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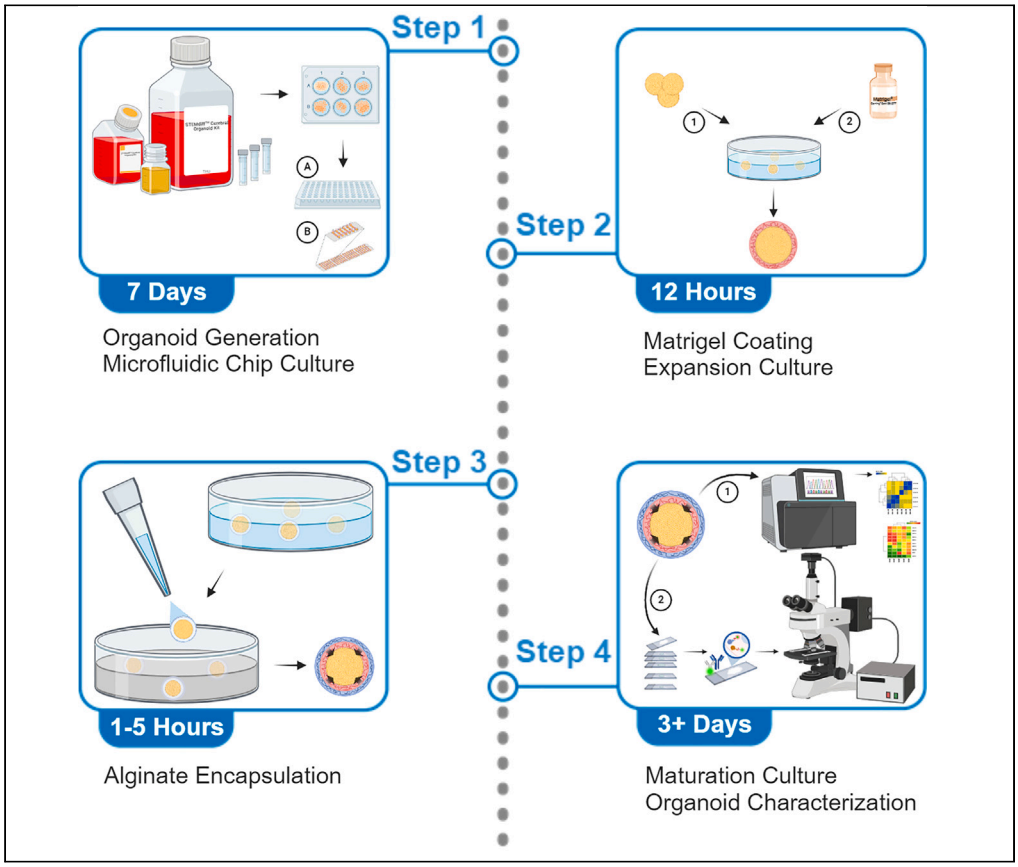
Protocol to encapsulate cerebral organoids with alginate hydrogel shell to induce volumetric compression



In vitro organoids, including cerebral organoids, are usually developed without mechanical compression, which may contribute to a delay in maturation. Here, we present a protocol for encapsulating cerebral organoids with a thin shell of low-concentration alginate hydrogel. We describe steps for organoid generation, microfluidic chip culture, Matrigel coating, expansion culture, and alginate encapsulation. We then detail procedures for maturation culture and organoid characterization. The moderate compressive stimulation that the shell provides promotes cell proliferation and neuronal maturation.

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

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Highlights

Instructions for enveloping unguided neural organoids with alginate hydrogel

Soft, thin, and bioinert alginate hydrogel is used for volumetric compression

Volumetric compression promotes organoid growth and neuronal differentiation

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Protocol to encapsulate cerebral organoids with alginate hydrogel shell to induce volumetric compression

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SUMMARY

***In vitro* organoids, including cerebral organoids, are usually developed without mechanical compression, which may contribute to a delay in maturation. Here, we present a protocol for encapsulating cerebral organoids with a thin shell of low-concentration alginate hydrogel. We describe steps for organoid generation, microfluidic chip culture, Matrigel coating, expansion culture, and alginate encapsulation. We then detail procedures for maturation culture and organoid characterization. The moderate compressive stimulation that the shell provides promotes cell proliferation and neuronal maturation.**

For complete details on the use and execution of this protocol, please refer to Tang et al.[1]

BEFORE YOU BEGIN

Protocols for the generation of organoids, such as unguided neural organoids and forebrain organoids,[2] have been well established for a long time and broadly studied.[3–6] However, these protocols focus on supplying guidance molecules into the culture medium instead of simulating the physical environment attributed to limited space and pressurized fluid of early cavities,[7] which persist during embryonic development.[8,9] Incorporating biomechanical cues in development and regeneration research reveals the important role of compressive force in directing cell position and fate.[9–11]

This protocol introduces a method to generate unguided neural organoids starting from a human embryonic stem cell line with a commercial kit and then encapsulate the organoids[5] in a thin shell of alginate hydrogel, which provides moderate volumetric compression mimicking the pressure of adjacent tissues as *in vivo*. When organoids are visible and have a diameter of less than 1000 μm, encapsulation can be achieved. Therefore, there is no limitation on enveloping other organoids such as forebrain organoids generated by either reported guided methods[3,4,12,13] or other commercial kits to generate dorsal (STEMCELL Technologies Cat# 08620), ventral (STEMCELL Technologies Cat# 08630) and midbrain organoid (STEMCELL Technologies Cat# 100-1096) by using both human and murine embryonic cell lines. Moreover, this encapsulation protocol can be applied to any type of organoids cultured in suspension.

Generation of unguided neural organoid

Timing: 9 days

1. Fabrication of polydimethylsiloxane (PDMS) microfluidic chip using photo- and soft-lithography.[14,15]
   a. Use photolithography to pattern SU-8 2075 (Kayaku Advanced Materials Inc.; Cat# NC9463827) into 100 μm-deep microstructures (Figure S1) on a silicon wafer as per manufacturer's instructions.
   b. Use soft lithography to create a PDMS replica of the microstructures.
      i. Pour a mixture of PDMS prepolymer and curing agent (Sylgard 184; Dow Corning; 10:1 (w/w) ratio) onto the silicon mold.
      ii. Degas in a vacuum chamber for 30 min.
      iii. Cure for 1 h at 80°C on a hotplate.
   c. Peel the cured PDMS chip off the mold then create (3 mm diameter) organoid chambers and (0.8 mm diameter) inlets/outlets using a biopsy puncher.
   d. Bond the PDMS slab onto a glass substrate by applying oxygen plasma treatment on both surfaces and then bringing them into contact.
   e. Autoclave the PDMS chip at 121°C for 20 min and dry it in a heating oven at 60°C for 16 h.
   f. Insert organoids into the chambers using a pipette, then culture under static or dynamic conditions (rocker, shaker, perfusion with a syringe pump, or gravity as desired).

