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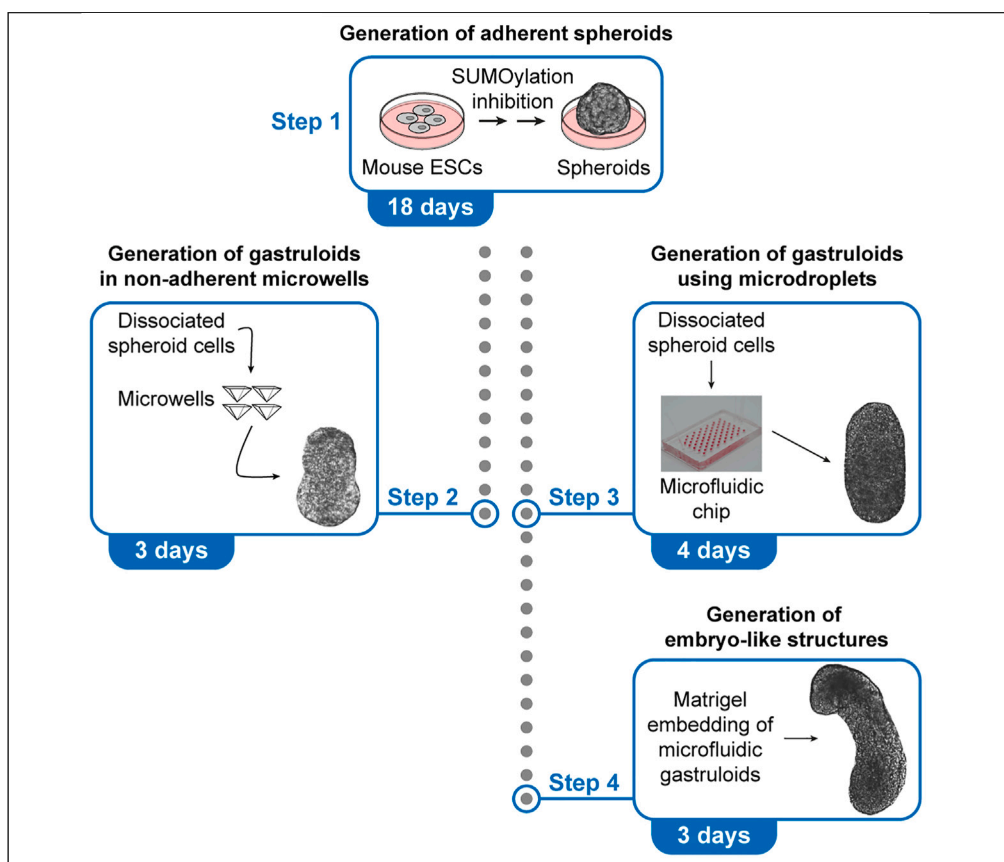
Generation of embryo-like structures from mouse embryonic stem cells treated with a chemical inhibitor of SUMOylation and cultured in microdroplets



The field of stem cell-based embryo-like models is rapidly evolving, providing *in vitro* models of *in utero* stages of mammalian development. Here, we detail steps to first establish adherent spheroids composed of three cell types from mouse embryonic stem cells solely treated with a chemical inhibitor of SUMOylation. We then describe procedures for generating highly reproducible gastruloids from these dissociated spheroid cells, as well as embryo-like structures comprising anterior neural and trunk somite-like regions using an optimized microfluidics platform.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Rounds of chemical SUMOylation inhibition in mouse ESCs generate adherent spheroids

Generation of gastruloids in non-adherent microwells

Dissociated spheroid cells self-organize into gastruloids in microfluidic droplets

Microdroplets-derived gastruloids embedded in Matrigel form embryo-like structures

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Generation of embryo-like structures from mouse embryonic stem cells treated with a chemical inhibitor of SUMOylation and cultured in microdroplets

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SUMMARY

The field of stem cell-based embryo-like models is rapidly evolving, providing *in vitro* models of *in utero* stages of mammalian development. Here, we detail steps to first establish adherent spheroids composed of three cell types from mouse embryonic stem cells solely treated with a chemical inhibitor of SUMOylation. We then describe procedures for generating highly reproducible gastruloids from these dissociated spheroid cells, as well as embryo-like structures comprising anterior neural and trunk somite-like regions using an optimized microfluidics platform.
For complete details on the use and execution of this protocol, please refer to Cossec et al. (2023).[1]

BEFORE YOU BEGIN

The protocol below describes the steps to generate spheroids, gastruloids and embryo-like structures (ELSs) from the embryonic stem cell (ESC) line ES R1. However, we have also successfully used these procedures with the CGR8 and E14 cell lines.

The protocol is defined by four major steps: (1) establishment of adherent spheroids, (2) generation of gastruloids in non-adherent AggreWell plates, (3) generation of gastruloids in optimized droplet microfluidic chips, and (4) embedding of the microdroplet gastruloids in Matrigel to generate head-and-trunk ELSs.

Before implementing this protocol, users should prepare all media, aliquot stock solutions and any necessary materials and store them as instructed in the materials and equipment section.

Chip microfabrication

Timing: ~ 2 days

This section describes the fabrication of molds and the procedure to generate the droplet microfluidics platform for the 3D culture of mESCs.

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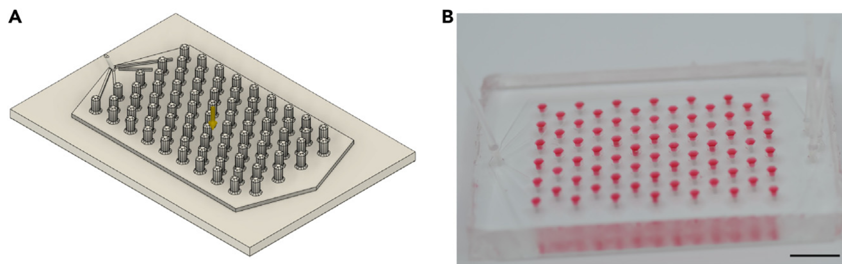

Figure 1. Microfluidic chip

(A) Schematic of the mold for the fabrication of the microfluidic chip. For details about chip dimensions, please refer to Cossec et al. (2023).[1]

(B) Picture of a loaded chip. The inlet can be seen on the left and the three outlets on the right. Scale bar, 1 cm.

1. Fabricate the molds of the microfluidic droplet devices from the design built using Autodesk Fusion 360 (Figure 1A). The design is fully detailed in Cossec et al. (2023).[1] The main channel height is 1 mm, the bottom part of the traps is 2.4 mm and the median part is 1.2 mm in diameter, while the height of the traps is 4 mm.
   a. 3D print the molds using a ClearV4 resin (Formlabs) and an SLA 3D printer (Form3, Formlabs).
   b. Right after the printing, wash the mold with isopropanol and dry it.
   c. Cure the resin for 30 min in a UV oven (Form Cure, Formlabs) set up at 60°C.
   d. Cover the mold with 5 mL of Novec 1720 (3M) and heat it at 60°C, until the solution is completely dried.

