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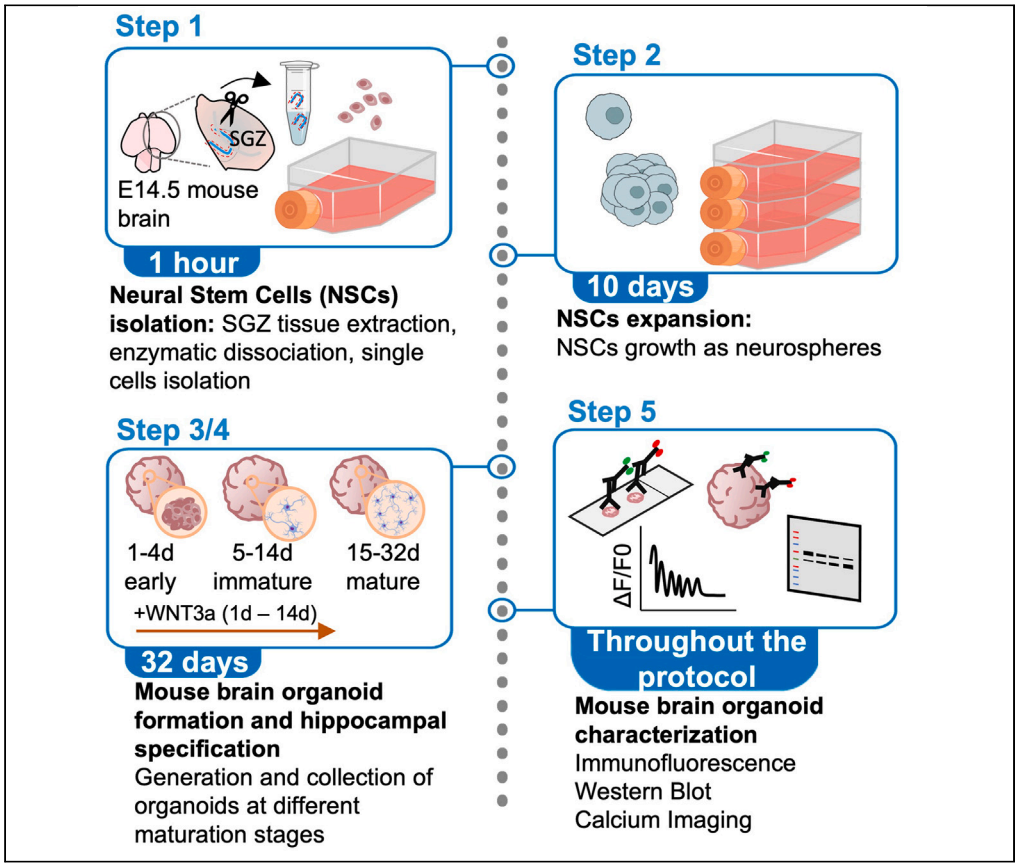
Protocol

Generation of mouse hippocampal brain organoids from primary embryonic neural stem cells



Here we present a protocol to generate standardized cerebral organoids with hippocampal regional specification using morphogen WNT3a. We describe steps for isolating mouse embryonic (E14.5) neural stem cells from the brain subgranular zone, preparing organoids samples for immunofluorescence, calcium imaging, and metabolic profiling. This protocol can be used to generate mouse brain organoids for developmental studies, modeling disease, and drug screening. Organoids can be obtained in one month, thus providing a rapid tool for high-throughput data validation.

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

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Highlights

Step-by-step protocol to generate and characterize mouse brain organoids

Isolation of neural stem cells from the subgranular zone of E14.5 mouse embryos

Steps to generate mature mouse brain organoids from neural stem cell in 32 days

Generation of mouse brain organoids with hippocampal signature using WNT3a

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Generation of mouse hippocampal brain organoids from primary embryonic neural stem cells

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SUMMARY

Here we present a protocol to generate standardized cerebral organoids with hippocampal regional specification using morphogen WNT3a. We describe steps for isolating mouse embryonic (E14.5) neural stem cells from the brain subgranular zone, preparing organoids samples for immunofluorescence, calcium imaging, and metabolic profiling. This protocol can be used to generate mouse brain organoids for developmental studies, modeling disease, and drug screening. Organoids can be obtained in one month, thus providing a rapid tool for high-throughput data validation.

