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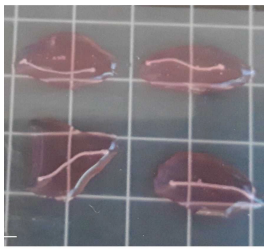
Detailed culture protocol for the generation of human polarized cortical assembloids (polCA)



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Abstract

Cortical organoids generating all major cortical cell types in distinct compartments are widely used to mechanistically study cortical development, evolution and related disorders. However, the lack of morphogen gradients imparting cortical positional information and topography in current systems hinders the solid investigation of complex cortical phenotypes. Here, we describe an assembloid culture protocol that combines an organizer-like structure expressing the morphogen FGF8 with an elongated organoid, thus introducing a polarized source of FGF8 morphogen within individual organoids. This system, referred to as polarized cortical assembloid (polCA), allows for the establishment of FGF8-dependent positional identity along the longitudinal axis of individual organoids and the generation of spatially oriented populations with distinct transcriptional signatures relevant for the cortical protomap. This protocol can be adapted to include organizer-like structures expressing various morphogens, allowing for a reliable analysis of their effect on cortical positional identity.

Image Attribution

Image showing the assembloids in Matrigel droplets

Guidelines

Human Pluripotent Stem Cell (hPSC)-derived brain organoids that recapitulate the transcriptional programs and cellular identities of the human fetal brain are extensively used to gain insights into molecular mechanisms underpinning human features of cortical development and disease-relevant alterations (Sidhaye & Knoblich, 2021, Amin et al., 2018). The cerebral cortex is the main site of neuronal activity integration and processing of the human brain (Yeo et al., 2011) and it is organized in functional cortical areas (Triarhou, 2021, Triarhou, 2020, Loukas et al. 2011). A certain degree of arealization is specified before connections arise by the concerted action of morphogen gradients that result in the expression of fate-determining transcription factors in distinct domains of the developing cortex, thus forming a protomap already at the radial glia stage (Bayatti et al., 2008; O'Leary et al.2007; Rubenstein et al. 1998; Vieira et al., 2010). Several studies described the ability of various factors, in particular FGF8, to modulate cortical fates in hPSC-derived cultures (Imaizumi et al., 2018; Astick & Vanderhaeghen, 2018;) and the emergence of area-related signatures in organoid-derived neurons (Cederquist et al., 2020). Recent attempts to introduce some level of positional information in brain organoids described a dorso-ventral axis through fusions of independently patterned organoids (Bagley et al. 2017; Birey et al., 2017; Xiang et al., 2017), a cortico-striatal axis (Xiang et al., 2017), a forebrain-to-hindbrain axis through microfluidic devices that modulate WNT signaling (Rifes et al., 2020), and a mosaic inducible SHH (iSHH) spheroid-organoid fusion strategy (Cederquist et al., 2019). Yet, individual organoids that reproducibly generate and spatially orient cell types with signatures relevant for specific cortical area have not been reported. In this protocol, we describe the assembly of an organizer-like structure expressing the morphogen FGF8 and tdTomato (OrEB) with an organoid with a linear shape to establish FGF8-dependent positional information along the longitudinal axis of individual organoids. This method, referred to as polarized cortical assembloid (polCA) allows for the emergence of spatially oriented populations with signatures relevant for the cortical protomap. As such, this approach can be used as a platform for understanding the early steps of protomap formation and their dysregulation in diseas, as well as adapted to test the role of various morphogens on inducing protomap-relevant signatures.
Some key procedures and reagents of this protocol are based on previous protocols. The culture of stem cells, stem cells engineering and generation of cerebral organoids are based on the original cerebral organoid method (Lancaster et al., 2013; Lancaster & Knoblich, 2014; Bagley et al., 2017). The time required for obtaining the polCA is around 75 days. The protocol should reliably produce elongated organoids with spatially oriented forebrain cells and progenitor populations with prefrontal and temporal gene enrichment. Final efficiency on cortical specification within these cultures will depend on the hESC line used, reproducibility of Fgf8-expressing OrEB formation and the specific patterning factors used in culture media. In addition, as reported in the original manuscript, this method can be used with various hESC and hPSCs, including hESCs carrying mutations in the FGF pathway to dissect the requirement of FGF8 gradient in establishing spatial orientation.