   ⚠ CRITICAL: Novec surface treatment should be done under a chemical hood.

   e. Store molds at 18°C–25°C.

   Note: Molds can be used repeatedly to fabricate chips over a period of 12 months.

2. Fabricate the chips:
   a. Fold aluminum foil around the mold to create a 'container' with raised sides of several cm.
   b. Prepare a mixture of polydimethylsiloxane (PDMS, SYLGARD 184) base and a curing agent at a ratio of 10:1 (about 50–60 mL per chip).
   c. Mix thoroughly with a spatula.
   d. Pour the PDMS mixture on the mold (about 1 cm thickness).
   e. Put the mold with poured PDMS in a vacuum desiccator for 20–30 min to remove air bubbles.
   f. Place the mold in an oven set up at 65°C for at least 4 h.

   ⏸ **Pause point:** Molds with cured PDMS can be stored at 18°C–25°C for at least one year.

   g. After curing, cut off the aluminum container with a scalpel and separate the PDMS imprinted with the top of the chip from the mold with a spatula.
   h. Use a 1.5 mm diameter punch to create an angled entry hole on one side (the chip inlet) and three straight exit holes on the other (the chip outlets) (Figure 1B).
   i. Clean all sides of the chip, as well as inlet and outlets, with ethanol then dry with an air jet.
   j. Remove dust from the surface of the chip and from a 75 × 50 mm glass slide (Corning) by using sticky tape.
   k. Plasma treat the top of the chip and the 75 × 50 mm glass slide for two rounds of 40 s (Cute, Femto Science).
   l. Place the PDMS device on the glass slide and press the sides to bond the two materials together.

   ⚠ CRITICAL: Make sure that bonding is complete to avoid any liquid leakage at the time of the cell loading.

m. Place the chips into an oven set up at 80°C for at least 2 h.

Note: Chips can be stored at 18°C–25°C for several days.

Preparation of feeder mouse embryonic fibroblasts (MEFs)

Timing: ~ 2 weeks

3. Thaw a vial of cryopreserved MEFs isolated from inbred *C57BL/6* mice:
   a. Pre-warm complete DMEM culture medium in a 37°C water bath.
   b. Transfer a vial of MEFs from liquid nitrogen to a cell culture hood.
   c. While cell suspension is thawing, add 9 mL of complete DMEM culture medium to a 15 mL Falcon tube.
   d. Add 1 mL of complete DMEM culture medium to the thawing cell suspension in the vial.
   e. Gently pipet the cell suspension and transfer it to the Falcon tube.
   f. Centrifuge the resuspended cells at 1,000 g for 5 min at 18°C–25°C.
   g. Add 14 mL of complete DMEM culture medium to a T75 flask.
   h. After centrifugation, discard the supernatant and resuspend cell pellet in 1 mL of complete DMEM culture medium.
   i. Add cell suspension to the T75 flask.
   j. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
   k. The next day, check that cells have adhered.
4. Allow cells to grow to confluence then passage them:
   a. Aspirate culture medium.
   b. Wash cells with PBS.
   c. Add 1 mL of Trypsin-EDTA and incubate cells at 37°C in a humidified incubator with 5% CO_2 until they detach (approximately 5 min).
   d. Add 9 mL of complete DMEM culture medium and pipet cell suspension until homogeneous.
   e. Transfer cells to a T175 flask containing 15 mL of complete DMEM culture medium.
5. Allow cells to grow to confluence then passage them, dividing the content of one T175 flask to three 150 mm culture plates.
6. Once MEFs reach confluence, treat cells with Mitomycin C.
   a. Prepare complete DMEM culture medium containing 10 μg/mL Mitomycin C as described in the materials and equipment section.
   b. Aspirate culture medium from the 150 mm culture plates and add 20 mL of complete DMEM culture medium containing 10 μg/mL Mitomycin C per plate.
   c. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 2.5–3 h.
7. Make stocks of Mitomycin C-treated MEFs:
   a. Wash cells with PBS.
   b. Add 3 mL of Trypsin-EDTA and incubate cells at 37°C in a humidified incubator with 5% CO_2 until they detach (approximately 5 min).
   c. Add 13 mL of complete DMEM culture medium and pipet cell suspension until homogeneous.
   d. Transfer cells to a 50 mL Falcon tube and pool contents of all three treated 150 mm culture plates.
   e. Count treated MEFs.
   f. Centrifuge cells at 1,000 g for 5 min at 18°C–25°C.
   g. Discard supernatant and resuspend cell pellet in cryopreservation medium (FBS with 10% of sterile DMSO) in order to have 4 million cells/mL.
   h. Distribute 1 mL of this feeder MEFs cell stock mixture to labeled cryovials and transfer them to an appropriate freezing container.
   i. Store at −80°C then transfer to liquid nitrogen for longer term storage.

ES R1 cell culture

Timing: ~ 10 days

8. Prepare gelatin coated cultureware:
   a. Coat one 60 mm culture plate and one 100 mm culture plate with 3 mL and 8 mL of 0.1% Gelatin respectively.
   b. Incubate at 37°C in a humidified incubator with 5% CO_2 for at least 30 min.

Note: Gelatin coated plates can be stored in the incubator for several days before use. Ensure that the entire surface of the plate is properly covered before use.

9. Prepare feeder MEFs coated cultureware:
   a. Pre-warm complete DMEM culture medium in a 37°C water bath.
   b. Transfer a vial of feeder MEFs from liquid nitrogen to a cell culture hood.
   c. While cell suspension is thawing, add 9 mL of complete DMEM culture medium to a 15 mL Falcon tube.
   d. Add 1 mL of complete DMEM culture medium to the thawing cell suspension in the vial.
   e. Gently pipet the cell suspension and transfer it to the Falcon tube.
   f. Centrifuge the resuspended cells at 1,000 g for 5 min at 18°C–25°C.
   g. Aspirate the gelatin from the 60 mm and 100 mm culture plates and add 2 mL and 7 mL of complete DMEM culture medium respectively.
   h. After centrifugation, discard the supernatant and resuspend cell pellet in 4 mL of complete DMEM culture medium.
   i. Add 1 mL of cell suspension to the 60 mm culture plate and the remaining 3 mL to the 100 mm culture plate.
   j. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
   k. The next day, check that cells have adhered and cover the entirety of the plate surface.

Note: Feeder MEFs coated plates can be stored in the incubator for up to 1 week before use. A 4 million cells/mL stock of feeders can also be used to coat two 6-well plates.