For complete details on the use and execution of this protocol, please refer to Ciarpella et al. "Murine cerebral organoids develop network of functional neurons and hippocampal brain region identity".[1]

BEFORE YOU BEGIN

Perform all the procedures for the isolation of neural stem cells from mouse embryos under sterile hood as well as all the procedures for cell expansion and the establishment of brain organoids culture. Mouse brain organoids are cultured on an orbital shaker in a humidified incubator at 37°C with 5% CO_2. NSCs isolation should take no more than 1 h, and at least 1–2 weeks are necessary for neural stem cells to grow before organoids generation.

The protocol describes the specific steps for using mouse NSCs derived from brain SGZ but the same method can be used to generate brain organoids from meningeal-derived and SVZ-derived NSCs, with comparable results.

Prepare all solutions following the recipes in the materials and equipment section. Some solutions can be prepared in advance and stored as indicated, others must be prepared fresh. Refer to the key resources table for a complete list of materials and equipment.

Institutional permissions

Animal housing and all experimental procedures were approved by the Istituto Superiore di Sanità (I.S.S., National Institute of Health; protocol n. C46F4.N.N4E, Italy) and the Animal Ethics Committee (C.I.R.S.A.L., Centro Interdipartimentale di Servizio alla Ricerca Sperimentale) of the University of Verona (Italy).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
SOX2 (1:200)	R&D Systems	Cat# AF2018, RRID: AB_355110
VIMENTIN (1:400)	Millipore	Cat# AB5733, RRID: AB_11212377
KI67 (1:200)	Abcam	Cat# ab16667, RRID: AB_302459
DCX (1:400)	Cell Signaling Technology	Cat# 4604, RRID: AB_561007
B3 TUBULIN (1:400)	Promega	Cat# G7121, RRID: AB_430874
MAP2 (1:200)	Sigma-Aldrich	Cat# M1406, RRID: AB_477171
GFAP (1:200)	Abcam	Cat# ab53554, RRID: AB_880202
SYNAPTOPHYSIN (1:200)	Synaptic Systems	Cat# 101 004, RRID: AB_1210382
ZBTB20 (1:200)	Genetex	Cat# GTX121616, RRID: AB_11177870
KA1 (1:200)	Abcam	Cat# ab67404, RRID: AB_1140942
PSD95 (1:200)	Millipore	Cat# MAB1596, RRID: AB_2092365
GEPHYRIN (1:200)	Synaptic Systems	Cat# 147 011, RRID: AB_887717
VGAT (1:400)	Synaptic Systems	Cat# 131 004, RRID: AB_887873
VGLUT (1:400)	Synaptic Systems	Cat# 135 303, RRID: AB_887875
GAD65/67 (1:400)	Santa Cruz Biotechnology	Cat# sc-365180, RRID: AB_10710523
NMDA (1:400)	Santa Cruz Biotechnology	Cat# sc-365597, RRID: AB_10847218
GLSYN (1:400)	Santa Cruz Biotechnology	Cat# sc-74430, RRID: AB_1127501
GSX2 (1:200)	Genetex	Cat# GTX129390; RRID: AB_288598
FOXG1 (1:200)	Abcam	Cat# ab18259; RRID: AB_732415
NKX2.1 (1:200)	Genetex	Cat# GTX34907
Prealbumin (TTR) (1:200)	Genetex	Cat# GTX85112; RRID: AB_10723946
FRIZZLED 9 (1:200)	Genetex	Cat# GTX71581; RRID: AB_375823
OCT6 (1:200)	Abcam	Cat# ab272925, RRID: AB_2927579
PROX1 (1:200)	Abcam	Cat# ab101851, RRID: AB_10712211
TO-PRO™-3 (1:3000)	Thermo Fisher Scientific	Cat# T3605
Donkey anti-rabbit Alexa Fluor 546 (1:1000)	Thermo Fisher Scientific	Cat# A10040, RRID: AB_2534016
Goat anti-chicken Alexa Fluor 546 (1:1000)	Thermo Fisher Scientific	Cat# A-11040, RRID: AB_2534097
Donkey anti-guinea pig CY3 (1:1000)	Jackson ImmunoResearch	Cat# 706-165-148, RRID: AB_2340460
Goat anti-mouse CY3 (1:1000)	Amersham	Cat# PA43002, RRID: AB_772235
Donkey anti-goat Alexa Fluor 546 (1:1000)	Thermo Fisher Scientific	Cat# A-11056, RRID: AB_142628
Goat anti-rabbit CY3 (1:1000)	Amersham	Cat# PA43004, RRID: AB_772236
Donkey anti-mouse Alexa Fluor 488 (1:1000)	Thermo Fisher Scientific	Cat# A-21202, RRID: AB_141607
Donkey anti-rabbit Alexa Fluor 488 (1:1000)	Thermo Fisher Scientific	Cat# A-21206, RRID: AB_2535792
OXPHOS (1:200)	Abcam	Cat# AB110413, RRID: AB_2629281
VDAC1 (1:200)	Abcam	Cat# 15895, RRID: AB_2214787
Laminin B1 (1:200)	Abcam	Cat# AB16048, RRID: AB_443298
Biological samples		
Embryonic (E14.5) mouse neural stem cells	N/A	N/A
Chemicals, peptides, and recombinant proteins		
DMEM-F12 GlutaMAX	Gibco	Cat#31331-028
Neurobasal Media	Gibco	Cat#21103-049
Penicillin/Streptomycin (P/S) 100×	Gibco	Cat#15140-122
N-2, 100×	Gibco	Cat#17502-048
B-27, 50×	Gibco	Cat#17504-044
bFGF	Peprotech	Cat#100-18B
EGF	Peprotech	Cat#AF-100-15
BDNF	Peprotech	Cat#450-02
WNT3a	Genetex	GTX109037-Pro
Accutase	Gibco	Cat#A1105-01
DNAse	Sigma-Aldrich	Cat#D4527
0.25% Trypsin – EDTA, 1×	Gibco	Cat#25200-056
Trypan blue	Gibco	Cat#15250-061

