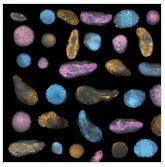

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Protocol to generate Gastruloids (LSCB, EPFL)

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1 Works for me dx.doi.org/10.17504/protocols.io.9j5h4q6

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ABSTRACT

Gastruloid generation protocol as performed in the Lutolf Lab, EPFL.

For previously published protocols, see:

Baillie-Johnson, Peter, et al. "Generation of aggregates of mouse embryonic stem cells that show symmetry breaking, polarization and emergent collective behaviour in vitro."*JoVE (Journal of Visualized Experiments)*105 (2015): e53252.

and

Mehmet, G., et al. "Generating Gastruloids from Mouse Embryonic Stem Cells."*Protocol Exchange* (2018).

GUIDELINES

This protocol assumes standard c

MATERIALS

NAME	CATALOG #	VENDOR
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher Scientific
PBS, pH 7.4	10010015	Thermo Fisher
50mL Reagent Reservoir White PS	613-1184	VWR international Ltd
96-well Clear Round Bottom Ultra-Low Attachment Microplate	7007	Corning
GSK-3 Inhibitor XVI	361559	Merck Millipore

MATERIALS TEXT

The following recipes were used to prepare the media used throughout the protocol:

10% Serum Medium (home made fresh):
to make 500mL

- 434mL DMEM, high glucose, with GlutaMAX™ [CAT# 61965059, Gibco™/Life Technologies]
- 50mL ES-grade Foetal Bovine Serum [CAT#16141-079, Gibco™/Life Technologies]
- 5mL Penicillin-Streptomycin 10000U/mL [CAT#15140122, Gibco™/Life Technologies]
- 5mL Non Essential Amino Acids 100X [CAT#11140035, Gibco™/Life Technologies]
- 5mL Sodium Pyruvate 100mM [CAT#11360039, Gibco™/Life Technologies]
- 1mL beta-mercaptoethanol 50mM [CAT#31350010, Gibco™/Life Technologies]

for final concentrations of:

- Penicillin: 100U/mL
- Streptomycin: 100ug/mL
- Non Essential Amino Acids: 0.1mM
- Sodium Pyruvate: 1mM
- GlutamaMAX™ (= L-Alanyl-Glutamine, included in DMEM): 3.97mM
- beta-mercaptoethanol: 0.1mM

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to make **10% Serum 2i/LIF Medium (made fresh)**, add:

- 3uM CHIR99021 (1:1000 from our 3mM stock,) [CAT#361559, Merck/Millipore]
- 1uM PD0325901 (1:500 from our stock) [CAT#S1036, Selleck Chemicals]
- 100u/mL LIF (1:1000 from our stock), [sourced in house]

N2B27 Medium (home made fresh):

to make 500mL

- 237mL Neurobasal™ Medium [CAT#21103049, Gibco™/Life Technologies]
- 237mL DMEM/F-12, with GlutaMAX™, [CAT#31331093, Gibco™/Life Technologies]
- 5mL Penicillin-Streptomycin 10000U/mL [CAT#15140122, Gibco™/Life Technologies]
- 5mL Non Essential Amino Acids 100X [CAT#11140035, Gibco™/Life Technologies]
- 5mL Sodium Pyruvate 100mM [CAT#11360039, Gibco™/Life Technologies]
- 2.5mL GlutaMAX™ Supplement, 200mM [= L-Alanyl-Glutamine, CAT#35050038, Gibco™/Life Technologies]
- 1mL beta-mercaptoethanol 50mM [CAT#31350010, Gibco™/Life Technologies]
- 5mL B27 Supplement, serum-free, 50X [CAT#17504001, Gibco™/Life Technologies]
- 2.5mL N-2 Supplement, 100X [CAT#17502001, Gibco™/Life Technologies]

for final concentrations of:

- Penicillin: 100U/mL
- Streptomycin: 100ug/mL
- Non Essential Amino Acids: 0.1mM
- Sodium Pyruvate: 1mM
- GlutamaMAX™ (= L-Alanyl-Glutamine, also included in DMEM/F-12): 1mM + 2.50mM = 3.50mM
- beta-mercaptoethanol: 0.1mM

BEFORE STARTING

Starting grounds: a healthy culture of adherent mouse Embryonic Stem Cells (mESCs), grown in DMEM-10%Serum, +2i +LIF, split every two or three days. Gastruloid generation is done at the time of splitting, and simultaneously with it.

Preparation of the cell suspension

1 Using a vacuum line+glass pasteur pipette, aspirate out the culture medium and replace with **3 ml** PBS-/- , for a short wash

When removing liquid during washes, do not completely dry out the cells. The surface of the well should still look glossy.

2 Aspirate out the PBS, and replace with **500 µl** Accutase.

3 Let Accutase act for ~ **00:03:00** , **Room temperature** , tapping the sides of the plate to ease dissociation, and until most cells are floating (as clumps or single cells)

4 Slightly tilt the plate, and use a P1000 to pipette the suspension up and down to further break down cell clumps. Use each ejection to wash the surface of the plate, so to collect as many cells as possible.