   Note: Use the multi-chamber microfluidic chip to 1) increase nutrient diffusion from the medium, 2) easily change the medium with minimal residual old medium and 3) reduce the medium consumption by saving approximately one-third of the medium following the instruction of using the STEMdiff Cerebral Organoid Kit (STEMCELL Technologies, Cat# 08570).

   Optional: Culturing embryoid bodies (EBs) in a U-bottom ultra-low attachment 96-well plate for the first 7 days instead of in the chip will not yield significant differences.

2. Culture of human embryonic stem cells (ESCs).
   a. Coat one well of a 6-well plate with 1 mL 1% Matrigel (Corning Cat# 354277) in DMEM/F12 and place it under room temperature or 37°C for 1 h.

      Note: The coated plate can be preserved at 4°C for no more than 1 week.

   b. Wash the attached ESCs culture in one well of a 6-well plate with DMEM/F12 or DPBS.
   c. Add 500 μL ReLeSR in the well and tilt the plate to make sure the cell colonies are all immersed and covered with ReLeSR solution, then aspirate and discard the ReLeSR solution within 1 min.
   d. Incubate the plate at 37°C for 1–2 min.
   e. Add 1 mL mTeSR plus complete medium in the well and hold the plate with one hand, then use another hand to tap the plate.

      Note: Detached colonies are easy to observe with the naked eye.

   f. Collect the detached colonies into a new 15 mL conical tube, and suspend the cell colonies with a 1 mL pipette to ensure the cell clusters are around 50 μm–150 μm in size and there are no large cell aggregates.
   g. Seed the ESCs in the Matrigel-coated well at the density of 1–2 clusters per 1 mm^2.
   h. Culture the cells with 2 mL of mTeSR plus medium with daily medium change (10 μM of Y-27632 is added for the first 24 h of culture).

Note: We tested other cell lines of human pluripotent stem cells, such as human ESC line H9 from WiCell (Cat# Wa09) and induced pluripotent stem cell line DXR0109B from ATCC (Cat# ACS-1023) in our laboratory.[16] Human pluripotent stem cells, such as the ESC line H1 from WiCell and the human induced pluripotent stem cell (hiPSC) line DYR0100 from ATCC, can also be cultured using mTeSR plus products according to this protocol.

⚠ CRITICAL: Do not triturate the cell suspension with a 1 mL pipette in step b more than two times. Breakup of large cell aggregates should be carried slowly. The physical pressure and shear stress created near the front opening of the pipette tip will increase the probability of spontaneous differentiation of PSCs.

3. Generation of embryoid body (EB).
   a. Wash the cells with DMEM/F12, and incubate the plate under 37°C with 1 mL of Accutase for each well for 2–3 min.
   b. Harvest the cells by transferring the cell suspension in a 15 mL conical tube and add an additional 9 mL of mTeSR plus complete medium in the same tube.
   c. Centrifuge the cell suspension at 250× *g* for 5 min.
   d. Resuspend the cell pellet in mTeSR plus complete medium and count the number of the cells.
   e. Transfer the cells in an ultra-low attachment U-bottom 96-well plate with a density of 6000 cells in 200 μL mTeSR plus complete medium containing 10 μM of Y-27632 per well.

Note: The initial number of cells for one well of the 96-well plate has the range from 1500[16] to 9000.[17] We recommend generating EB in the same medium that maintains PSCs. For example, if PSCs are maintained in mTeSR1, mTeSR plus, or E8 medium, the same type of medium should be used for the first 24 hours for PSCs to aggregate and form EBs. Do not use the E8 medium to culture mTeSR-maintained PSCs for this step. 24 hours after seeding the cells, change the mTeSR plus complete medium to EB formation medium of the STEMdiff Cerebral Organoid Kit (STEMCELL Technologies, Cat# 08570; relevant protocols) and regard it as day 0.

   f. Centrifuge the plate at 150× *g* for 5 min in a centrifuge machine. The aggregation of the cells at the bottom of the well is observed clearly (Figure 1A).

Note: Centrifugation is recommended here to assist cells in aggregating faster and achieving a more uniform aggregate shape, which ensures the formation of more regular organoids. Moreover, centrifuge makes more EBs show round morphology compared with natural aggregation. If a V-bottom plate is used, centrifugation is not necessary. As the V-bottom plate has a steeper slope, cells roll down to the bottom quickly to form an aggregation.

   g. Culture the cells in 200 μL mTeSR plus complete medium per well with 10 μM of Y-27632.

Note: After one day of culture, EBs are formed and show round morphology (Figure 1B).

   h. Transfer EBs to the chambers of a Polydimethylsiloxane (PDMS) microfluidic chip with a normal 100 μL pipette tip (Figure 1C).
   i. Culture EBs in the multi-chamber microfluidic chip with 60 μL of EB formation medium of STEMdiff Cerebral Organoid Kit for each row (connected channel for 3 organoids) (Figures 1C and 1D) on a rocking shaker in a humid cell culture incubator (Figures 1E and 1F) for 5 days with daily medium change.

Note: In our design of a microfluidic chip, we inserted truncated 10 μL pipette tips into both the left and right chambers of a row. Delicate transfer of EBs can be conducted either before or after pipette tip insertion.