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-Glutamine 200 mM	Gibco	Cat# 25030081
HBSS 10×	Gibco	Cat#14180-046
HEPES	Sigma	Cat# H3375-250G
PFA	Mondial	FM0622
Sucrose	Sigma-Aldrich	Cat#84100
Fetal bovine serum (FBS)	Gibco	Cat# 10270106
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#472301
Cryobloc (OCT)	Diapath	Cat#070130
Bovine serum albumin (BSA)	Sigma-Aldrich	A7906-10G
Triton-X	Sigma-Aldrich	T8787
Sucrose	PanReac AppliChem	A3935
Glucose	Sigma-Aldrich	47021
Glycerol	Thermo Fisher Scientific	17904
NaCl	PanReac AppliChem	A2942
KCl	Sigma-Aldrich	P9541
Na_2HPO_4	Sigma-Aldrich	S-3234
KH_2PO_4	Sigma-Aldrich	P9791
$MgCl_2$-$6H_2O$	J. T. Baker	0162
$CaCl_2$-$2H_2O$	Sigma-Aldrich	C3881
Tris base	PanReac AppliChem	A2264
EDTA	Sigma-Aldrich	E-5134
Sodium dodecyl sulfate (SDS)	J.T. Baker	2811
1,4-Diazabicyclo [2.2.2] octane (DABCO)	Sigma-Aldrich	D-2522
Proteinase K 20 mg/mL	Thermo Fisher Scientific	EO0491
NP-40	Fluka	74385
Sodium deoxycholate (DOC)	Sigma-Aldrich	D-6750
Dithiothreitol (DTT)	PanReac AppliChem	A2948
PMSF	Sigma-Aldrich	P7626
Protease inhibitor	Roche	11836153001
Phosphatase inhibitor	Roche	04906837001
Critical commercial assays		
Pierce BCA protein assay kit	Thermo Fisher Scientific	23227
PowerSYBR Green PCR Master Mix	Thermo Fisher Scientific	4367659
Fluo-4 Direct calcium assay kit	Thermo Fisher Scientific	F10472
Experimental models: organisms/strains		
c57Bl/6J pregnant female mice	Charles River	#001CD
Oligonucleotides		
Primer for ND1 forward: 5′ – CTAGCAGAAACAAACCGGGC-3′	Invitrogen; Quiros et al., 2015	N/A
Primer for ND1 reverse: 5′ – CCGGCTGCGTATTCTACGTT-3′	Invitrogen; Quiros et al., 2015	N/A
Primer for HK2 forward: 5′-GCCAGCCTCTCCTGATTTTAGTGT-3′	Invitrogen; Quiros et al., 2015	N/A
Primer for HK2 reverse: 5′- GGGAACACAAAAGACCTCTTCTGG-3′	Invitrogen; Quiros et al., 2015	N/A
Software and algorithms		
ImageJ	U.S. National Institutes of Health	https://imagej.nih.gov/ij/
Other		
ORBi shaker	Benchmark Scientific	#BT3001
96-well cell imaging plates	Eppendorf	Cat# 0030741030
24-well plates	Thermo Fisher Scientific	Cat#144530
Confocal Microscope LSM710	Zeiss	RRID:SCR_018063
Ti Eclipse Microscope	Nikon	RRID:SCR_021242
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	Thermo Fisher Scientific	#ND-ONE-W