10. Thaw a vial of cryopreserved ES R1 cells:
    a. Pre-warm Serum + Lif culture medium in a 37°C water bath.
    b. Transfer a vial of ES R1 from liquid nitrogen to a cell culture hood.
    c. While cell suspension is thawing, add 9 mL of Serum + Lif culture medium to a 15 mL Falcon tube.
    d. Add 1 mL of Serum + Lif culture medium to the thawing cell suspension in the vial.
    e. Gently pipet the cell suspension and transfer it to the Falcon tube.
    f. Centrifuge the resuspended cells at 1,000 g for 5 min at 18°C–25°C.
    g. Aspirate the culture medium from the 60 mm feeder coated culture plate and add 2 mL of Serum + Lif culture medium.
    h. After centrifugation, discard the supernatant and resuspend ES R1 cell pellet in 1 mL of Serum + Lif culture medium.
    i. Gently administer ES R1 cell suspension dropwise to the 60 mm feeder coated culture plate.
    j. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
11. The next day, check that small ES R1 cell colonies have formed atop the feeder MEFs layer and change the culture medium to pre-warmed Serum + Lif culture medium.
12. Allow the colonies to grow to ~80% confluency, replacing the culture medium every day, then passage them:
    a. Aspirate culture medium.
    b. Wash cells with PBS.

c. Add 500 μL of Trypsin-EDTA and incubate cells at 37°C in a humidified incubator with 5% CO_2 for 2–3 min.
d. Add 4.5 mL of Serum + Lif culture medium and pipet cell suspension until homogeneous.
e. Aspirate the culture medium from the 100 mm feeder coated culture plate and add 5 mL of Serum + Lif culture medium.
f. Transfer the entirety of the cell suspension from the 60 mm plate dropwise to the 100 mm feeder coated culture plate.
g. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.

13. The next day, check that ES R1 cell colonies have formed atop the feeder MEFs layer and change the culture medium to pre-warmed Serum + Lif culture medium.
14. Allow the colonies to grow to ~80% confluency, replacing the culture medium every day, then passage them (1:6 dilution) to gelatin coated 100 mm culture plates.
15. Passage ES R1 cells every other day on gelatin coated 100 mm culture plates, and replace culture medium on days without a passage.

Note: Post-thawing, passage ES R1 cells at least once on feeder coated plates, then at least twice on gelatin coated plates before proceeding with the protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
ClearV4 resin	Formlabs	Cat# RS-F2-GPCL-04
Novec 1720 (3M)	Inventec	Cat# 99688102
PDMS (SYLGARD 184)	Neyco	Cat# DC184-1.1
DMEM, high glucose, GlutaMAX supplement	Thermo Fisher Scientific	Cat# 61965-059
Fetal bovine serum (FBS)	Serana	Cat# S-FBS-SA-015
Penicillin-Streptomycin (100×)	Thermo Fisher Scientific	Cat# 15140-122
PBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat# 14190-169
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	Cat# 25300-054
Mitomycin C from *Streptomyces caespitosus*	Sigma-Aldrich	Cat# M0503
DMSO	Euromedex	UD8050
Gelatin from porcine skin	Sigma-Aldrich	Cat# G2500
KnockOut DMEM	Thermo Fisher Scientific	Cat# 10829-018
ES cell qualified FBS	Thermo Fisher Scientific	Cat# 16141-079
GlutaMAX supplement (100×)	Thermo Fisher Scientific	Cat# 35050-038
MEM non-essential amino acid solution (100×)	Thermo Fisher Scientific	Cat# 11140-050
2-mercaptoethanol (55 mM)	Thermo Fisher Scientific	Cat# 21985-023
Mouse LIF, premium grade	Miltenyi Biotec	Cat# 130-099-895
ML-792	CliniSciences	Cat# HY-108702
TAK-981	CliniSciences	Cat# HY-111789
Neurobasal medium	Thermo Fisher Scientific	Cat# 21103-049
Advanced DMEM/F-12	Thermo Fisher Scientific	Cat# 12634-010
N-2 supplement (100×)	Thermo Fisher Scientific	Cat# 17502-048
B-27 supplement (50×), minus vitamin A	Thermo Fisher Scientific	Cat# 12587-010
Bovine albumin fraction V (7.5% solution)	Thermo Fisher Scientific	Cat# 15260-037
Anti-adherence rinsing solution	STEMCELL Technologies	Cat# 07010
Fluorinert Electronic Liquid FC-40 (3M)	Inventec	Cat# 99687220
RAN - Neat FluoroSurfactants	RAN Biotechnologies	Cat# 008-FluoroSurfactant-10G
Matrigel basement membrane matrix	Corning	Cat# 354234
Perfluorooctanol (PFO)	Sigma-Aldrich	Cat# 370533
Novec 7500 (3M)	Inventec	Cat# 99687022

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Mouse embryonic fibroblast (MEFs) C57BL/6 – E13.5 – male or female	Cossec et al.[1]	N/A
ES R1 cells 129X1 × 129S1 – E3.5 – male	Cossec et al.[1]	N/A
Software and algorithms		
Autodesk Fusion 360	N/A	https://www.autodesk.com/
Other		
75 × 50 mm glass slides	Corning	Cat# 2947-75x50
AggreWell 800	STEMCELL Technologies	Cat# 34815
Idex 1507L chromatography tubing, natural PFA, 1/16″ OD × 0.040″ ID × 50 ft L	Cole-Palmer	Cat# FV-02012-62
PTFE tubing (0.56 mm ID and 1.07 mm OD) (Adtech Polymer Engineering BIOBLOCK/13)	Thermo Fisher Scientific	Cat# 11929445
Idex low-pressure cross body, natural PEEK, 0.050″ bore, 1/8″ OD tubing, 1/4-28 flat bottom; 1/EA	Cole-Parmer	Cat# FV-02023-80
Idex flangeless fitting, short head, natural PEEK, 1/16″ OD tubing, 1/4-28 flat bottom	Cole-Parmer	Cat# FV-02014-97
Silicone tubing (ID 1.0 mm)	Thermo Fisher Scientific	Cat# 10430313
96-well clear flat bottom ultra-low attachment microplate, individually wrapped, with lid, sterile	Corning	Cat# 3474

MATERIALS AND EQUIPMENT

Complete DMEM Culture Medium		
Reagent	Final concentration	Amount
DMEM, high glucose, GlutaMAX supplement	N/A	445 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Penicillin-Streptomycin (100×)	1×	5 mL
Total		**500 mL**
Store at 4°C for up to 1 month.		

Serum + Lif Culture Medium		
Reagent	Final concentration	Amount
KnockOut DMEM	N/A	490.8 mL
ES Cell Qualified FBS	15%	90 mL
GlutaMAX Supplement (100×)	1×	6 mL
MEM Non-Essential Amino Acids (100×)	1×	6 mL
Penicillin-Streptomycin (100×)	1×	6 mL
2-Mercaptoethanol (55 mM)	0.11 mM	1.2 mL
Mouse LIF (100 μg/mL)	10 ng/mL	60 μL
Total		**600 mL**
Filter on 0.22 μm PES membrane and store at 4°C for up to 1 month.		