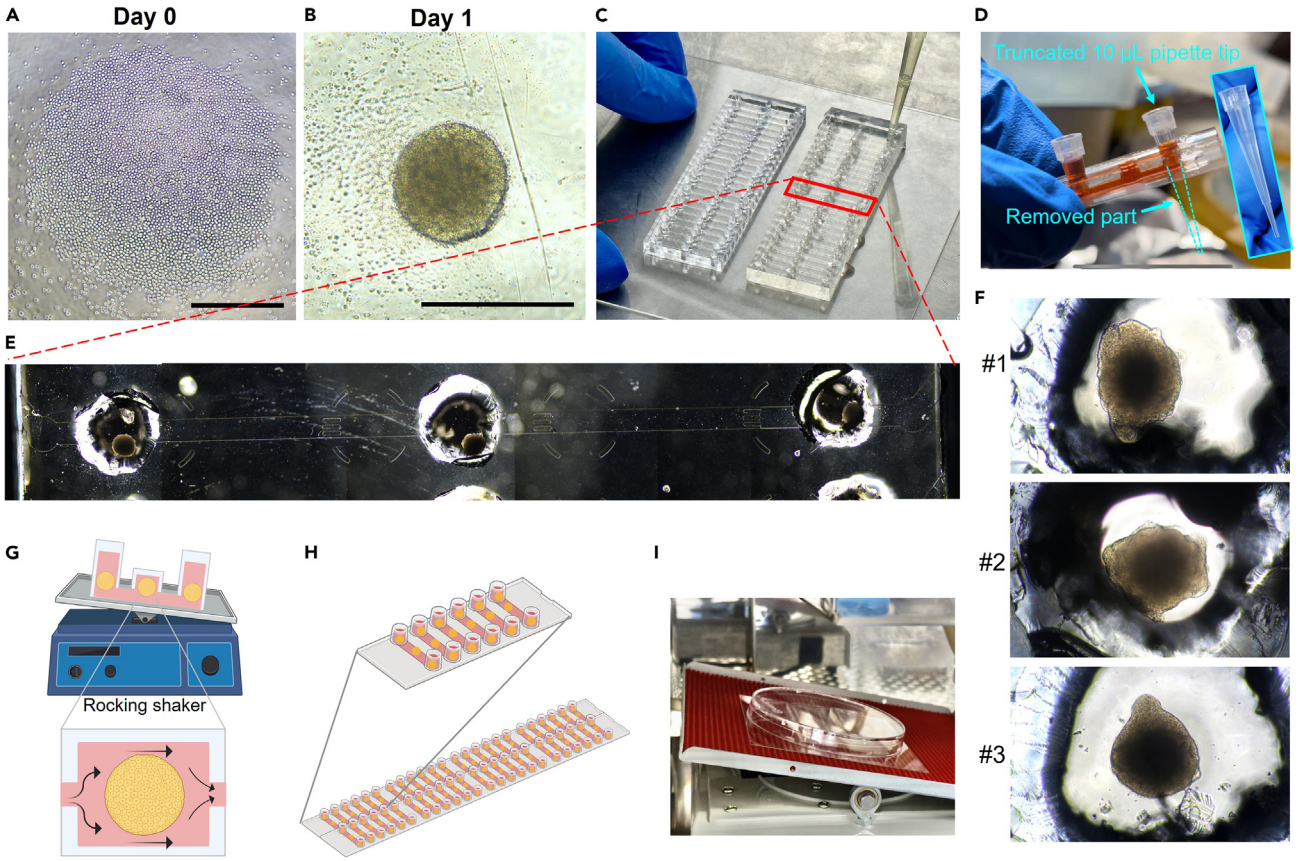

Figure 1. EB generation and culture in a microfluidic chip

(A) Stem cells in U-bottom 96-well plates, after centrifugation. Scale bar: 500 μm.

(B) EB formed and checked 24 h after seeding. Scale bar: 500 μm.

(C) Loading EBs onto a microfluidic chip.

(D) Bright-field images of three EBs cultured in one channel of a chip.

(E) Representative bright field images of EBs with 3 repeats.

(F) Enlarged images of 3 representative organoids cultured in chip.

(G) Schematic of culturing EBs in a microfluidic chip on a rocking shaker.

(H) Schematic of the microfluidic chip design. The upper panel shows the assembly of the chip, and the lower panel shows its transformation into a high-throughput chip.

(I) Photo of the chips on a rocking shaker in a cell culture incubator.

4. Organoid generation.
   a. For chip culture, make a daily medium change with 60 μL EB formation medium.
   b. For the 96-well plate culture, change the EB formation medium following the instruction of the manufacturer (STEMCELL Technologies, Cat# 08570; relevant protocols). Briefly, once the EBs are formed, apply EB formation medium for 5 days and Neural induction medium of STEMdiff Cerebral Organoid Kit for 2 days subsequently.
   c. At day 7, change the Neural induction medium to an Expansion Medium (STEMCELL Technologies, Cat# 08570; relevant protocols) containing 2% of Matrigel (Corning, Cat# 354230).

Note: The culture of organoids on a chip is ended after this step. From now on the organoids cultured either in a 96-well plate from the beginning or on a chip will be transferred to a U-bottom ultra-low attachment 96-well plate for a 3-day static culture with Expansion Medium for encapsulation (see steps below). In our experimental condition, we hardly found any significant differences in the organoids cultured in a 96-well plate or on a chip according to the morphological hallmark under a bright field view.

d. Encapsulate organoids with alginate hydrogel 12 h after culturing in an Expansion Medium with 2% Matrigel (indicated in step 1 in the step-by-step method details).
e. Culture the organoids statically in U-bottom ultra-low attachment 96-well plate in Expansion Medium until day 10.
f. From day 10, transfer organoids with a wide-bore pipette tip (either cut to a 1 mm opening or use commercialized products) to a 6-cm petri dish.

Note: We suggest no more than 16 organoids in one 6-cm petri dish to reduce the chance of organoid fusion.

g. If organoids are cultured with a 96-well U-bottom plate at the start, transfer 16 organoids by wide-bore pipette tip to a 6-cm petri dish at day 10.

Note: This differs from the method of the STEMdiff Cerebral Organoid Kit (relevant protocols), which indicates a transfer of EBs in an ultra-low attachment 24-well plate for a 2-day Neural induction period. We choose to leave the organoids in the U-bottom plate and change the neural induction medium daily during the 2-day culture period. We then use an Expansion Medium containing 2% Matrigel to culture 3 more days, according to the instruction of the STEMdiff kit (STEMCELL Technologies, Cat# 08570).

h. Culture the organoids in 6 mL of Maturation medium (STEMCELL Technologies, Cat# 08570, or Cat# 08571) on an orbital shaker from day 10 and change the medium every 3–4 days.

Producing alginate hydrogel

Timing: 8–10 h

5. Preparation of solutions.
   a. Weigh 0.05 *g* of sodium alginate powder (0.1%, w/v) and dissolve it in 50 mL of physiological saline at room temperature on a shaker for 3–5 h.

Note: Prepared solutions can be kept in the 4°C refrigerator for up to 2 weeks, provided sterile storage is ensured.

   b. Weigh 0.5 *g* of calcium chloride powder (1.0%, w/v) and dissolve it in 50 mL of ddH_2O.

Note: (1) Usually, $CaCl_2$ dissolves in water very quickly. (2) Prepared solutions can be stored in a 4°C refrigerator for up to 4 weeks, provided sterile storage is ensured.

Note: All the solutions should be filtered through 0.22 μm filters to eliminate the contaminants.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken polyclonal anti-microtubule-associated protein 2 (MAP2), 1:1,000	Abcam	Cat# ab92434 and ab5392; RRID: AB_2138147 and AB_2138153
Chicken polyclonal anti-neurofilament Heavy (NF), 1:1,000	Sigma-Aldrich	Cat# AB5539; RRID: AB_11212161
Mouse monoclonal IgG1 antibody against human nestin, 1:200	STEMCELL Technologies	Cat# 60091; RRID: AB_2905494