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
QuantStudio™ 3 Real Time PCR System	Applied Biosystems	N/A
Stirrer	Bibby	HB501
pH meter	Crison	Basic20
Centrifuge	Eppendorf	5417R
Centrifuge	MPW	223e
Cryostat	Leica	CM1860
Forceps	FST	11255–2055
SuperFrost Plus Glass Slides	Epredia™ J1800AMNZ	10149870
Slides coverslips	Diapath	061051
Termoblock	FALC	
Silicon mold	N/A	N/A
Stereomicroscope	N/A	N/A
gentleMACS Dissociator	Miltenyi Biotec	
gentleMACS C Tubes	Miltenyi Biotec	130-093-237
Cell strainer 40 mm	Corning	352340
Syringe filter 0.22 mm	Sartorius	
For comprehensive key resources table also see Ciarpella et al.[1]		

MATERIALS AND EQUIPMENT

Prepare solution for mouse embryonic (E14.5) SGZ-NSCs isolation

Timing: 1 h

Prepare the solutions necessary for NSCs isolation and culture, as described in the tables below.

Expansion medium without growth factors (can be prepared and stored for 3–4 weeks at 4°C)		
Reagent	Final concentration	Amount
DMEM/F12/Glutamax	N/A	240 mL
P/S, 100×	1×	2.5 mL
N-2, 100×	1×	2.5 mL
B-27, 50×	1×	5 mL
Total		**250 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 μm filter.

Note: The suitability of the solution can be quickly check by looking at the color of the media. DMEM/F12/Glutamax contains Phenol Red thus providing indication about pH solution. Under suitable condition, growth medium will have a warm pink-red color. After long storage, the indicator color turns to bright pink. If contamination occurred during storage, the indicator color may turn into yellow.

Expansion Medium (to be prepared fresh)		
Reagent	Final concentration	Amount
Expansion medium	N/A	50 mL
bFGF	20 ng/mL	50 μL
EGF	20 ng/mL	50 μL
Total		**50 mL**

HEPES (can be prepared and stored for 3–4 weeks at 4°C) pH 7.3		
Reagent	Final concentration	Amount
HEPES	0.3 M	7.15 g
dH_2O	N/A	80 mL
Total		**100 mL**

⚠ CRITICAL: check and adjust the pH before each use; sterilize by filtering with a 0.22 μm filter.