N2B27 + Lif Culture Medium		
Reagent	Final concentration	Amount
Neurobasal Medium	N/A	235.3 mL
Advanced DMEM/F-12	N/A	235.3 mL
N-2 Supplement (100×)	1×	5 mL

(*Continued on next page*)

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Reagent	Final concentration	Amount
B-27 Supplement (50×), minus vitamin A	1×	10 mL
GlutaMAX Supplement (100×)	1×	5 mL
Bovine Albumin Fraction V (7.5% solution)	0.05%	3.33 mL
Penicillin-Streptomycin (100×)	1×	5 mL
2-Mercaptoethanol (55 mM)	0.11 mM	1 mL
Mouse LIF (100 μg/mL)	10 ng/mL	50 μL
Total		500 mL

Filter on 0.22 μm PES membrane and store at 4°C for up to 1 month.

- **10 μg/mL Mitomycin C:** Dissolve 2 mg of Mitomycin C in 1 mL of complete DMEM culture medium. Add 100 μL of this 2 mg/mL stock solution to 19.9 mL of complete DMEM culture medium for a final concentration of 10 μg/mL. Use immediately.
- **Cryopreservation medium:** FBS with 10% of sterile DMSO. Use immediately.
- **0.1% Gelatin:** Add 1 g of Gelatin from porcine skin in 1 L ddH_2O. Sterilize by autoclaving at 120°C for 20 min and store at 18°C–25°C for up to 2 months.
- **100 μg/mL Mouse LIF:** Dissolve 100 μg of LIF in 1 mL of sterile culture-grade PBS. Store aliquots at −20°C for up to 1 year.
- **10 mM ML-792 stock solution:** Dissolve 5 mg of ML-792 (MW: 551.41 Da) in 906.77 μL sterile DMSO to constitute a 10 mM ML-792 stock solution. Store aliquots at −20°C for up to 1 year.
- **20 mM TAK-981 stock solution:** Dissolve 5 mg of TAK-981 (MW: 578.10 Da) in 432.45 μL sterile DMSO to constitute a 20 mM TAK-981 stock solution. Store aliquots at −20°C for up to 1 year.
- **0.25 mM TAK-981 intermediate dilution:** Add 1 μL of 20 mM TAK-981 stock solution to 79 μL of Serum + Lif culture medium. Use immediately.
- **30% w/w RAN stock solution:** Add 12.5 mL FC-40 to about 15 g RAN, sonicate until complete dissolution. Store at 18°C–25°C for up to one year.
- **0.5% v/v RAN working solution:** Dilute 250 μL of the 30% w/w RAN stock solution into 14.75 mL of FC-40. Store at 18°C–25°C for up to one year.

> ⚠ CRITICAL: Fluorosurfactants and fluorinated oils may pose a risk of bio-accumulation. Handle with gloves and wear protective glasses.

- **10% PFO:** Add 2 mL of PFO to 18 mL of Novec 7500 (3M). Store at 18°C–25°C for up to one year.

STEP-BY-STEP METHOD DETAILS

Establishment and culture of adherent spheroids

Timing: 18 days

This section describes the experimental steps required to generate spheroids composed of three distinct cell types by treating mouse ESCs with sequential rounds of SUMOylation inhibition (Figure 2A). The treatment regimen gives rise to epiblast-like cells and primitive endoderm-like cells, similar to their E4.5-E5.5 *in vivo* counterparts, as well as a third ESC-like cell population. These cell types self-assemble into adherent spheroids in the culture dish and can be maintained in culture for up to four weeks. The hypoSUMOylation-instructed spheroid cells will be used to generate gastruloids and ELSs in the subsequent steps of the protocol.

For details about the characterization of the spheroid cell types and structure, please refer to Cossec et al. (2023).[1]

Note: Prepare gelatin coated 100 mm culture plates before each cell passage. Pre-warm culture medium in a 37°C water bath before use.

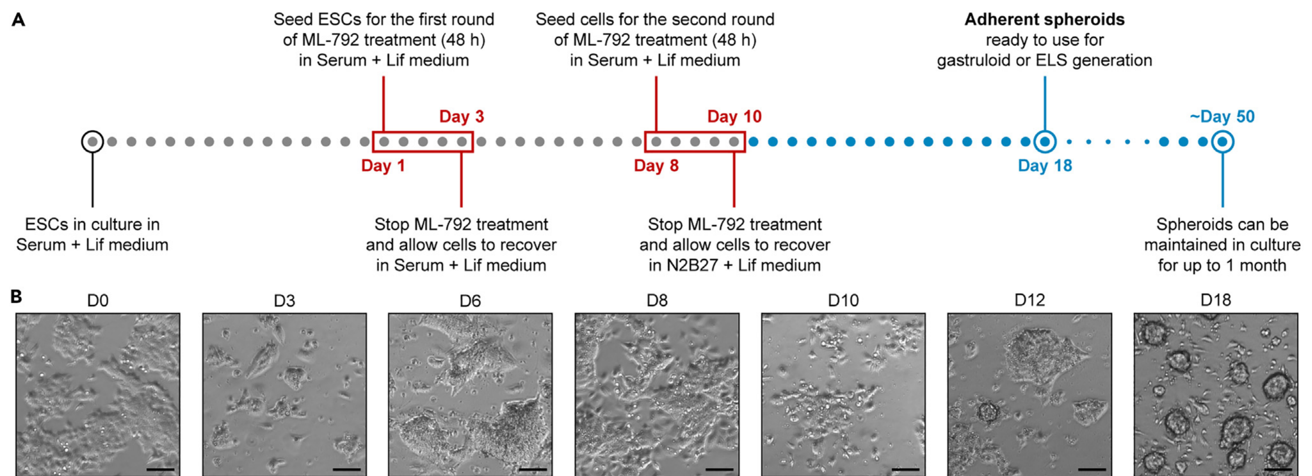

Figure 2. Generation of adherent spheroids from mESCs

(A) Experimental timeline.

(B) Representative images of cells at different time points. Scale bars, 100 μm.

1. Treat ES R1 cells with ML-792[2] for the first hypoSUMOylation round (day 1):
   a. Aspirate culture medium from a confluent ES R1 100 mm culture plate.
   b. Wash cells with PBS.
   c. Add 1 mL of Trypsin-EDTA and incubate cells at 37°C in a humidified incubator with 5% CO_2 for 1–2 min.
   d. Add 9 mL of Serum + Lif culture medium and pipet cell suspension until homogeneous.
   e. Transfer cells to a 15 mL Falcon tube.
   f. Count cells.
   g. Seed 2.5 million cells in 10 mL of Serum + Lif culture medium, supplemented with 2.5 μM of ML-792 (2.5 μL of a 10 mM stock solution) on a gelatin coated 100 mm culture plate.
   h. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.

   Note: Protocol can be scaled down to a 6-well plate format (500,000 cells in 2 mL culture medium per well) or scaled up to a 150 mm culture plate format (7 million cells in 20 mL culture medium).