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse monoclonal anti-PKCζ, 1:50	Santa Cruz	Cat# sc-17781; RRID: AB_628148
Rabbit monoclonal anti-Ki67, 1:200	Abcam	Cat# ab16667; RRID: AB_302459
Rabbit polyclonal anti-laminin, 1:500	Abcam	Cat# ab11575; RRID: AB_298179
Donkey polyclonal anti-Mouse IgG H&L (Alexa Fluor 488), 1:1,000	Abcam	Cat# ab150105; RRID:AB_2732856
Donkey polyclonal anti-rabbit IgG H&L (Alexa Fluor 488), 1:1000	Abcam	Cat# ab150073; RRID:AB_2636877
Donkey polyclonal anti-mouse IgG H&L (Alexa Fluor 594), 1:1,000	Abcam	Cat# ab150108; RRID:AB_2732073
Donkey polyclonal anti-rabbit IgG H&L (Alexa Fluor 568), 1:1,000	Abcam	Cat# ab175470; RRID:AB_2783823
Goat polyclonal anti-chicken IgY H&L (Alexa Fluor 488), 1:1,000	Abcam	Cat# ab150169; RRID:AB_2636803
Goat polyclonal anti-chicken IgY H&L (Alexa Fluor 647), 1:1,000	Abcam	Cat# ab150171; RRID:AB_2921318
Chemicals, peptides, and recombinant proteins		
mTeSR Plus complete medium	STEMCELL Technologies	Cat# 100–0276
STEMdiff Cerebral Organoid Kit	STEMCELL Technologies	Cat# 08570
DMEM/F12	Gibco	Cat# 11320033
Matrigel hESC-qualified matrix, LDEV-free, 5 mL	Corning	Cat# 354277
Matrigel growth factor reduced (GFR) basement membrane matrix, LDEV-free	Corning	Cat# 354230
ReLeSR	STEMCELL Technologies	Cat# 05872
Accutase	Sigma-Aldrich	Cat# A6964
Y27632	MCE	Cat# 146986-50-7
Calcium chloride	Sigma-Aldrich	Cat# C5670
Sodium alginate	Macklin	Cat# S817374
Monodisperse fluorescent polystyrene microspheres	Aladdin	Cat# M122073
Polydimethylsiloxane (PDMS)	Dow Corning	Cat# SYLGARD 184 Silicone Elastomer kit
Paraformaldehyde (4% PFA)	Servicebio	Cat# G1101-500ML
Normal donkey serum	Jackson Laboratory	Cat# 017-000-121
DAPI (4′,6′-diamidine-2′-phenylindole dihydrochloride)	Sigma-Aldrich	Cat# D9542
Triton X-100	Sigma-Aldrich	Cat# T8787
Critical commercial assays		
Yefluor 488 EdU Imaging Kit	Yeasen	Cat# 40275ES60
Experimental models: Cell lines		
Human embryonic stem cell (hESC) line H1	MeisenCTCC	CTCC-0805-PC
Other		
Incubator	Esco	Cat# CCL-170T-8
Biological safety cabinet	Esco	Cat# 2010655
Orbital shaker	NEST	Cat# 105008
Rocking shaker	NuoMi	Cat# NMYC-60
U-bottom ultra-low attachment 96-well plate	Corning	Cat# 7007
Sterile microcentrifuge tubes	Axygen	Cat# MCT-150-C-S
6-cm Petri dish	Biofil	Cat# TCD000060
Sterile filter pipette tips	Kirgen	Cat# KG1313; KG1212; KG1111

STEP-BY-STEP METHOD DETAILS

Encapsulation of unguided neural organoid with alginate hydrogel

Timing: 2 h

These steps outline the process for enveloping unguided neural organoids with alginate hydrogel and identifying the transparent alginate hydrogel using fluorescent microspheres (Figure 2).

1. Alginate encapsulation (See Methods video S1).
   a. On the 7th day after EB generation, collect the organoids in a 6 cm petri dish using pipette tips with a larger diameter than the organoids, and then discard the medium.

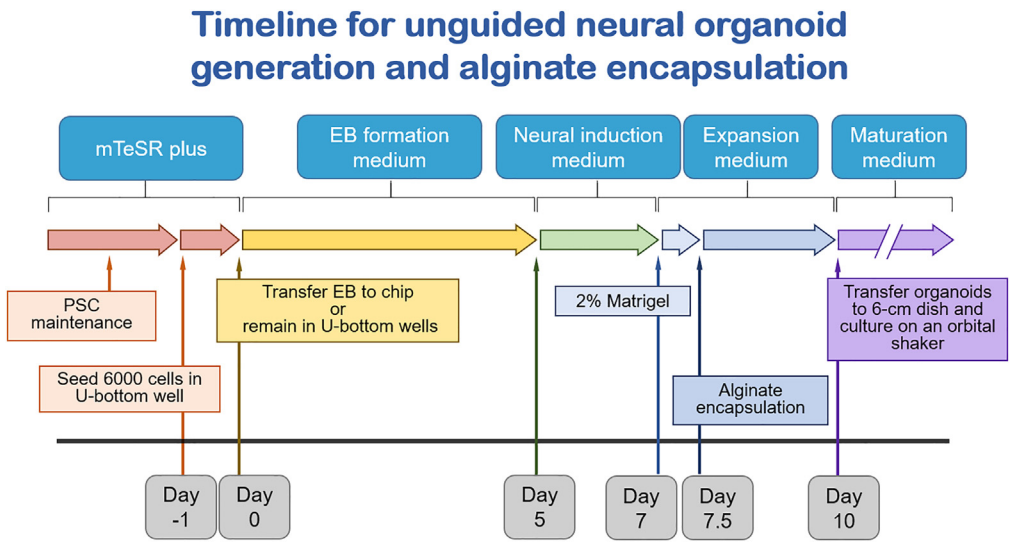

Figure 2. Scheme of unguided neural organoid generation and alginate encapsulation

Note: (1) Given that the organoids in our experiments have a diameter of less than 1 mm, we used 1 mL tips (KG5313, KIRGEN). (2) There is no limit on the number of organoids in a 6-cm dish unless the alginate shell has formed on the first organoid after 1 minute of incubation with the calcium chloride solution; afterward, the organoid needs to be taken out (see steps below).

b. Add 3 mL of sodium alginate solution and gently mix it with the organoids.
c. Aspirate the organoids using a 1 mL pipette. If the organoids are too large, cut the pipette to make the opening slightly larger than the organoids to avoid damage (or use commercialized tips).
d. Place the 1 mL pipette tip 1–2 cm above the liquid surface of 1.0% (w/v) calcium chloride solution, and quickly press the pipette and blow the droplet into the calcium chloride solution. Operate on one individual organoid at a time (Figure 3A–3C).
e. Incubate the droplet at room temperature for 1 min for alginate gelation.

Note: A transparent shell can be observed under a microscope (Figure 3D).

f. Discard the calcium chloride solution using pipette tips.
g. Add 3–4 mL DMEM/F12 to rinse the alginate gel encapsulated organoids twice.
h. Transfer the organoids in a U-bottom ultra-low attachment 96-well plates and culture them in Expansion Medium (STEMCELL Technologies, Cat# 08570; relevant protocols).

Note: The effect of physical confinement causes the encapsulated organoid to exhibit a smooth and round surface (Figure 3E).

2. Labeling of alginate hydrogel with fluorescent microspheres.
   a. Mix the fluorescent microsphere suspension (Aladdin, M122073; 1: 100 v/v) into the alginate solution by stirring with a pipette before usage.
   b. Add 3 mL sodium alginate solution and gently mix it with the organoids.
   c. Encapsulate the organoid with the alginate hydrogel in the same manner as described in step 1c.