We provide below a Troubleshooting guide:

a) No formation of OrEB or elongated organoids (Section 3)
- Check the cell viability before seeding. A high cell viability (>80%) is critical for the correct generation.
- Check the moulds preparation procedure. Make sure to perform the pluronic treatment on day 0 (same day of organoid set up) and to remove all bubbles before use. Increase the number of DPBS washes if needed.
- After seeding the cells, spin the ULA plate at 150 g for 3 minutes to promote cell aggregation.
- Check for mycoplasma contaminations.

b) Low efficiency of in-mould assembly (Section 4)

- Verify OrEB presence in the tip before transferring to the mould.
- Check OrEB positioning under a fluorescent microscope after transfer. We recommend doing some practice in manipulating EBs and the use of a fluorescence microscope under the hood to facilitate OrEB positioning.

c) Lower recovery of assembloids (Section 5)

- Check organoid thickness before mould extraction. Thinner assembloids are fragile and tend to break during the extraction procedure. If necessary, start with a higher number of cells.
- Check timing of the procedures. Assembloids should be extracted and embedded at day 7. Make sure to following this timing and straighten them up as soon as they are in the matrigel droplet. Also, make sure to work rapidly to avoid matrigel polymerization; position the plate on ice if embedding timing takes more than 5 minutes.

d) Suboptimal differentiation of PolCA (Section 6)

- Changing the medium more frequently to avoid media exhaustion.
- Adjust the shaking speed to ensure polCA are maintained as floating cultures. If needed, rinse culture plates with Anti-adherence rinsing solution before use, to minimize adhesion to the bottom of the dish.
- Changing the batch of hESCs and/or rule out major genome integrity loss in the lines used.
- Increasing the number of cells seeded for elongated organoids.

Materials

Cell lines

- Human embryonic stem cells (hESCs) H9 (WiCell, WA09)
- Human embryonic stem cells (hESCs) H9 (WiCell, WA09) engineered to constitutively produce and secrete FGF8; this will be used for the generation of mosaic organizer-like organoids (at 1% contribution)
- Human embryonic stem cells (hESCs) H9 (WiCell, WA09) engineered to express tdTomato; these cells will be used for the generation of mosaic organizer-like organoids (99 % contribution) to provide positional reference upon assembly.

General reagents:

- Pluronic F127 (Sigma-Aldrich, Cat. P2443)
- Anti-adherence rinsing solution (StemCell Technologies, Cat. 07010)
- Matrigel hES-qualified (Corning, Cat. 354277) for hES culture only
- Matrigel Basement Membrane Matrix (Corning, Cat. 354234) for organoid embedding
- mTeSR1 medium (StemCell Technologies, Cat. 85850)
- 0.5 M EDTA (Sigma-Aldrich, Cat. E8008)
- Accutase (Sigma-Aldrich, Cat. A6964)
- DPBS without Ca-Mg (Gibco, Cat. 14190-250)
- DMEM-F12 (Invitrogen, Cat. 11330032)
- N-2 Supplement (100X) (Invitrogen, Cat.17502048)
- 1µg/ml Heparin solution (Sigma-Aldrich, Cat. H3149-10KU)
- GlutaMAX Supplement (Invitrogen, Cat. 35050038)
- MEM Non-essential Amino Acid Solution (100X) (Sigma, Cat. M7145)
- Rock inhibitor (RI) Y27632 (Sigma, Cat. SCM075)
- NeurobasalTM Medium (Invitrogen, Cat.21103049)
- B-27 Supplement (50X), minus vitamin A (Invitrogen, Cat. 12587010)
- Human Insulin 10 mg/ml Solution (Sigma-Aldrich, Cat. I9278-5ML)
- 0.05 mM BME Solution (Merck Millipore, Cat. 8057400005
- Antibiotic-Antimycotic (ThermoFisher, Cat. 15240062)
- Human BDNF (StemCell Technologies, Cat. 78005.3)
- Human GDNF (StemCell Technologies, Cat. 78058.3)

Culture media:

- Neural Induction (NI) Medium (500 ml): DMEM-F12, 5 mL N-2 Supplement
1 µg/ml heparin solution, 5 mL GlutaMAX Supplement, 5 mL MEM Non-essential Amino Acid Solution, 50 µM Rock inhibitor

- Differentiation -A Medium (Diff-A) (500 ml): 250 mL DMEM-F12, 250 mL Neurobasal Medium, 5 mL N-2 Supplement, 10 mL B-27 Supplement minus vitamin A, 2.5 µg/ml Human Insulin Solution, 0.05 mM BME Solution, 5 mL GlutaMAX Supplement, 5 mL MEM Non-essential Amino Acid Solution, 5 mL Antibiotic-Antimycotic.

- Differentiation+A Medium (Diff+A) (500 ml): 250 mL DMEM-F12, 250 mL Neurobasal Medium, 5 mL N-2 Supplement, 10 mL B-27 Supplement, 2.5 µg/ml Human Insulin Solution, 0.05 mM BME Solution, 5 mL GlutaMAX Supplement, 5 mL

MEM non-essential Amino Acid Solution, 5 mL Antibiotic-Antimycotic.