   Alternatives: All 2.5 μM ML-792 treatment steps can be replaced with 0.1 μM TAK-981[3] treatments, by supplementing 10 mL of Serum + Lif culture medium with 4 μL of 0.25 mM TAK-981 intermediate dilution to treat the cells.

2. Treat ES R1 cells with ML-792 (day 2):
   a. Aspirate culture medium.
   b. Add 10 mL of Serum + Lif culture medium, supplemented with 2.5 μM of ML-792 (2.5 μL of a 10 mM stock solution).
   c. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
3. Stop the first hypoSUMOylation round (day 3):
   a. Aspirate culture medium.
   b. Wash cells with PBS twice.
   c. Add 10 mL of Serum + Lif culture medium.
   d. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.

   Note: High cell mortality is observed after 48 h of treatment with a SUMOylation inhibitor (Figure 2B).

4. Allow cells to recover from hypoSUMOylation in Serum + Lif culture medium:
   a. The next day (day 4), wash cells with PBS once.
   b. Add 10 mL of Serum + Lif culture medium.
   c. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
   d. Passage cells (1:2 dilution on a gelatin coated plate) when they reach ~80% confluence (usually at day 6) and replace culture medium on days without a passage.
5. Treat ES R1 cells with ML-792 for the second hypoSUMOylation round (day 8):
   a. Count the cells.
   b. Seed 2.5 million cells in 10 mL of Serum + Lif culture medium, supplemented with 2.5 μM of ML-792 (2.5 μL of a 10 mM stock solution) on a gelatin coated 100 mm culture plate.
   c. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.

   Note: The interval between the two rounds of hypoSUMOylation can be extended to more than 5 days with no effect on the efficacy of spheroid formation. We tested up to 15 days of recovery time between the two rounds with a successful outcome.

6. Treat ES R1 cells with ML-792 (day 9):
   a. Aspirate culture medium.
   b. Add 10 mL of Serum + Lif culture medium, supplemented with 2.5 μM of ML-792 (2.5 μL of a 10 mM stock solution).
   c. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
7. Stop the second hypoSUMOylation round (day 10):
   a. Aspirate culture medium.
   b. Wash cells with PBS twice.
   c. Add 10 mL of N2B27 + Lif culture medium.
   d. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
8. Allow cells to recover from hypoSUMOylation in N2B27 + Lif culture medium:
   a. The next day (day 11), wash cells with PBS once.
   b. Add 10 mL of N2B27 + Lif culture medium.
   c. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
9. Passage cells in N2B27 + Lif culture medium (day 12):
   a. Aspirate culture medium.
   b. Wash cells with PBS.
   c. Add 1 mL of Trypsin-EDTA and incubate cells at 37°C in a humidified incubator with 5% CO_2 for 30–60 s.
   d. Add 9 mL of N2B27 + Lif culture medium and pipet cell suspension until homogeneous.
   e. Transfer cells to a 15 mL Falcon tube.
   f. Centrifuge cells at 1,000 g for 5 min at 18°C–25°C.
   g. Discard the supernatant and resuspend cells in 3 mL of N2B27 + Lif culture medium.
   h. Transfer 1 mL of resuspended cells to a gelatin coated 100 mm culture plate containing 9 mL of N2B27 + Lif culture medium.
10. Passage cells every other day (1:3 dilution) and replace medium on days without a passage.

    Note: By day 16, three-dimensional adherent spheroids should be visible (Figure 2B).

11. Make stocks of adherent spheroid cells:
    a. Count spheroid cells dissociated as in Step 9 in N2B27 + Lif culture medium.
    b. Centrifuge cells at 1,000 g for 5 min at 18°C–25°C.
    c. Discard supernatant and resuspend cell pellet in cryopreservation medium (ES Cell Qualified FBS with 10% of sterile DMSO) in order to have 3 million cells/mL.
    d. Distribute 1 mL of this spheroid cell stock mixture to labeled cryovials and transfer them to an appropriate freezing container.
    e. Store at −80°C then transfer to liquid nitrogen for longer term storage.

12. Thaw a vial of cryopreserved spheroid cells:
    a. Coat a 6-well plate with 2 mL of 0.1% Gelatin per well.
    b. Pre-warm N2B27 + Lif culture medium in a 37°C water bath.
    c. Transfer a vial of spheroid cells from liquid nitrogen to a cell culture hood.
    d. While cell suspension is thawing, add 9 mL of N2B27 + Lif culture medium to a 15 mL Falcon tube.
    e. Add 1 mL of N2B27 + Lif culture medium to the thawing cell suspension in the vial.
    f. Gently pipet the cell suspension and transfer it to the Falcon tube.
    g. Centrifuge the resuspended cells at 1,000 g for 5 min at 18°C–25°C.
    h. Aspirate the gelatin from one well of the 6-well plate and add 1 mL of N2B27 + Lif culture medium.
    i. After centrifugation, discard the supernatant and resuspend spheroid cell pellet in 1 mL of N2B27 + Lif culture medium.
    j. Gently administer spheroid cell suspension dropwise to the well.
    k. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 h.
    l. The next day, check that cells have adhered.
13. Passage spheroid cells every other day on gelatin coated wells and replace culture medium on days without a passage.

Note: Post-thawing, passage spheroid cells at least twice before proceeding to the next steps of the protocol. Spheroid cells can be maintained in culture for up to 4 weeks, during which time they can be used to generate gastruloids and ELSs.

Note: As previously reported,[1] ES R1 cells cultured in ground state condition (MEK inhibition and GSK3 inhibition with Lif)[4] are unable to form spheroids.

Alternatives: Two other ESC lines (CGR8 and E14) have been tested to generate spheroids under comparable conditions, yielding a similar efficiency.

Generation of gastruloids in non-adherent microwells

Timing: 3 days

This section describes the experimental steps required to generate gastruloids from dissociated spheroid cells seeded in non-adherent AggreWell plates (Figure 3A). The spheroid cells aggregate in the microwells and self-organize to mimic the gastrulation stage (~E6.75) of the embryonic development, both at a transcriptomic and a morphological level. These gastruloids present a properly positioned primitive streak and establish proximal-distal and anteroposterior axes.

For details about the characterization of the gastruloid cell types and structure, please refer to Cossec et al. (2023).[1]

14. Prepare the AggreWell plate:
    a. Add 1 mL of Anti-Adherence Rinsing Solution per well of an AggreWell 800 plate.
    b. Centrifuge the plate at 300 g for 5 min at 18°C–25°C.
    c. Incubate the plate at 18°C–25°C in a cell culture hood for 30 min.
    d. Aspirate the Anti-Adherence Rinsing Solution.
    e. Wash each well with 2 mL of PBS twice.
    f. Add 500 μL of N2B27 + Lif culture medium per well.
    g. Store plate at 37°C in a humidified incubator with 5% CO_2 until use.

Note: AggreWell plate can be prepared and stored at 37°C for up to 2 days before cell seeding.