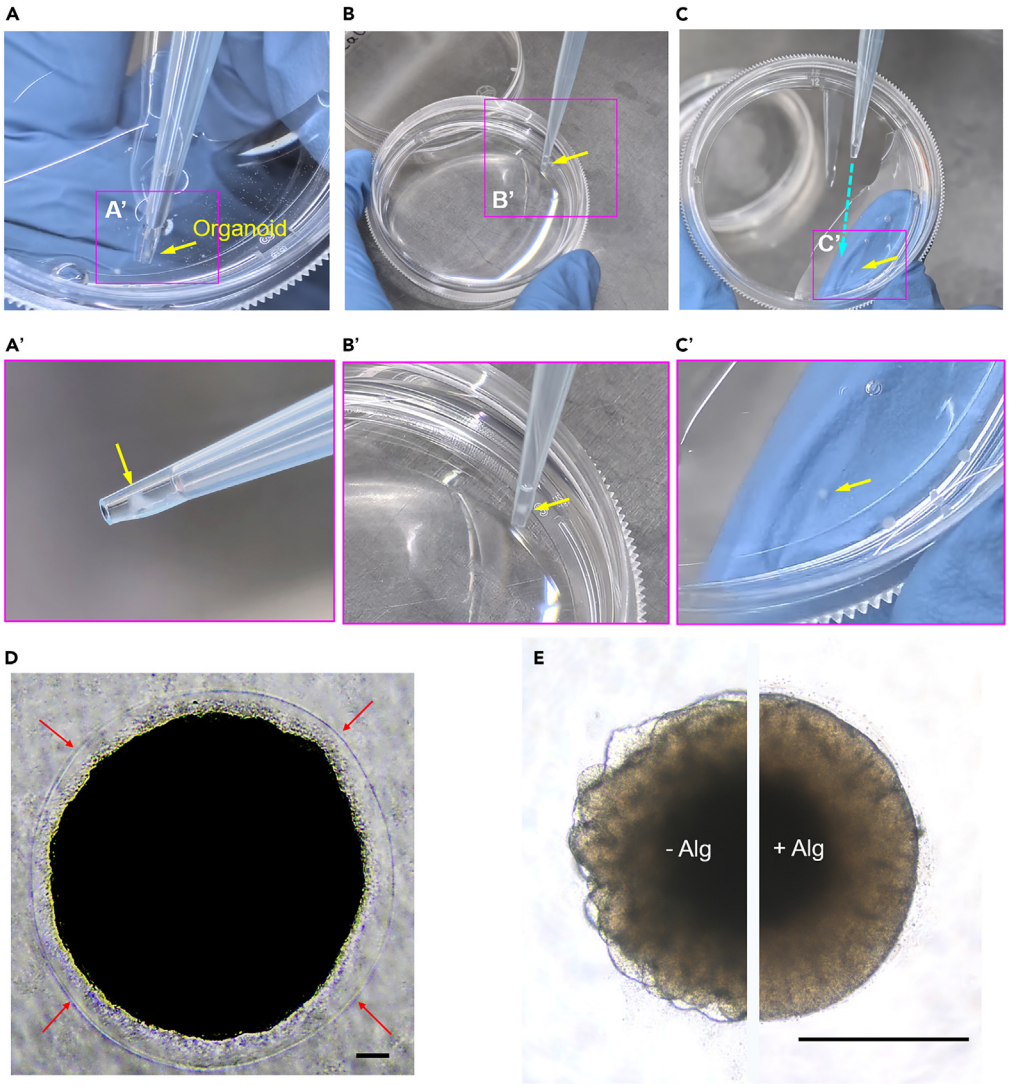

Figure 3. Procedure and results of encapsulating an organoid within an alginate hydrogel

(A) An organoid submerged in the alginate solution aspirated into the pipette tip.

(B) The pipette containing the organoid and alginate solution positioned above the calcium chloride solution.

(C) The organoid released into the calcium chloride solution. (A′), (B′) and (C′) depict corresponding magnified areas from (A), (B), and (C), respectively.

(D) A bright-field image with enhanced contrast showing the boundary of the alginate shell (indicated by red arrows). Scale bar: 50 μm.

(E) Comparison of encapsulated organoids and the control group. Scale bar: 500 μm. Figure reprinted with permission from Tang et al.[1]

Note: Step 2, as described here, is not necessary to be performed every time. Labeling the shell with fluorescent microspheres enables tracking the shape and integrity changes of the alginate shell.

⚠ CRITICAL: The thickness of the alginate shell depends on the volume of alginate solution aspirated into the pipette (in step 1c) and can be adjusted according to the requirements, making it an operator-dependent step. For example, if stronger confinement of the organoid is expected in the experiments, using more alginate solution will make the shell

thicker, thus needing stronger force to break it. On the other hand, if a thinner shell is required on the organoid, reduce the volume of the alginate solution in the 1 mL pipette tip before blowing it into the calcium chloride solution.

Evaluation of organoid encapsulation

Timing: 3 days (excluding imaging time)

These steps describe how to characterize organoids after alginate hydrogel encapsulation via immunohistochemical staining. This is the general protocol to stain either 35-day-old organoids or organoids of any age.

3. Unguided neural organoid fixation.
   a. Collect organoids with a Pasteur pipette to a new conical tube.

   Note: The front tip of the Pasteur pipette can be cut to make a larger opening to avoid damaging the organoid.

   b. Rinse the organoid with 1× PBS to remove the original culture medium.
   c. Discard the PBS and incubate the organoid with 1 mL of 4% (w/v) paraformaldehyde at 4°C for 16 h.
   d. Incubate the organoid with 1 mL of 2 mg/mL lysine solution at room temperature for 1 h to neutralize paraformaldehyde.
   e. Wash the organoid with 1× PBS containing 3% BSA for 10 min twice.
4. Cryosection of organoid.
   a. Incubate organoid with 10% sucrose, 20% sucrose and 30% sucrose solution in sequence under 4°C.

   Note: Move the organoid into the respective sucrose solution with a higher concentration till the organoid sinks to the bottom of the sucrose solution. If the organoid sinks in 10% sucrose solution, 20% sucrose solution can be directly applied; the sinking process usually takes no more than 12 hours.

   b. Incubate organoids in OCT at 4°C for 16 h.
   c. Move the organoid to a cryomold and store it at −80°C for 16 h.

   Note: Samples can be stored under −80°C for at least 2 months.

   d. Section organoid for thickness of 30 μm using a freezing microtome.
   e. Adhere the slices to a glass slide that has undergone positive charge treatment on the surface, and dry the slides for 1 h at room temperature.
   f. Store the slides at −20°C.

   Note: Process the slides within one month. The slides can be stored for at least 2 months if kept at −80°C.