Large equipment:
- Autoclave
- Gamma irradiator or in alternative a UV lamp (VWR, UVLS-26 EL Series UV Lamp)
- Tissue culture humidified CO_2 incubator
- Biological safety cabinet
- Bright field and fluorescence microscope
- Gilson Pipetman (P1000, P200 and P10)
- Orbital shaker (Infors Celltron orbital shaker, cat. no. INF-69222)
- Automated cell counter and counting slides
- Vacuum pump
- Benchtop centrifuge
- Water bath, 37 °C

Small items:
- Polydimethylsiloxane (PDMS) multi-array 15 mm-long organoid moulds
- Steel dish
- Tissue culture dishes in various formats
- Sterile filter pipette tips
- Sterile microcentrifuge tubes (1.5-ml size; Fisher Scientific, cat. no. 05-408-129)
- Stericup 0.2-µm filter units (500 and 250 ml; Millipore, cat. nos. SCGVU02RE SCGVU05RE, respectively)
- Steriflip 50 mL filter unit: (Millipore, SCGP00525)
- U-bottom ultra-low attachment plates, 96 well (Corning, cat. no. 7007)
- Conical tubes, 15 ml or 50 mL
- Parafilm (Sigma-Aldrich, cat. no. P7793)
- Pipetboy (Integra Biosciences, cat. no. 155 000)
- Serological pipettes (5, 10, 25 ml)
- Sterilized scissors, forceps and spoons/spatulas
- Trypan blue
- Sterile Plastic Transfer pipet, 3mL (Thermo Fisher Scientific, cat. no. PP89SA)
- Reservoir 50 ml individually packaged sterile

Before start

Note that appropriate consent procedure and administrative regulations must be followed for work involving hESCs. Please consult with your institution to adhere with national and institutional regulations. Also, note that the procedure requires the use of polydimethylsiloxane (PDMS) multi-array 15 mm-long organoid moulds. The file for 3D mould printing can be found in the original publication (Bosone, Castaldi et al. 2024).

Protocol Overview

1 The whole procedure (outlined in Figure 1) consists of the following 6 major steps:
Step 1 | Maintenance of hESC cultures
Step 2 | Mould Preparation
Step 3 | Set up of organizer-like embryoid bodies (OrEBs) and elongated organoids
Step 4 | PolCA in-mould assembly
Step 5 | PolCA extraction from the mould and embedding in large Matrigel droplets
Step 6 | Post-embedding organoid culture

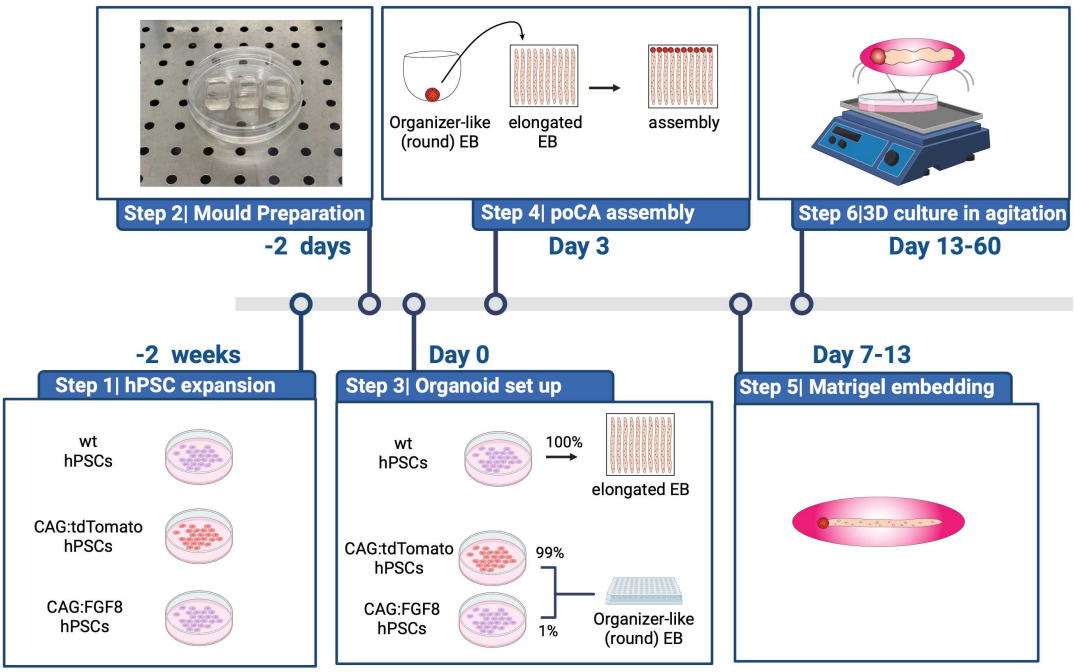


Figure 1. Protocol overview for the generation of 60-days-old polarized cortical assembloids (polCA). Illustration was created with Biorender.

