solution to a sterile reservoir, and gently transfer 150μl to each well of the 96-well gastruloid plate using a multichannel micropipette, ejecting the medium at the side of the well, above the 40μl volume (Fig. 1B; 48h).

3.4.19 Incubate the plate in a humidified incubator (37°C; 5% CO_2) for 24h.

72h after aggregation: Removal of Chiron pulse and medium change

3.4.20 Pre-warm the required volume of N2B27/NDiff227 in a water-bath set to 37°C.

3.4.21 Assess the quality and progression of the gastruloids on an inverted benchtop microscope. Gastruloids should still have a smooth appearance, but, depending on the cell line, they may have advanced to an ovoid morphology. For instance wild-type (E14-Tg2A) gastruloids tend to maintain a spherical appearance at this stage, whereas for $Bra^{+/GFP}$ gastruloids start elongating by 72h. In the particular case of $Bra^{+/GFP}$ gastruloids, the expression of the reporter will be highly polarised to the 'posterior' region (Fig. 1B; 72h)

3.4.22 Remove 150μl from each well with a multichannel micropipette, holding the pipette at an angle and carefully removing the medium from the side of the well at the interface between the vertical side and the inverted dome of the well (Fig. 1B; 72h). Note, it is important to leave 40μl in the wells to prevent gastruloids from drying out.

3.4.23 As an optional control measure to ensure the gastruloids have not been aspirated, gently place the side of the pipette (still holding the aspirated medium) on top of a tip box to maintain the sterility of the tip ends, and confirm the presence of gastruloids using the bench-top microscope. Eject the medium if gastruloids have not been aspirated, or replace the medium and return to these wells after the rest of the plate's secondary medium has been removed.

3.4.24 Transfer a sufficient quantity of fresh, warm N2B27/NDiff to a reservoir and pipette 150μl directly into each well of the 96-well plate as in section 3.4.18. Note, that the medium should be ejected into the wells with sufficient force to agitate the gastruloids, preventing them from

adhering to the bottom of the wells. *Note: forceful pipetting is not necessary when using low-adherence 96-well plates (see 2.4.5).*