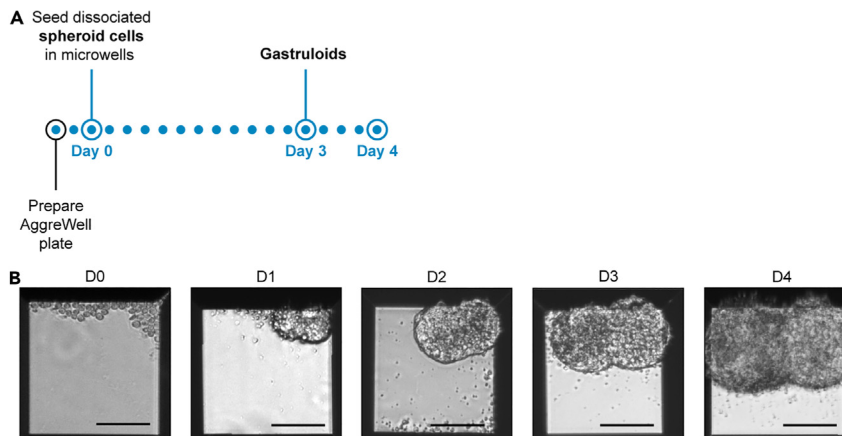

Figure 3. Generation of gastruloids in non-adherent microwells
(A) Experimental timeline.
(B) Representative images of the gastruloids at different time points. Scale bars, 100 μm.

15. Seed spheroid cells in the AggreWell plate:
    a. Count dissociated spheroid cells in N2B27 + Lif culture medium.
    b. Seed 30,000 cells in 1 mL of N2B27 + Lif culture medium per well of the AggreWell plate (∼100 cells per microwell).

Note: Cell number can be increased up to 40,000 cells per well (∼133 cells per microwell).

Optional: Centrifuge AggreWell at 300 g for 2 min at 18°C–25°C after cell seeding.

16. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 3 days to obtain gastruloids (Figure 3B).

Note: Cells can be incubated for a 4th day if bigger gastruloids are required (i.e., for staining experiments) or if longer kinetics are needed (i.e., for treatment, compound testing…) (Figure 3B).

Optional: For the initial three days, refreshing the culture medium is unnecessary. However, if cells are incubated for a 4th day or if medium needs to be changed to apply a treatment, carefully aspirate 750 μL of cell culture medium from each well, taking care not to disturb the gastruloids. Gently add 750 μL of fresh N2B27 + Lif culture medium by pipetting slowly against the wall of the well.

Generation of gastruloids using microdroplets

Timing: 4 days

This section describes the experimental steps required to generate gastruloids from dissociated spheroid cells seeded in the microfluidic chip (Figure 4A). The spheroid cells aggregate and self-organize to form elongated structures, with a mesodermal posterior pole and a neuroectodermal anterior pole. These gastruloids contain properly patterned cell types from all three embryonic germ layers.

For details about the characterization of the gastruloid cell types and structure, please refer to Cossec et al. (2023).[1]

Note: Fabrication of the chip was described in the before you begin section.

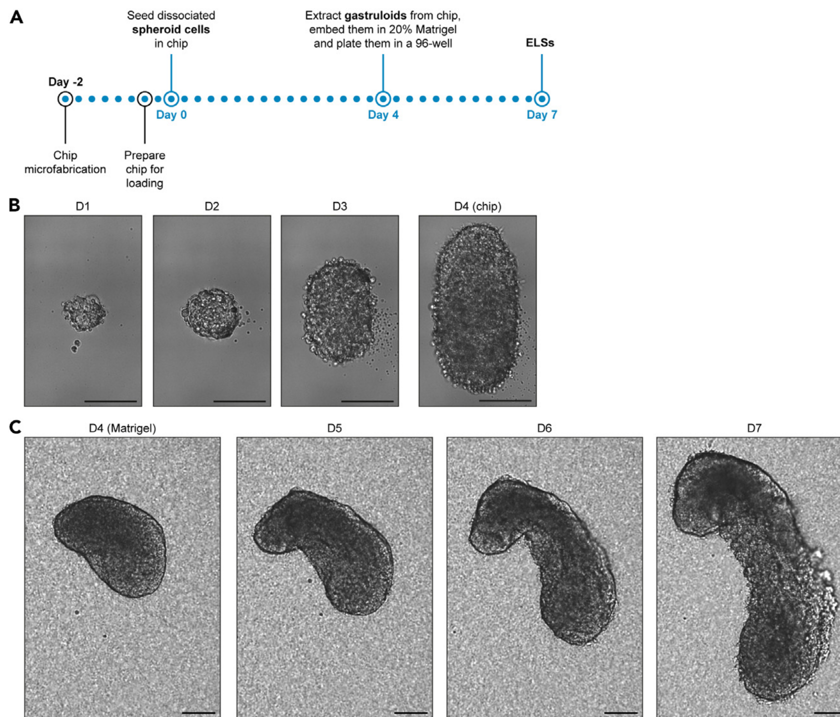

Figure 4. Generation of gastruloids and ELSs using microdroplets

(A) Experimental timeline.

(B) Representative images of the gastruloids in the chip at different time points.

(C) Representative images of the ELSs embedded in 20% Matrigel in a 96-wells plate at different time points. Scale bars, 100 μm.

17. Render the chip fluorophilic:
    a. Equip the inlet and the three outlets of the chip with PFA resin tubing (Idex 1507L) with an inner diameter of 0.040″ and outer diameter of 1/16′′.
    b. Hold the chip vertically under a chemical hood and use a syringe to insert 4–6 mL of Novec 1720 (3M) in the inlet to fill the chip.
    c. Heat the chip at 110°C for 1 h to ensure that the Novec solvent has fully evaporated.

⚠ CRITICAL: Novec surface treatment should be done under a chemical hood.

18. Prepare the chip, fluidic connectors and droplet generator:
    a. Hold the chip vertically and use a syringe to insert ~5 mL of fluorinated oil (FC-40, 3M) containing 0.5% RAN surfactant (RAN biotechnology) in the inlet to fill the chip and remove air bubbles.

Alternatives: 0.5% RAN can be replaced by a 0.5% FluoSurf neat (Emulseo) solution.

    b. Fill two 1 mL glass syringes (SGE), equipped with PTFE tubing (0.56 mm ID and 1.07 mm OD, Adtech), with a FC-40/0.5% RAN solution.
    c. Equip a low-pressure cross body (Idex, 1/4–28 flat-bottom ports) with Idex Flangeless Short Head Fitting Assemblies (1/4–28 flat-bottom connection for 1/16″ OD Tubing) and with PFA resin tubing (Idex 1507L) with an inner diameter of 0.040″ and outer diameter of 1/16″, connected with three silicone tubing segments (ID 1.0 mm, Thermo Fisher Scientific). This cross junction assembly constitutes the droplet generator (Figure 5A).

Note: The cross junction can be reused by washing it first with a 70% ethanol solution, then with DI water, and leaving it to dry in an oven set at 70°C for ~1 h.