Step 1 | Maintenance of hESC cultures

2w

2 Grow all three hESC lines onto 6-well plates coated with hES-qualified Matrigel in mTeSR1 medium at 37°C and 5% CO_2. 2w

3 Passage cells 3-4 days before organoid set up using 0.5 mM EDTA solution treatment and mechanical
dissociation. To obtain approximately 6 x 10^6 cells for seeding elongated organoids in one mould, we recommend to prepare a full 6-well plate. 1h

Step 2 | Mould Preparation 2d

4 Place up to five PMDS moulds in a steel dish and sterilize with one autoclaving cycle, followed by a gamma irradiation treatment.
As alternative sterilization procedure, fully immerge the moulds in a 70% ethanol solution in a sterile jar overnight (Figure 2a). Transfer moulds into a petri dish and irradiate them with a UV lamp (254 nm) for 30 minutes by positioning the lamp a few centimeters from the moulds (Figure 2b-c). 1d

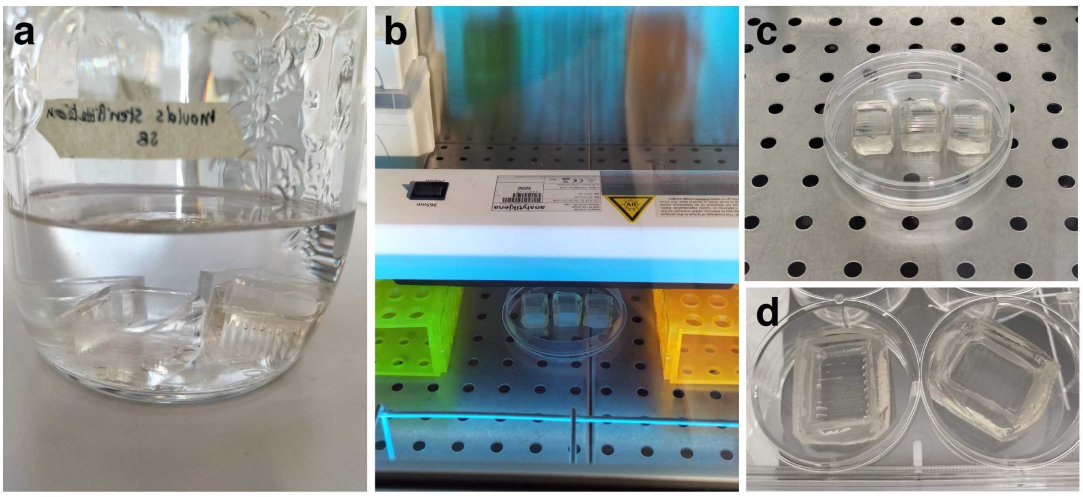


Figure 2. Mould Preparation. Moulds are covered with ethanol in a closed jar overnight (a), then transferred into a sterile petri dish and sterilized under UV light (b, c). In d, sterlized moulds after treatment with pluronic solution. Bubbles that form after treatment (d, left) are manually removed (d, right).

5 Right before use, treat sterilized moulds with 1% Pluronic F127 solution in DPBS for 30 minutes. Remove bubbles after treatment (Figure 2d). 45m

6 Wash three times with 1x DPBS (15 minutes for each wash) and keep sterile till use. 45m

Step 3 | Set up of organizer-like embryoid bodies (OrEBs) and elongated organoids 1d

7 Proceed with the mosaic Organizer Embryoid Body (OrEB) generation as follows (this will be day 0 of the organoid culture). 1h

7.1 Treat both CAG>FGF8 and CAG>tdTOMATO hESCs with Accutase at 37°C for 5-10 minutes, to obtain single cell suspensions.

7.2 Dilute Accutase by adding mTeSR1 supplemented with 10 µM Rock inhibitor and transfer cells into a 15 ml tube.

7.3 Add 1:1 Trypan Blue to 10 ul of cell suspension to determine viability and count cells using the counting chamber.

7.4 Meanwhile, centrifuge cells at 200 g for 5 minutes. Eliminate the supernatant and resuspend cells in mTeSR1 supplemented with 50 µM RI. For CAG>FGF8 hESCs, we recommend a concentration lower than 10^5 cells/ml.

7.5 Mix CAG>FGF8 and CAG>tdTOMATO hESCs at a proportion of 1% and 99% respectively in a 15 ml tube. For a full 96-well ultra-low attachment (ULA) plate, mix 3000 CAG>FGF8 hESCs and 297000 CAG>tdTOMATO hESCs in 15 ml of mTeSR1 medium supplemented with 50 µM RI.

7.6 Transfer the cell suspension into a reservoir and aliquot 150 µl/well (corresponding to 3000 cells/well) in a 96 well-ULA plate using a multi-channel micropipette. Place in the incubator at 37°C and 5% CO_2.