5. Immunohistochemical staining.
   To identify and assess the developing neural tissues, we conducted immunohistochemical staining of relevant markers on day 35 organoids, including Ki67 for proliferation, Nestin for neural progenitor cells, PKCζ for apical structures, Neurofilament (NF) for neuronal axons, and microtubule-associated protein 2 (MAP2) for neuronal cell bodies and dendrites.
   a. Incubate the slide, which has attached sectioned slices, with −20°C pre-chilled absolute methanol. Do this for 10 min while shaking at 40 rpm on an orbital shaker.

b. Discard the methanol, and wash the slide with 1× PBS in three separate 5-min intervals to eliminate the OCT.
c. Draw hydrophobic circles around samples attached to glass slides with a PAP pen.
d. Block samples with 10% normal donkey serum in PBS containing 0.5% Triton at 4°C for 16 h.
e. Incubate samples with a primary antibody diluted in 10% normal donkey serum (Jackson lab) in 1x PBS containing 0.5% Triton at 4°C for 16 h.
f. Wash the slides with PBS 3 times, each time for a duration of 10 min.
g. Incubate samples with a secondary antibody diluted in 10% normal donkey serum (Jackson lab) in 1x PBS containing 0.5% Triton at room temperature for 1 h.
h. Wash the slides with PBS 3 times, each time for a duration of 10 min.
i. Incubate samples with DAPI solution for 30 min at room temperature.
j. Mount the glass slide with glycerol and a coverslip.
k. Seal the coverslip with transparent nail polish and allow it to dry.
l. Capture samples under a confocal microscope.

6. EdU labeling on day 35 organoids.

The EdU (5-ethynyl-2-deoxyuridine) incorporation test is a reliable new technique to detect cell proliferation, as a supplement to immunohistochemical staining of Ki67.

a. Dilute 2× EdU working solution (component A with 10 mM stock concentration) with a Maturation medium to achieve a concentration of 40 μM.
b. Dilute the pre-warmed 2× EdU working solution by adding the same volume of Maturation medium to achieve a 1× EdU working solution (20 μM final concentration).
c. Culture organoids with 1× EdU working solution at 37°C for 3 h.
d. Wash the organoid with 1× PBS to remove the solution.
e. Discard the PBS and incubate the organoid with 1 mL of 4% (w/v) paraformaldehyde at 4°C for 16 h.
f. Perform the immunohistochemical staining procedure as outlined in Step 6.

EXPECTED OUTCOMES

To understand the pivotal role of biomechanical factors in organogenesis, especially concerning the development of the central nervous system, we introduce a method designed to apply compressive force to unguided neural organoids cultured *in vitro*. This method builds on studies demonstrating the value of mechanical force in differentiation and maturation.[7–11,18–20] The process involves utilizing alginate hydrogel to create a thin envelope around the organoid sphere; since alginate is a well-characterized natural biopolymer with no batch-to-batch variabilities,[21] and it is easily crosslinked by calcium ions, creating smaller than typical-cell porous mesh,[22] it has seen uses in organoid technology and mechanoregulation.[23–26] One-time embedded alginate hydrogel shell effectively confines the unguided neural organoids, thereby fostering their growth and maturation at a critical 3-day expansion culture period under the Expansion Medium. Our protocol demonstrates the manipulation of the alginate hydrogel to form a thin shell, showing 33.00 μm ± 8.63 μm in thickness, exerting approximately 6 micro-Newtons (μN) of mechanical force on a 500 μm diameter sphere. This encapsulation restricts the extrusion of neural rosette buds, ensuring their uniform alignment within the spheroid and resulting in a smooth organoid surface. The alginate hydrogel shell remains intact for a minimum of 5 days following encapsulation (Figures 4A–4C), with 60%, 40%, and 0% of intact shells at day 12, day 18, and day 21 respectively. Measurement of organoid diameters 35 days after encapsulation reveal an increase in size, suggesting that temporal physical confinement augments organoid growth (Figure 4D). Robust signals of EdU labeling and immunostaining for the cell proliferation marker protein Ki67, emanating from the rosette structure indicate enhanced cell proliferation in unguided neural organoids post-alginate encapsulation (Figures 5A–5C). Staining of Nestin, a neural stem cell marker, is extensively observed within the compressed organoids (Figures 5D and 5E). Additionally, immunostaining of the apical marker PKC ζ reveals larger rosette formations in the alginate-treated organoids

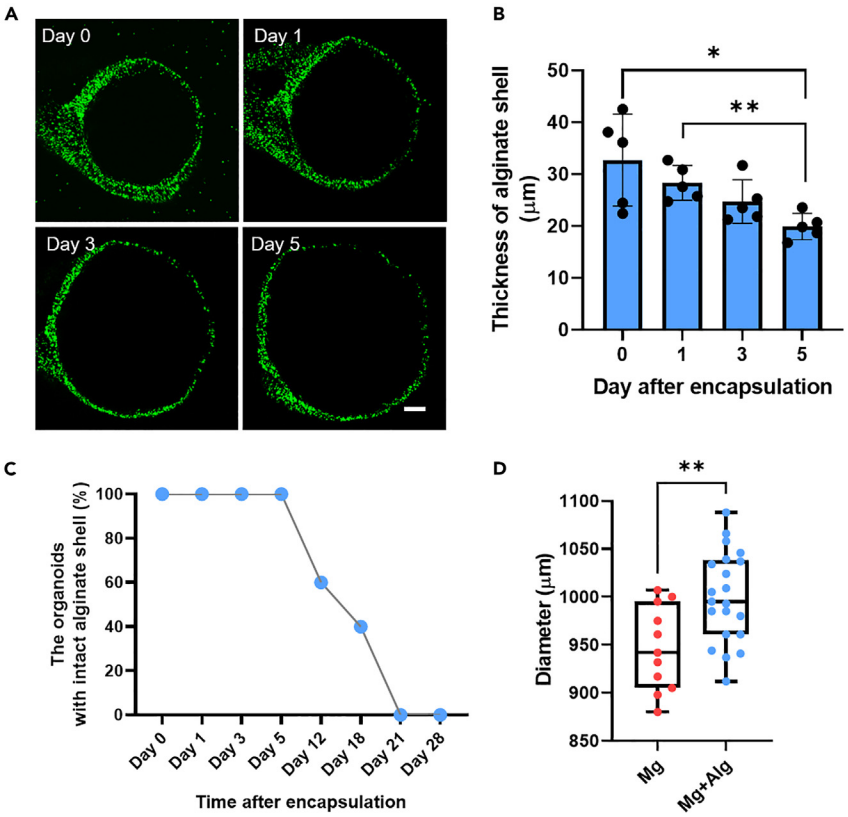

Figure 4. Evaluation of the compressive duration of alginate shell

(A) Alginate shells marked with GFP fluorescent particles. Scale bar: 100 μm.

(B) Quantification of alginate shell thickness during the culture period at days 0, 1, 3, and 5. An unpaired two-tailed Student's t-test was used (n ≥ 5/group), averaging 33 μm in thickness. Significance indicators * and ** correspond to $P < 0.05$ and $P < 0.01$, respectively.

(C) The ratio of the intact shell remaining throughout the culture period of 28 days.