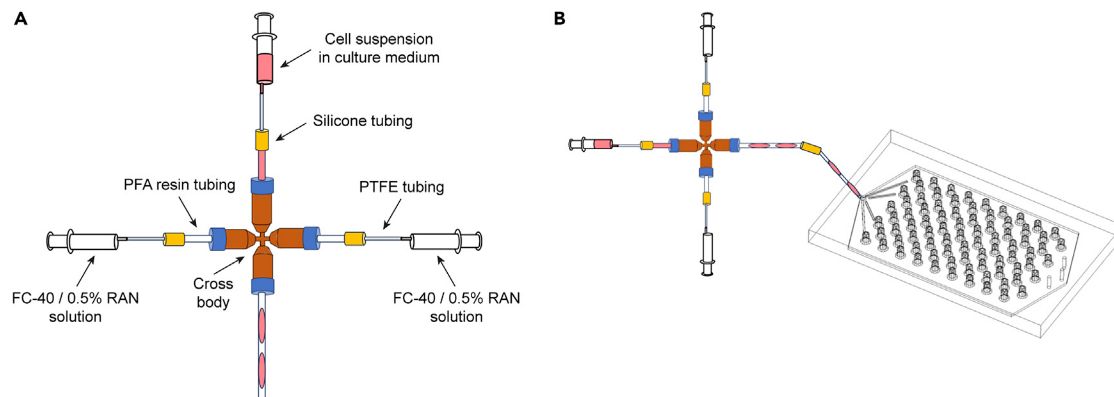

Figure 5. Schematic of the droplet generator

(A) Cross junction assembly.

(B) Connection of the droplet generator to the chip.

19. Prepare a spheroid cell suspension for microfluidic chip loading:
    a. Count dissociated spheroid cells in N2B27 + Lif culture medium.
    b. Prepare a spheroid cell suspension at 16,800 cells/mL in N2B27 + Lif culture medium (~120 cells per 7 μL droplets) in a Falcon tube.

    Note: 1.5 mL of spheroid cell suspension is required to load each chip.

    Note: A range of 8,400 to 25,200 spheroid cells/mL can be used.

    Note: Spheroid cell suspension can be stored at 37°C in a humidified incubator with 5% CO_2 for up to 1 h before chip loading.

    c. Homogenize cell suspension and prepare 1.5 mL aliquots in 2 mL Eppendorf tubes.
    d. Aspirate an aliquot of cell suspension into a 1 mL plastic syringe (BD), equipped with PTFE tubing.
20. Prepare the fluidic system for chip loading:
    a. Install the two syringes filled with FC-40/0.5% RAN and the one filled with cells and culture medium in the syringe bay of a Nemeysis syringe pump (Cetoni).
    b. Connect the PTFE tubes to the cross junction through the silicone tubing connectors, as shown in Figure 5A.
    c. Prime the droplet generator by flowing the cell suspension at 300 μL/min and the FC-40/0.5% RAN from the two glass syringes also at 300 μL/min, until all of the tubes are completely filled with liquid and all the air has been evacuated.
21. Load the chip:
    a. Connect the fourth PFA tube of the droplet generator to the chip inlet through the silicone tube connector (Figure 5B).
    b. Fill the device with FC-40/0.5% RAN by flowing from the two glass syringes in order to remove any remaining air bubbles in the traps and the chamber.
    c. Tilt the chip at an angle of 30°–45° in order to use buoyancy to help the fluid flow.
    d. Droplet containing cells are generated by injecting 7 μL plugs of the cell suspension at 1,000 μL/min then stopping the aqueous flow, then injecting 6 μL of FC-40/0.5% RAN segments generated at 1,000 μL/min. This alternating operation is then repeated to obtain the desired number of drops. By choosing a sufficiently long outlet tube, the drops can be stored in the tubing before reaching the microfluidic chip.
    e. Flow the water-in-oil segments down the tubing and into the microfabricated device.
    f. Once the drops are in the microfluidic device, tilt the chip to guide the droplets towards the individual traps.

g. Trap one droplet in each trap.

Note: Droplet guiding can be additionally facilitated by orienting the chip in the direction of targeted traps.

h. Allow the droplets to move to the upper part of the traps by gravity.
i. Disconnect the droplet generator from the chip.
j. Wash any remaining extra droplets by flowing pure FC-40 from the inlet at 300 μL/min.

22. Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 4 days to obtain gastruloids (Figure 4B).

Matrigel embedding of microdroplet gastruloids to generate ELSs

Timing: 3 days

This section describes the experimental steps required to generate ELSs from the microfluidic-derived gastruloids (Figure 4A). Once embedded in Matrigel, the gastruloids elongate much further, reaching lengths of over 1 mm, and adopt a head-and-trunk morphology. These ELSs contain properly patterned derivatives of the neuroectodermal (midbrain-hindbrain, spinal cord), mesodermal (somites, mesenchyme) and endodermal (gut) lineages.

For details about the characterization of the ELS cell types and structure, please refer to Cossec et al. (2023).[1]

23. Prepare Matrigel:
    a. Thaw a bottle of Matrigel on ice in a 4°C fridge for 16–24 h.
    b. The next day, prepare 1 mL aliquots of thawed Matrigel.
    c. Prepare N2B27 + Lif culture medium with 20% Matrigel by adding 1 mL of Matrigel to 4 mL of N2B27 + Lif culture medium.

CRITICAL: The Matrigel handling steps should be carried out on ice to prevent premature gelling.

    d. Store remaining Matrigel aliquots at −20°C for later use.
24. Recover microdroplet-derived gastruloids:
    a. Connect a 10 mL syringe containing pure FC-40 to the inlet of the chip.
    b. Flip the chip vertically.
    c. Allow the droplets to move out of the traps.
    d. Flush ∼10 mL of FC-40 from the inlet to facilitate droplets removal from the chip.
    e. Collect droplet contents in a 15 mL Falcon tube.
    f. Separate droplet contents from oil by aspirating with a 1,000 μL tip which has been beveled at its extremity.
    g. Place droplet contents in a 2 mL Eppendorf tube.
    h. Add 100 μL of a 10% PFO solution and mix content by inversion.
    i. Centrifuge at 900 g for 2 min at 18°C–25°C in order to break the emulsion.

Alternatives: Remaining emulsion can be broken by filtration on PTFE membrane (Thermo Fisher Scientific #F2517-9).

    j. Transfer droplet contents to a 1.5 mL Eppendorf tube and allow the gastruloids to settle by gravity.
    k. Discard the supernatant and mix the gastruloids with 1 mL of 20% Matrigel in N2B27 + Lif culture medium.

l. Transfer the gastruloids in Matrigel to the stock tube of 20% Matrigel in N2B27 + Lif culture medium.
m. Mix by inversion.
n. Seed the gastruloids in Matrigel in a 96-well plate with flat bottom and treated for ultra-low adhesion, by dispersing 100 μL of the stock solution using a 1,000 μL beveled tip.
o. Mix stock tube by inversion after seeding each line of the 96-well plate.