HBSS 1× (can be prepared and stored for 3–4 weeks at 4°C) pH 7.4		
Reagent	Final concentration	Amount
dH_20	N/A	428.5 mL
HBSS 10×	1×	50 mL
0.3 M HEPES, pH 7.3	0.01 M	16.5 mL
P/S, 100×	1×	5 mL
Total		**500 mL**

⚠ CRITICAL: check and adjust the pH before use; sterilize by filtering with a 0.22 μm filter.

Note: The suitability of the solution can be quickly check by looking at the color of the media. HBSS 10× contain Phenol Red thus providing indication about pH solution. Under suitable condition, growth medium will have a warm pink-red color. After long storage, the indicator color turns to bright pink. If contamination occurred during storage, the indicator color may turn into yellow.

PBS 10× (can be prepared and stored for 3–4 weeks at 4°C)		
Reagent	Final concentration	Amount
NaCl	1.37 M	80 g
KCl	27 mM	2 g
Na_2HPO_4	81 mM	11.5 g
KH_2PO_4	14.7 mM	2 g
dH_2O	N/A	~1000 mL
Total		**1000** mL

⚠ CRITICAL: Sterilize by filtering with a 0.22 μm filter.

Note: PBS 10× is a stock solution. PBS 1× should be prepared by dilution in dH_2O.

SGZ-NSCs Extraction Solution (to be prepared the day before use and stored at 4°C, trash out any solutions' residual after usage)		
Reagent	Final concentration	Amount
Trypsin/EDTA 1×	0.2×	600 μL
DNAse, 120 U/mL	0.4 U/mL	10 μL
PBS 1×	N/A	2390 μL
Total		**3 mL**

Note: The final total volume of 3 mL of "SGZ-NSCs Extraction solution" is necessary and sufficient for one litter. Adjust the volume of solution accordingly to litters that have to be used.

Prepare solution for mouse brain organoids generation and further analysis

Timing: 1 h

All the solutions necessary for mouse brain organoids generation, culture maintenance and further processing are described in the tables below.

Expansion medium without growth factors (can be prepared and stored for 3–4 weeks at 4°C)		
Reagent	Final concentration	Amount
DMEM/F12/Glutamax	N/A	240 mL
P/S, 100×	1×	2.5 mL
N-2, 100×	1×	2.5 mL
B-27, 50×	1×	5 mL
Total		**250 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 μm filter.

Note: The suitability of the solution can be quickly check by looking at the color of the media. DMEM/F12/Glutamax contains Phenol Red thus providing indication about pH solution. Under suitable condition, growth medium will have a warm pink-red color. After long storage, the indicator color turns to bright pink. If contamination occurred during storage, the indicator color may turn into yellow.

Neuron Chow without differentiation factor (can be prepared and stored for 3–4 weeks at 4°C)		
Reagent	Final concentration	Amount
Neural Basal Medium	N/A	242 mL
P/S, 100×	1×	2.5 mL
L-Glutamine 200 mM	4 mM	500 μL
B-27, 50×	1×	5 mL
Total		**250 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 μm filter.

Note: The suitability of the solution can be quickly check by looking at the color of the media. Neural Basal Medium contains Phenol Red thus providing indication about pH solution. Under suitable condition, growth medium will have a warm pink-red color. After long storage, the indicator color turns to bright pink. If contamination occurred during storage, the indicator color may turn into yellow.

Expansion Medium (to be prepared fresh) (for brain organoids culture from day 0 to day 4)		
Reagent	Final concentration	Amount
Expansion medium	N/A	50 mL
bFGF	20 ng/mL	N/A
EGF	20 ng/mL	N/A
(optional WNT3a)	(5 ng/mL)	(N/A)
Total		**50 mL**

Induction medium I (to be prepared fresh) (for brain organoids culture from day 5 to day 6)		
Reagent	Final concentration	Amount
Expansion medium	N/A	50 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
bFGF	10 ng/mL	N/A
EGF	10 ng/mL	N/A
(optional WNT3a)	(5 ng/mL)	(N/A)
Total		**50 mL**