(D) Quantification of the diameters of unguided neural organoids embedded with Matrigel in the absence (Mg group) and presence (Mg+Alg group) of external alginate embedding at day 35. An unpaired two-tailed Student's t-test was used (n ≥ 5/group). Figure reprinted with permission from Tang et al.[1]

(Figures 5F and 5G). Notably, an increased presence of axons and dendrites, marked respectively by neurofilament (NF) and Microtubule-associated protein 2 (MAP2), indicates the promotion of neuronal maturation (Figures 5H–5K).

QUANTIFICATION AND STATISTICAL ANALYSIS

Calculation of the force generated by alginate encapsulation.

1. Employ a theoretical model that assumes a plane state of stresses in an infinitesimal element in the material (Figure 6A). In this model, the normal and shear stresses on any section are related to σ_x, σ_y, τ_{xy} and the angle θ.

$$\begin{cases} \sigma_{x'} = \dfrac{\sigma_x+\sigma_y}{2} + \dfrac{\sigma_x - \sigma_y}{2}\cos 2\theta - \tau_{xy}\sin 2\theta \\ \tau_{x'y'} = \dfrac{\sigma_x - \sigma_y}{2}\sin 2\theta + \tau_{xy}\cos 2\theta \end{cases}.$$

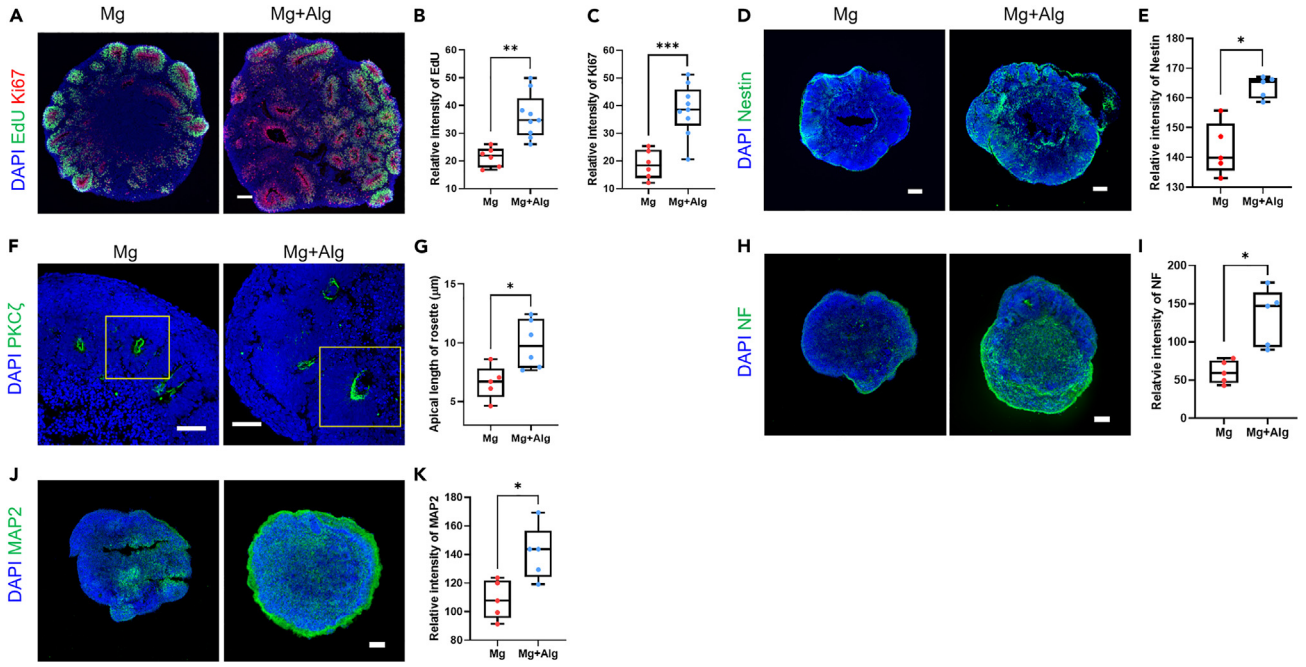

Figure 5. Immunofluorescent staining characterization

(A) Fluorescent images highlighting the presence of cell division markers EdU and Ki67 at day 35. Scale bar: 100 μm.

(B) A quantified measure of EdU signal intensity.

(C) A quantified measure of Ki67 signal intensity.

(D) Expression of neural stem cell marker Nestin in unguided neural organoids embedded with Matrigel (Mg) or Matrigel-core-alginate-shell (Mg+Alg) at day 35. Scale bar: 100 μm.

(E) Quantification of the relative intensity of the Nestin signal.

(F) Confocal images of the apical marker (PKCζ) of rosettes at day 35. Scale bar: 50 μm.

(G) Quantification of the apical length of rosettes positive with PKCζ protein.

(H) Expression of axon marker neurofilament (NF) at day 35. Scale bar: 100 μm.

(I) Quantification of the expression level of NF.

(J) Expression of microtubule-associated protein-2 (MAP2) at day 35. Scale bar: 100 μm.

(K) Quantification of the expression level of MAP2. An unpaired two-tailed Student's t-test was used (n ≥ 5/group). Significance indicators * correspond to $P < 0.05$. Figure reprinted with permission from Tang et al.[1]

2. Under the rheology test conditions, the normal stresses σ_x and σ_y are equal to zero, and only shear stress τ_{xy} was measured. Hence, the normal and shear stresses on any section are given by:

$$\begin{cases} \sigma_{x'} = -\tau \cdot \sin 2\theta \\ \tau_{x'y'} = \tau \cdot \cos 2\theta. \end{cases}$$

The theoretical assumption is that calcium alginate hydrogels with a network structure are homogeneous in composition and undergo only elastic deformation before their strength decreases. Therefore, yielding in rheology tests is primarily due to the normal stress, which is maximized when τ is at maximum and $\theta = 45°$. At this point, $\sigma_{max} = \tau_{max}$, which can be directly measured by the MCR302 rheometer (Anton Paar, Austria; Figure 6B).

3. Calculate the pressure (P) exerted by the alginate shell on the organoid using the theory of thin shells:

$$P = \frac{4\sigma_m \delta}{D}.$$

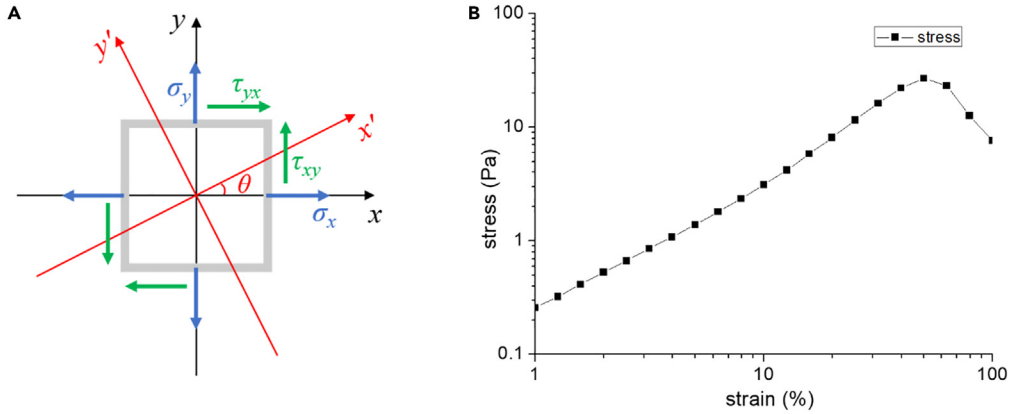

Figure 6. Plane state of stresses in an infinitesimal element of the material for the calculation of compressive stress
(A) Representation of an infinitesimal element subjected to a planar stress state.
(B) Depiction of shear stress as measured by the MCR302 rheometer (manufactured by Anton Paar, Austria).
Figure reprinted with permission from Tang et al.[1].

where δ is the thickness of the alginate shell, and D is the diameter of the organoid.