3.4.25 Incubate the plate in a humidified incubator (37°C; 5% CO_2) for 24h.

96h after aggregation: Medium Change

3.4.26 Repeat steps 3.4.20 – 3.4.25 to exchange 150µl medium with fresh, warm N2B27/NDiff227.

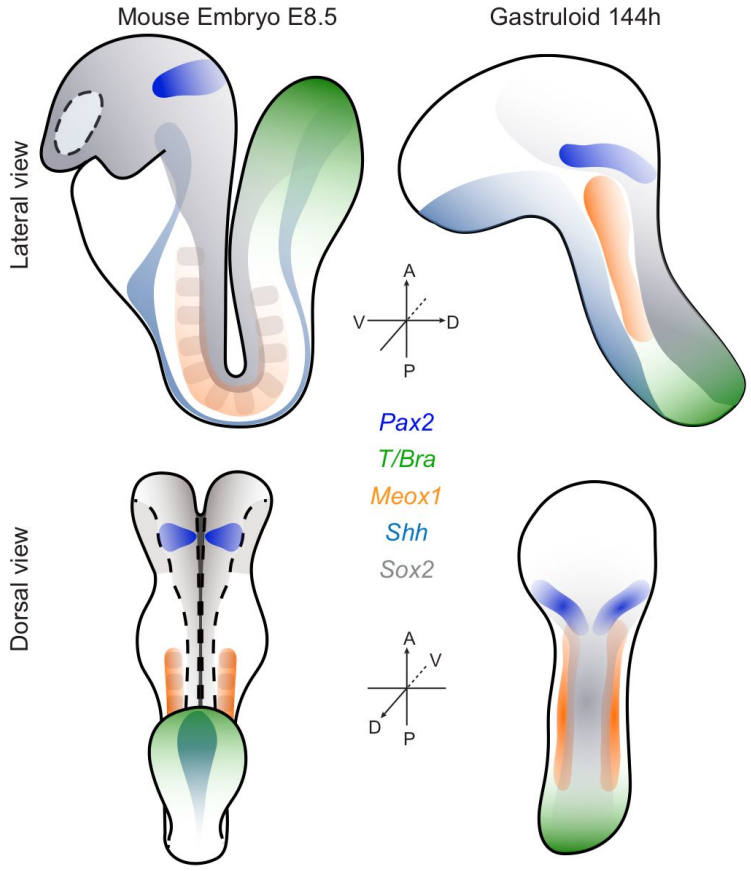

Figure 2: Overview of gene expression progression and axes formation in gastruloids. Schematic diagrams showing candidate genes denoting the three axes *(T/Bra denoting anteroposterior [AP], Shh denoting dorsoventral [DV], Meox and Pax2 denoting medio-lateral [ML] axes)* and the three germ layers *(T/Bra denoting mesoderm, Sox2 and Pax2 denoting ectoderm, Shh denoting endoderm)* in relation to their expression in mouse embryo at E8.5. A, Anterior; P, Posterior; D, Dorsal; V, Ventral; Axis lines without arrowheads denote medio-lateral axis.

120h after aggregation: Medium change and continued culture

3.4.27 At this stage, Gastruloids ought to be highly elongated, and display polarised expression of Brachyury in the posterior *(**23-26**, Figure 2)*, as well as expression of anterior markers

localised to the opposite pole of Brachyury expression such as GATA6 *(**26**)*. Typically, the gastruloid culture is halted at this point for imaging, fixation, or other downstream techniques (see section 3.5). *Note: For some cell lines, these expression domains might be visible earlier or later than 120h and so this needs to be confirmed individually.*

3.4.28 If continuing the culture to 144h repeat steps 3.4.20 – 3.4.25 to exchange 150µl medium with fresh, warm N2B27/NDiff227. When using standard round bottom 96-well plates (see 2.4.4.) and if culture is to be prolonged to 144h, gently withdraw the entire volume of the well using a p1000 micropipette set to 200µl, and individually transfer gastruloids to a fresh, low-adherence 96-well plate, and incubate in a humidified incubator (37°C; 5% CO_2) for 24h. *Note: using a p1000 tip cut ~5mm from the end with sterile scissors may help reduce damage to individual gastruloids.*

3.4.29 If the culture is to be continued to 168h, individually remove the gastruloids from the wells as in section 3.4.28, but transfer them into low-attachment 24-well plates holding 700µl fresh N2B27/NDiff227 (*Note: one gastruloid per well*). Incubate on an incubator-compatible horizontal shaker (e.g. Infors Celltron 69222) in a humidified incubator (37°C; 5% CO_2) for 48h at ~40 rpm (***27***), replacing 400µl medium with fresh, warm N2B27/NDiff227 at 144h.

3.5 Removing Gastruloids for down-stream applications

3.5.1 Pipette a suitable volume of $PBS^{-/-}$ into a bacterial-grade $10cm^2$ dish (~5 ml).

3.5.2 At the required time-point, remove the 96-well gastruloid plate from the incubator and replace 150µl medium with 200µl warm $PBS^{-/-}$ using the method described in steps 3.4.20 – 3.4.25.

3.5.3 Using a multichannel micropipette with the pipette-tips cut 5mm from the end, pipette up and down once, and then transfer the whole contents of the wells to the $10cm^2$ dish. Check that all gastruloids have been removed from the wells by sampling each well rapidly on an inverted bench-top microscope; collect any that have not been removed using a p200 set to 200µl with the tips cut as above.