5 Transfer the Accutase-cell suspension to a clean 15mL Falcon tube labelled "cells"

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6 Use ~ **4.5 ml** DMEM-10%Serum to further wash the surface of the well, again, pipetting up and down and hoping to collect any previously missed cells. Transfer this secondary cell suspension in the same tube as before (total volume **5 ml**)

7 Centrifuge **200 x g** (**1000 rpm , (15.5 cm rotor radius)**) **00:04:00** min **4 °C**

8 Using a vacuum line+glass pasteur pipette, aspirate the supernatant without disturbing the pellet, redissolve the pellet in **10 ml** PBS-/-, and centrifuge again **200 x g** (**1000 rpm , (15.5 cm rotor radius)**) **00:04:00** min **4 °C**

9 Repeat **go to step #8** for a second PBS-/- wash

10 Using a vacuum line+glass pasteur pipette, aspirate the supernatant without disturbing the pellet, and resuspend the pellet in **1 ml** N2B27

It is important to thoroughly resuspend the pellet at this point, as a single cell suspension is needed for accurate counting later on. Resuspend the pellet by pipetting several times (~20) with a P1000, then switching to a P200, and then to a P20

Cell counting and Gastruloid seeding

11 Load **10 µl** of the N2B27-cell suspension into a manual cell counter (Neubauer chamber/haemocytometer), and calculate the concentration of cells in your suspension.

Each well of the 96well plate will receive one **40 µl** drop of N2B27. Each drop should contain 300 cells, i.e. a concentration of 300 cells / **40 µl** = 7.5 cells/uL

To fill an entire 96well plate with **40 µl** drops, **40 µl** x 96 well = **3.840 ml** N2B27 are needed.
For simplicity, **5 ml** N2B27 suspension are prepared: at 7.5 cells/uL, this requires 37500 cells.

At this point, cells can also be used to seed a new culture and keep propagating the line. We plate from 65000 to 75000 cells in a well of a tissue-culture-treated 6well plate, filled with 2mL prewarmed DMEM/10%Serum+2i/LIF

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12 To **5 ml** fresh N2B27, add the volume of cell suspension carrying 37500 cells, as calculated before. (If you want to prepare more than one plate of gastruloids, scale the volume of N2B27 and of cells accordingly)

13 Load the **5 ml** N2B27-cell suspension into a multichannel pipette reservoir, and dispense **40 µl** of this solution to each well of an ultra-low-adhesion 96well plate

> Make sure that the cell suspension in the reservoir is always well mixed, to ensure homogeneous dispensing in the wells. At each transfer, pipette up and down several time with the multichannel, and slightly agitate the reservoir from time to time.

14 Lightly tap the plate against the surface of the hood to make sure all drops are at the very bottom of each well, doublecheck under the microscope for the presence of cells in the drops, and leave the plate in a humidified incubator, 5%CO2, undisturbed **24:00:00** h

This is considered the beginning of Day 1 (D1), t=0h-24h

Growing Gastruloids

15 **Beginning of Day 2 (D2), t=24h-48h**

No intervention needed at this timepoint. The Gastruloids will keep developing in their **40 µl** of N2B27. Leave to grow **24:00:00** h more

> Gastruloids should look like a small (**100 µm**) cluster, still in the process of aggregating. Individual cells might still be visibile in the surroundings

16 **Beginning of Day 3 (D3), t=48h-72h**

Prepare **16 ml** N2B27, adding CHIR99021 to a final concentration of **3 Micromolar (µM)** . Load this into a multichannel pipette reservoir, and dispense **150 µl** of this solution to each well of the plate (i.e. total volume of **190 µl** per well). Place back in the incubator for **24:00:00** h

> Gastruloids should look like a small, clean, translucent sphere of around **200 µm** diameter. No additional (satellite) spheres should be seen around it.

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17 **Beginning of Day 4 (D4), t=72h-96h**

Using a multichannel pipette, remove **150 µl** of the medium from each well and replace with **150 µl** fresh N2B27. Place back in the incubator for **24:00:00** h

Gastruloids are round spheres of around **300 µm** in diameter. Extensive cell shedding is expected as a consequence of the CHIR pulse.

18 **Beginning of Day 5 (D5), t=96h-120**

Using a multichannel pipette, remove **150 µl** of the medium from each well and replace with **150 µl** fresh N2B27. Place back in the incubator for **24:00:00** h

Gastruloids morphology is no longer symmetrical: they look like a ovoid with length of around **600 µm** . The protruding part is more translucent than the rest, and this is the part that will elongate in the next 24h.

At t=120h, the Gastruloid looks like a bowling pin with length of around **700 µm** - **800 µm** . A thinner extension protrudes at the "posterior", more translucent than the denser anterior (spherical).

Day 6 (D6) and Day 7 (D7), t=144h, 168h

Culture can be extended up to 168h (i.e. 7 days total). This can be done by continuing the daily medium changes as above, or by transferring individual Gastruloids to separate wells of a 24well plate, in **800 µl** fresh N2B27, and replacing half of the medium one day later. The plate is kept shaking on an orbital shaker, in the incubator for both additional days. If keeping the Gastruloids in the original 96well plate, no shaking is required but there is an increased risk of the Gastruloids adhering to the sides of the well and degenerating.

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