8 On the same day of mosaic OrEB generation, proceed with the generation of elongate organoids as follows. 1h

8.1 Treat wt hESCs with Accutase at 37°C and 5% CO_2 for 5-10 minutes to obtain single-cell suspensions.

8.2 Dilute Accutase by adding mTeSR1 supplemented with 5 µM RI and transfer all the cells in a 50 ml tube.

8.3 Add 1:1 Trypan Blue to 10 µl of cell suspension to determine viability and count cells using the counting chamber. Meanwhile, centrifuge cells at 200 g for 5 minutes.

8.4 Eliminate the supernatant and resuspend cells, to obtain 5,5 x 10^6 cells in 1,5 ml of mTeSR1 supplemented with 50 µM RI.

8.5 Transfer the whole cell suspension (1,5 ml) into a single sterilized and pre-treated mould placed in single well of a 6-well plate (used as a support). Place in the incubator at 37°C and

5% CO_2.

9 One day later (day 1), EB should have formed. Carefully remove medium from one corner of the mould and replace with 1,5 ml Neural Induction (NI) Medium (See Video 1 showing the procedure for polCA feeding within the mould positioned in a well of a 6-we plate). Incubate at 37°C and 5% CO_2. 30m

10 One day later (day 2), feed both cultures as follows. 45m

10.1 Carefully remove medium from one corner of the mould and replace with 1,5 ml NI medium (See Video 1).

10.2 Feed mosaic OrEBs with 180 µl/well of NI medium.

Step 4 | PolCA in-mould assembly 2d

11 On Day 3, both OrEB and elongated organoids should have formed and can be used for the assembly (Figure 3a-b). 2h

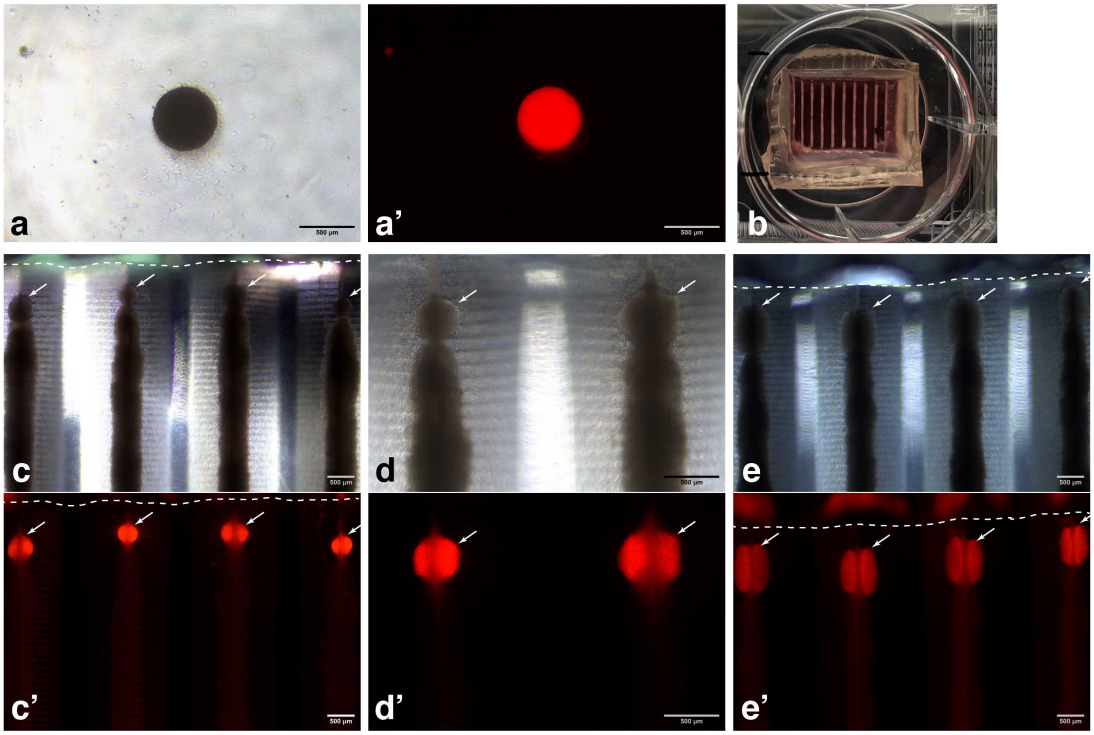


Figure 3. In-mould assembly. a-a') Brightfield image of an organizer-like EB (OrEB) generated from 3000 cells at day 3 and showing strong tdTomato expression (a'). b) Image of one mould containing 10 elongated organoids at day 3. c-e) Brightfield (c, d, e) and fluorescent image (c',d',e') of polCA just after (c-c'), one day (d-d') or 4 days (e-e') after OrEB deposition on the upper side (arrows). Dotted lines indicate the mould top border. Scale bars, 500 mm.