4. From this, calculate the force exerted on any small region on the surface of the organoids:

$$F = PS.$$

where S represents the area of the corresponding regions. Estimating the average thickness of the alginate shell as 33 μm, in our experiments we assume that the shell exerted approximately 6 micro-Newtons (μN) of mechanical force on a 500 μm diameter sphere.

The results are derived from a relatively simplified theoretical model in engineering mechanics. Taking into account the discrepancies between this model and real-world conditions, especially that protruding structures withstand higher pressure than other areas, the calculation offers only a rough estimate of the maximum average pressure the shell can exert.

LIMITATIONS

Cerebral organoids generated by the unguided method, here referred to as unguided neural organoids, contain randomly developed early neural tube structures, known as neural rosettes. The typical aggregation and alignment of neural stem cells in neural rosettes creates specific physical properties. Applying compressive confinement to an unguided neural organoid introduces an anisotropic distribution of mechanical force, which may lead to differential cellular responses such as the invagination of neural rosettes after alginate gel encapsulation, along with an increase in the number of neural stem cells.

The encapsulation of an unguided neural organoid with alginate hydrogel depends on several factors during manual manipulation, such as the width of the front opening of a pipette tip, the volume aspirated in the pipette tip, the speed at which the organoid covered with alginate solution spurts into a calcium chloride solution and others, making it an operator-dependent process. These factors may result in an uneven thickness of the alginate shell. One possible way to fix the width of the front opening of a pipette tip, thus streamlining the organoid embedding process, is by using commercially available wide-bore pipette tips. Although the increase in embedding reproducibility through the usage of wide-bore pipette tips is yet to be confirmed, it might reduce crucial variables of this protocol. Due to the physical properties of alginate hydrogel, the shell may crack and fail to provide

long-term encapsulation with the rapid growth of the organoid. There is a need for the development of novel biocompatible hydrogel materials with higher elasticity and extensibility, along with high-throughput automatized encapsulation systems.

TROUBLESHOOTING

Problem 1

EB quickly turns dark under a microscope with a bright field view.

Potential solution

- Make sure PSCs are not differentiated and are expressing the putative markers such as OCT3/4, NANOG, and SSEA proteins.
- Seed 1500–6000 cells in one well of a U-bottom ultra-low attachment-96 well plate. A higher number of cells will increase the density of EB, decreasing transparency, and increasing the risk of spontaneous differentiation.

Problem 2

The alginate solution has a high viscosity and is not easy to filter through a 0.22-μm membrane.

Potential solution

- Produce the alginate solution with the correct concentration.
- Ensure that the room temperature is not lower than 20°C.
- Apply more force during filtration. If filtration is stopped, change the filter to a new one.

Problem 3

EBs disintegrate during transfer.

Potential solution

- Cut the front opening of a pipette tip and heat the tip briefly to smooth it, then use the cut pipette tip to transfer EBs.
- Use a commercialized wide-bore pipette tip to transfer EBs.
- Do not let the pipette tip touch the bottom of the well in a 96-well plate, which may injure the EBs.
- Eject a small amount of EB into a pipette tip, forming a droplet at the front opening. Gently touch the small droplet to the opening of a chamber on the PDMS microfluidic chip; the medium solution will automatically fill the chamber, and EB will naturally sink into it due to gravity.
- Aspirate EB slowly to avoid shear stress at the opening of the pipette tip, which can disintegrate EBs.
- Transfer EB one at a time.

Problem 4

The alginate shell on an unguided neural organoid is too thick.

Potential solution

- When aspirating organoids into a pipette tip before spurting into the calcium chloride solution, try to reduce the volume of the alginate solution until the alginate solution is just visible with the organoid in the pipette tip.
- Position the spurt of the alginate solution together with the organoid at a higher level (~1–2 cm) above the calcium chloride solution.
- Use a 1 mL pipette tip which contains more air inside it, and quickly push out the alginate solution with the organoid.

Problem 5
The alginate shell on an unguided neural organoid is thin and easily broken.

Potential solution

- Immerse the organoids in the alginate solution for a longer period (e.g., 3–5 min) before transferring them into calcium chloride.
- Incubate the alginate-covered organoid in calcium chloride for no less than 30 s.
- Ensure that the alginate powder is fully dissolved.

Problem 6
The alginate shell has successfully formed but breaks in a short amount of time.

Potential solution

- Make sure the formed shell is intact and structural integrity is not compromised.
- If the organoids grow rapidly in size, they will break the shell more easily.
- It is possible to enhance the strength of the shell by increasing the alginate concentration.
- Try using a higher alginate concentration. For instance, consider changing the concentration from 0.1% (w/v) to 0.2% or 0.5% (w/v), it will result in a thicker, more robust shell.

Problem 7
The formation of an alginate hydrogel shell does not occur because the alginate solution-covered unguided neural organoid always floats on the surface of the calcium chloride solution after ejection.

Potential solution

- The position for ejecting the organoid should be high (1–2 cm) above the surface of the calcium chloride solution.
- Quickly eject the organoid into the calcium chloride solution by pushing the pipette rapidly.
- Ensure that the alginate powder is fully dissolved and the correct concentration is maintained.

Problem 8
There are nontransparent impurities on the organoids or in the medium after encapsulation.

Potential solution

- Calcium ions tend to precipitate when encountering phosphate ions or some other ions. It is unsuitable to wash the organoids in PBS or DPBS (related to step-by-step method details, step 1e).
- It is helpful to wash the organoids adequately with a solution that does not contain phosphate ions (or other ions that will cause precipitation) before and after encapsulation. DMEM/F12 and DMEM are suitable, for example.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shaohua Ma (ma.shaohua@sz.tsinghua.edu.cn).

Technical contact
Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contacts: Zitian Wang, (zitian-wang@sz.tsinghua.edu.cn), and Xiaowei Tang, (txw18@mails.tsinghua.edu.cn).

Materials availability

This protocol did not generate new materials.

Data and code availability

This protocol did not generate new data or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.102952.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.M.; methodology, Z.W. and X.T.; investigation, Z.W., X.T., G.S., E.A.G., and J.W.; writing – review and editing, Z.W., X.T., and D.K.; supervision, S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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