25. Incubate structures at 37°C in a humidified incubator with 5% CO_2 for 3 days to obtain ELSs (Figure 4C).

EXPECTED OUTCOMES

This protocol provides a reproducible method to generate models of mouse gastrulation and early organogenesis from ESCs solely treated with a chemical inhibitor of SUMOylation.

The treatment regimen (Step 1) in ES R1 cells yields adherent spheroids with 100% success rate (Figure 2). These spheroids are composed of peri-implantation cell types (epiblast-like, primitive endoderm-like) and ESC-like cells that can self-organize to form gastruloids and ELSs in non-adherent culture conditions.

Seeding dissociated spheroid cells in AggreWell plates (Step 2) generates gastruloids (~E6.75 stage) with an 83% efficacy (Figure 3). Day 3 gastruloids are ~200 μm in length, present an anteroposterior axis and express primitive streak, nascent mesoderm and definitive endoderm markers, as well as neuromesodermal progenitors and neuroepithelium markers.

Seeding dissociated spheroid cells in a custom droplet microfluidic device (Step 3) produces gastruloids containing cell types of all three embryonic germ layers patterned along an anteroposterior axis (Figure 4). The day 4 gastruloids have a major axis length of ~350 μm. Matrigel embedding of the microdroplet-derived gastruloids (Step 4) generates elongated ELSs with a 74% efficacy (Figure 4). These ELSs have an enlarged pole expressing anterior neural markers and a trunk with somites-like segments. 31% of ELSs reach lengths of over 1 mm.

LIMITATIONS

This protocol describes the generation of spheroids, gastruloids and ELSs from the ES R1 cell line. While we have reproduced these results with other cell lines (CGR8 and E14), the efficacy for each step varies from cell line to cell line. The choice of cell line might thus need to be optimized depending on the aims of one's study.

The gastruloids and ELSs are not perfect mimics of the *in vivo* embryonic development. They notably lack extraembryonic ectoderm derivatives, primordial germ cells, a notochord and the hematopoietic lineage. The microwell gastruloids are partially desynchronized with a premature expression of neuromesodermal and neuroepithelial markers. The ELSs present a predominantly dorsalized phenotype and an underdeveloped gut endoderm relative to the mesoderm and neuroectoderm lineages.

While the gastruloids and ELSs recapitulate some aspects of gastrulation and early organogenesis quite faithfully, other developmental pathways appear to be hindered. Studying these defects could shed some light on the morphogenesis of certain tissues.

TROUBLESHOOTING

Problem 1

Massive cell death or a high percentage of differentiation is achieved following the two days of treatment with the SUMOylation inhibitor (related to step #3).

Potential solutions

- Some cell lines are more sensitive to the chemical inhibitors than others. The treatment regimen can be performed on ESCs cultured on feeder MEF-coated plates to increase cell viability and maintenance of pluripotency after the first round of treatment. Cells can be maintained on feeders until day 12, after which time they can be passaged on gelatin coated plates to observe spheroid formation.
- Alternatively, the concentration of the chemical inhibitor of SUMOylation could be optimized depending on the cell line.

Problem 2

Spheroids cells fail to properly reattach and/or don't reassemble after passage (related to step #13).

Potential solution

Check the expiration dates of the N2B27 + Lif culture medium components. Preparing a new bottle of medium with a fresh solution of 2-Mercaptoethanol could resolve this issue.

Problem 3

Low success rate of gastruloid or ELS formation (related to steps #16 and #25).

Potential solutions

- Passage spheroids on adherent plates a few more times to allow proper recovery after thawing before using cells for gastruloid or ELS generation.
- Alternatively, if spheroid cells have been in culture for close to a month, thaw a new vial and allow cells to recover before using them for gastruloid or ELS generation.

Problem 4

The microwell gastruloids are too small (related to step #16).

Potential solution

Increase dissociated spheroid cell number at the seeding step (#15) to improve aggregation and elongation in the microwells.

Problem 5

ELSs don't elongate following Matrigel embedding (related to step #25).

Potential solutions

- Check the expiration dates of the N2B27 + Lif culture medium components. Preparing a new bottle of medium with a fresh solution of Bovine Albumin Fraction V (7.5% solution) could resolve this issue.
- Check the expiration dates of the fluorinated oil, surfactant and Novec used during the chip preparation and loading steps (#17 to 21). Replacing them with new lots could resolve this issue.

Problem 6

Droplets break as they enter into the chip or as they get trapped in the individual trapping positions.

Potential solutions

- Angle the inlet tube at about 45° into the chip.
- Try lower flow rates of the injection of the water-oil suspension.
- Design traps with rounded edges at the point where they reach the wide microfluidic chamber.

Problem 7

The flow in the chamber is too slow and the droplets are stuck near the channel inlet.

Potential solution

Punch several outlets at the downstream end of the channel to favor oil flow.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jack-Christophe Cossec (jcossec@pasteur.fr).

Materials availability

This study did not generate new unique reagents. The printing file for the chip mold can be sent upon request.

Data and code availability

This published article did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, J.-C.C. and A.D.; experiment design and execution, T.T., S.S., and J.-C.C.; droplet microfluidic platform design, S.S. and C.N.B.; writing – review and editing, T.T., S.S., and J.-C.C., with input from C.N.B. and A.D.; funding acquisition and supervision, C.N.B. and A.D.

DECLARATION OF INTERESTS

Authors are designated as inventors of the patent application WO/2023/002057 covering aspects of the *in vitro* generation of organized 3D cell structures and the microfluidic chip described in the article.

REFERENCES

1. Cossec, J.C., Traboulsi, T., Sart, S., Loe-Mie, Y., Guthmann, M., Hendriks, I.A., Theurillat, I., Nielsen, M.L., Torres-Padilla, M.E., Baroud, C.N., and Dejean, A. (2023). Transient suppression of SUMOylation in embryonic stem cells generates embryo-like structures. Cell Rep. *42*, 112380. https://doi.org/10.1016/j.celrep.2023.112380.
2. He, X., Riceberg, J., Soucy, T., Koenig, E., Minissale, J., Gallery, M., Bernard, H., Yang, X., Liao, H., Rabino, C., et al. (2017). Probing the roles of SUMOylation in cancer cell biology by using a selective SAE inhibitor. Nat. Chem. Biol. *13*, 1164–1171. https://doi.org/10.1038/nchembio.2463.
3. Langston, S.P., Grossman, S., England, D., Afroze, R., Bence, N., Bowman, D., Bump, N., Chau, R., Chuang, B.C., Claiborne, C., et al. (2021). Discovery of TAK-981, a First-in-Class Inhibitor of SUMO-Activating Enzyme for the Treatment of Cancer. J. Med. Chem. *64*, 2501–2520. https://doi.org/10.1021/acs.jmedchem.0c01491.
4. Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519–523. https://doi.org/10.1038/nature06968.