11.1 Feed elongated organoids with 1,5 ml of NI medium.

11.2 Cut the sterile tip of a p200 micropipette and collect a single OrEB from the ULA, let it move down by gravity.

11.3 By touching the inner wall of the top side of the mould, carefully insert the tip into the medium and transfer the OrEB at the tip of a single elongated organoid (top side). Verify the presence and the localization of the OrEB with a fluorescence microscope (Figure 3c).

11.4 Repeat Steps 10.3 and 10.4 for all the available organoids. Put all the OrEBs on the same side of the mould and mark the positions of each OrEBs to facilitate subsequent feedings.

11.5 Put the plate containing the mould in the incubator at 37°C and 5% CO_2 for one day.

12 On day 4, OrEB should be visible at the tip of all organoids (Figure 3 d). Careful feed assembloids daily with NI medium (1,5 ml/mould) by placing the micropipette delivering media

30m

on the side of the mould opposite to OrEB location till day 7.

Step 5 | PolCA extraction from mould and embedding in large matrigel droplets

13 On day 7, OrEB should have fused with the elongated organoids (Figure 3e-e') and assembloids can be prepared for matrigel embedding as follows. 4h

13.1 Thaw matrigel Basement Membrane Matrix in ice 2 hours before starting.

13.2 Transfer the mould to a 10 mm dish containing 10 ml of Differentiation -A medium (Diff-A) medium using forceps (Figure 4a-c).

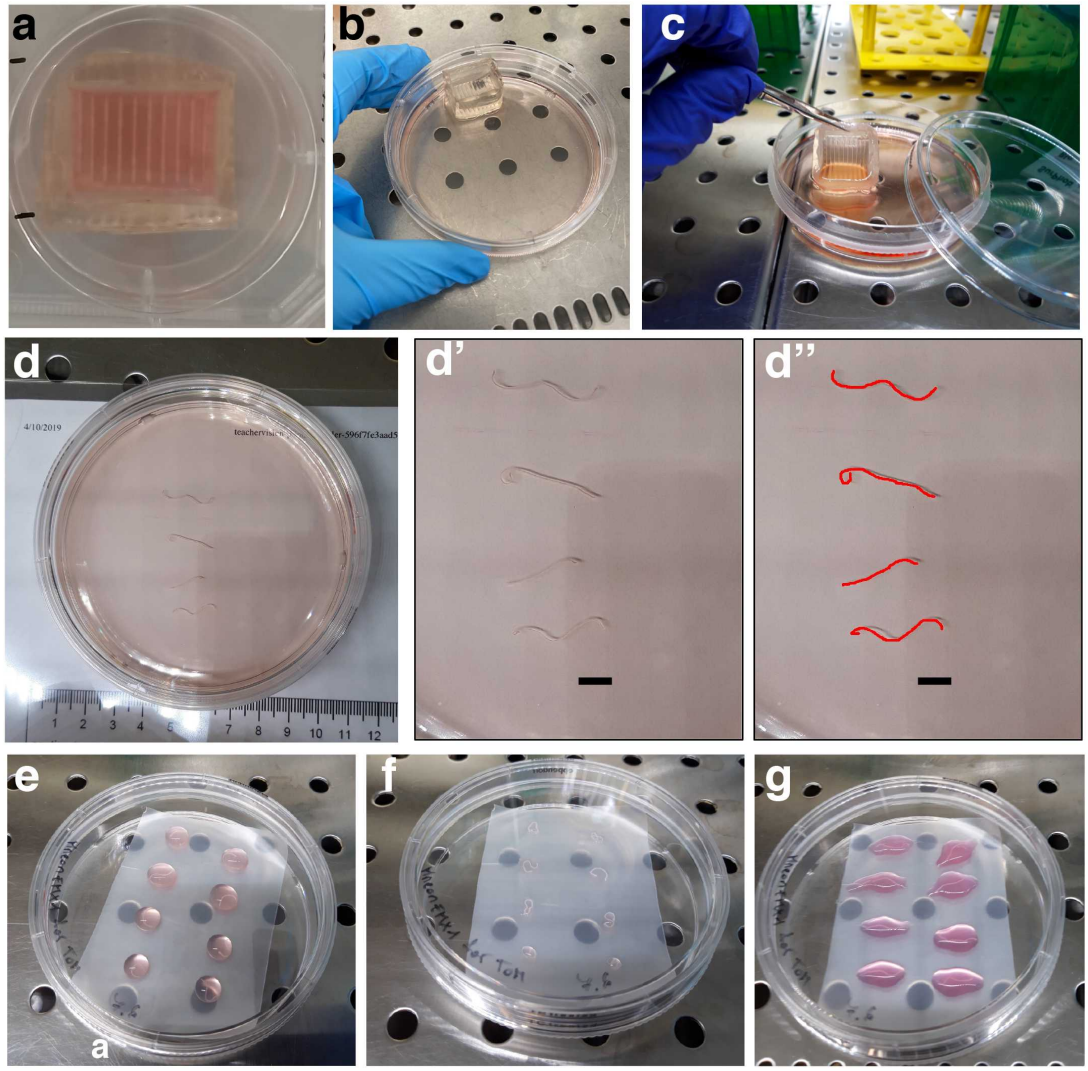
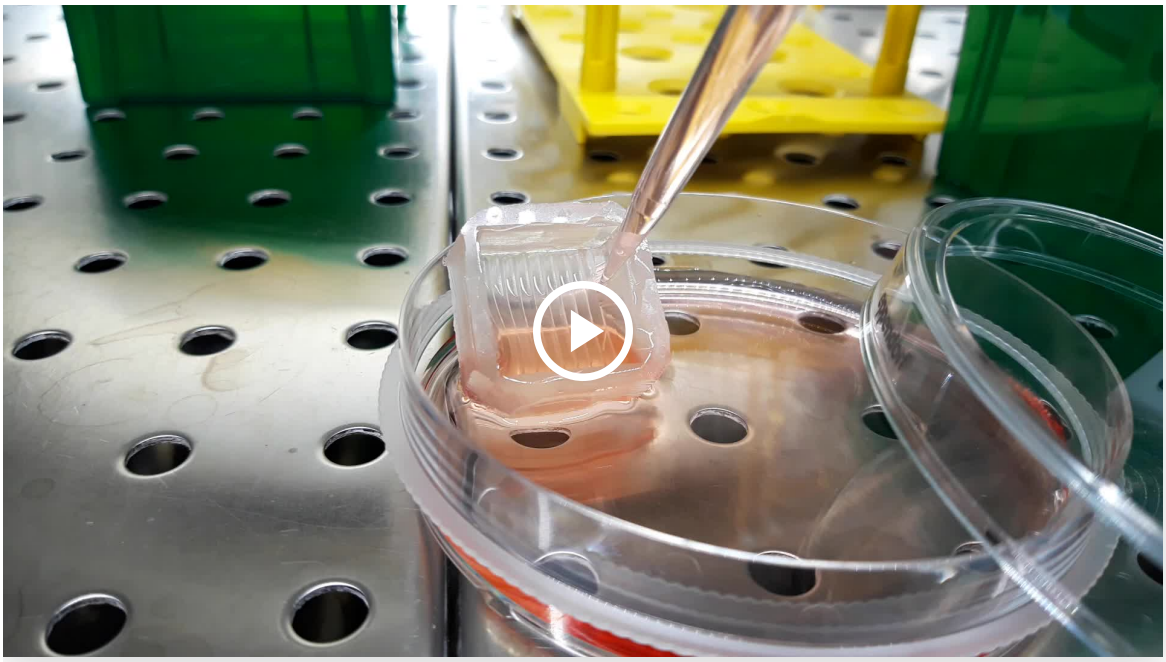