3.5.4 Swirl the $10cm^2$ dish to drive all the gastruloids to the centre of the well, collect and transfer to 1.5ml or 2ml microcentrifuge tubes for down-stream processing (e.g. fixation for immunofluorescence or *in situ* hybridisation). To prevent gastruloids sticking to the inside of the tubes and pipette tips, coat with FBS or detergent (e.g. 2% Pluronic® F-127 in PBS) prior to use.

*Note: As an alternative to steps 3.5.1- 3.5.4 one can also use recently designed collector plates for pooling together gastruloids from a plate **(33)**.*

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4. Notes

4.1 *Composition of basal medium.* Some cell lines require DMEM, GMEM, or other formulations depending on their growth conditions. Grow cells for 2 passages in ESL containing different basal medium compositions, form gastruloids and assess their aggregation, growth, and morphology throughout the time-course.

4.2 *Pretreatment of mESCs in culture with 2iL [ESL-2iL] medium prior to gastruloid protocol*: Test the effect of either [ESL-ESL] or [ESL-2iL] pre-culture on gastruloid formation. Some lines do not require the 2iL pulse before passaging, and for others 2iL pulse might be essential for robust

formation of gastruloids. The concentration of Chi/PD03 and LIF might need to be optimised depending on the cell line.

4.3 *Number of mESCs used for making gastruloids:* Different cell lines require optimisation of the number of cells needed for making robust gastruloids (i.e. >70% consistent elongation in a plate by 120h). Test the effect of different plating densities of cells during gastruloid formation from 200-500 cells/well

4.4 *Genetic background of mESCs used for making gastruloids*: For reasons not clear yet from a molecular point of view, genetic background of the mESCs (i.e. source mouse strain from which mESCs were derived) can impact the formation of gastruloids. As a result, it will be necessary to optimise the culture conditions for uncharacterised cell lines, as well as cell lines that have recently been derived following genetic manipulation (i.e. insertion of reporter genes).

4.5 *Dosage of Chi pulse on gastruloid formation and progression:* We have found that the dose of Chi during the 48-72h pulse to be cell line dependent, resulting in fewer or more elongated gastruloids by 120h ***(34)***. Test the effect a range of Chi concentrations has on gastruloid formation and progression.

4.6 *General procedural precautions*: Avoid plating more than four 96-well plates at the same time, as this will increase the time cells are out of the incubator, to potentially detrimental effect.

4.7 *Analysis*: For qualitative assessment, gastruloids are scrutinized by visual inspection under an inverted tissue culture microscope. For quantitative assessment, images from a 96 well plate can be analysed to identify shape, size (length, width, aspect ratio, etc), localization and intensity of gene expression following in situ hybridisation or immunofluorescence, etc.

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Table 1: Cell lines that have been successfully tested with this protocol and specific requirements. The following table details the specific cell lines we have tested with respect to the gastruloid protocol. Some cell lines, as highlighted in the text, require a 24h pre-treatment with 2iLthe day before gastruloid formation [ES-2iL] and that information has been indicated here. The number of cells needed for 'successfully' making gastruloids is around 300, however depending upon specific conditions in the lab and composition of ESL (GMEM or DMEM based), starting cell numbers might need to be optimized (See Section 4, note 4.3).

Cell Line	**Strain/Background**	**Requirement of [ESL-2iL] Pre-gastruloid**
E14-Tg2A ***(35)***	129/Ola	Yes
$Bra^{+/GFP}$ ***(36)***	129P2/OlaHsd	No
$Nodal^{+/YFP}$ ***(37)***	129S2/SvPas	No
$Gata6^{H2BVenus}$ ***(38)***	(C57BL/6 x 129S4/SvJae)F1	Yes
$Sox1^{eGFP}$;$Bra^{mCherry}$ ***(39)***	129P2/OlaHsd	No
AR8::mCherry ***(40)***	129S6/SvEvTac	No
$FoxA2^{+/YFP}$	(C57BL/6J x 129S6/SvEvTac)F1	Not Tested
Sox1GFP (46C) ***(41)***	129/Ola	Not Tested

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