Figure 4. PolCA extraction from mould and embedding in a large matrigel droplet. On day 7, the polCA containing mould is transferred from a 6-well plate (a) to a 10 cm dish containing some medium (b-c). Individual assembloids are extracted from the mould (see also video 2) into culture medium, when they curl up a bit (d, scale bars 5 mm). Assembloids are then carefully positioned in media droplets onto a parafilm sheet (e). After media removal (f), individual assembloids are embedded in large matrigel droplets and straightened longitudinally (g).

13.3 Aspirate NI medium from the mould and extract assembloids from the mould individually by first spraying some medium on the side of the assembloid opposite to the OrEB fusion, to detach its bottom part; then spray some medium along its entire length to completely detach it and bring it into suspension in the dish (Figure 4d, Video 2 showing the procedure for polCA extraction from the mould).



13.4 By using a cut sterile tip of a p1000 micropipette, transfer individual assembloids from the dish to a piece of parafilm as individual droplets (Figure 4e). Remove the excessive Diff-A medium as much as possible and add a droplet of Matrigel along the entire length (Figure 4f-g). Assembloids tend to curl up
once extracted from the mould so make sure to straighten them up as soon as they are in the matrigel droplet. Typically, 200 ul of Matrigel is sufficient for one assembloid.

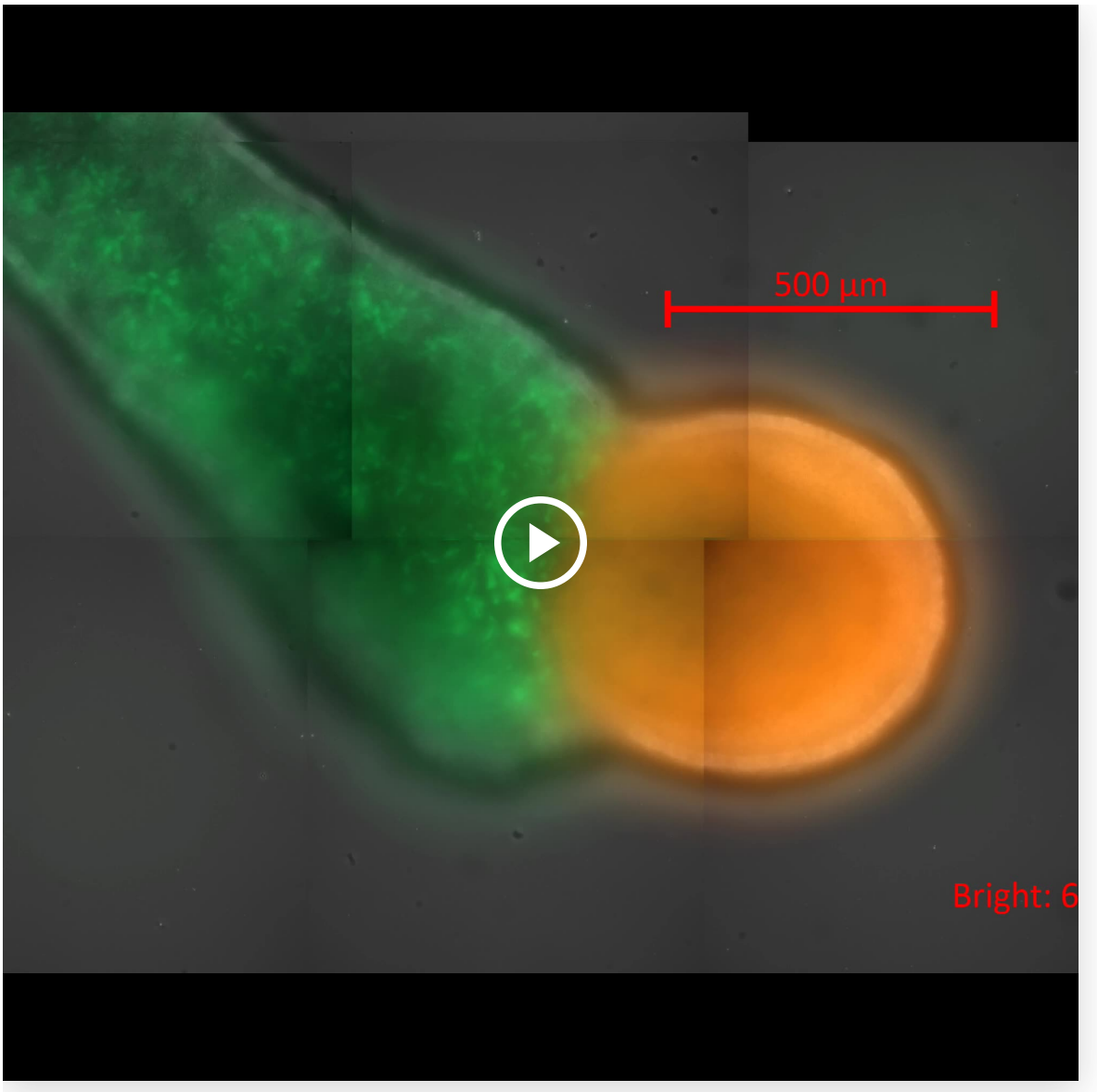
13.5 Repeat Steps 12.3-12.4 for all remaining assembloids.

14 Incubate at 37°C and 5% CO_2 for 30 minutes to allow Matrigel polymerization.

30m

15 When Matrigel has polymerized, detach embedded assembloids from parafilm by spraying Diff-A medium on them and transfer one assembloid per well of a 6-well plate.

16 Put the plate in the incubator at 37°C and 5% CO_2. Assembloids should continue to grow in Matrigel. See Video 3 below showing an example of OrEB and elongated organoid (SP8>GFP line) fusion growth upon embedding in Matrigel (over 4 hours, time frame 6 hours).OrEB and elongated organoid (SP8>GFP line) fusion growth upon embedding in Matrigel (over 4 hours, time frame 6 hours).



Step 6 | Post-embedding organoid culture (day 8-60)

8w

17 Till day 15, feed with Diff-A medium every 2-3 days (5 ml/well of a 6-well plate).

18 On day 15**,** move plates onto an orbital shaker at 52 rpm rotating speed at 37°C and 5% CO_2.

19 Feed with Differentiation +A medium (Diff+A) medium every 2-3 days till day 30 (5 ml/well of a 6-well plate).

20 Feed with Diff+A medium supplemented with 1% (v/v) Matrigel Basement Membrane Matrix, 20 ng/ml Human BDNF, 20 ng/ml Human GDNF every 2-3 days till day 60 (5 ml/well of a 6-well plate).

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