Induction medium II (to be prepared fresh) (for brain organoids culture from day 7 to day 14)		
Reagent	Final concentration	Amount
Expansion medium	N/A	50 mL
bFGF	5 ng/mL	N/A
(optional WNT3a)	(5 ng/mL)	(N/A)
Total		**50 mL**

Differentiation media (to be prepared fresh) (for brain organoids culture from day 15 to day 32)		
Reagent	Final concentration	Amount
Neuron Chow	N/A	50 mL
BDNF	50 ng/mL	N/A
Total		**50 mL**

Fixing solution (to be prepared fresh)		
Reagent	Final concentration	Amount
PFA	4%	100 mL
Sucrose	4%	4 g
Total		**100 mL**

⚠ CRITICAL: PFA is hazard and the "Fixing solution" must be prepared under a chemical hood.

Triton-X stock solution (0.5%) (can be prepared and stored for 2–3 months at 4°C)		
Reagent	Final concentration	Amount
Triton-X	0.5%	5 mL
PBS 1×	N/A	995 mL
Total		**1000 mL**

⚠ CRITICAL: Triton-X is viscous, a p1000 cut tip should be used to draw it. Triton-X takes time to be solubilized, use a magnetic stirrer and leave the tip inside the bottle to allow complete Triton-X dissolution.

Blocking solution 0.5% (to be prepared fresh) (for nuclear marker staining)		
Reagent	Final concentration	Amount
BSA	2%	0.2 g
Triton-X stock solution	0.5%	10 mL
Total		**10 mL**

⚠ CRITICAL: BSA takes minutes to be solubilized, use a magnetic agitator to ensure proper dissolution. Keep on ice during usage.

Blocking solution 0.25% (to be prepared fresh) (for cytosolic marker staining)		
Reagent	Final concentration	Amount
BSA	2%	0.2 g
Triton-X stock solution	0.25%	5 mL
PBS 1×	N/A	5 mL
Total		**10 mL**

⚠ CRITICAL: BSA takes minutes to be solubilized, use a magnetic agitator to ensure proper dissolution. Keep on ice during usage.

DABCO stock (prepare and store 2–3 months at 4°C)		
Reagent	Final concentration	Amount
DABCO powder	10 mg/mL	10 mg
dH_2O	N/A	1 mL
Total		**1 mL**

⚠ CRITICAL: DABCO is light sensitive, wrap the Eppendorf in tin foil and keep away from light.

DABCO solution (to be prepared fresh and stored 3–4 weeks at 4°C)		
Reagent	Final concentration	Amount
PBS 1×	N/A	5 mL
Glycerol 100%	50%	5 mL
DABCO stock	0.1 mg/mL	100 µL
Total		**10 mL**

⚠ CRITICAL: DABCO is light sensitive, wrap the falcon in tin foil and keep away from light. Do not use it if the solution remains at 20°C–22°C for more than 2 h.

PSS solution (can be prepared and stored 3–4 weeks at 4°C)		
Reagent	Final concentration	Amount
NaCl	140 mM	4 g
KCl	5 mM	0.18 g
Na_2HPO_4	1.2 mM	0.08 g
$MgCl_2$-$6H_2O$	1.4 mM	0.14 g
$CaCl_2$-$2H_2O$	1.8 mM	0.13 g
Glucose	11.5 mM	1.03 g
HEPES	10 mM	1.19 g
Total		**500 mL**

⚠ CRITICAL: Check and adjust the pH before use; sterilize by filtering with a 0.22 µm filter.

Lysis buffer		
Reagent	Final concentration	Amount
TrisHCl pH 8.5–8.8 1 M	100 mM	100 mL
EDTA 0.5 M	5 mM	10 mL
SDS 10%	0.2%	20 mL

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Continued

Reagent	Final concentration	Amount
NaCl 5 M	200 mM	40 mL
dH_2O	N/A	830 mL
Total		**1000 mL**

Proteinase K solution

Reagent	Final concentration	Amount
Proteinase K 20 mg/mL	10 mg/mL	500 µL
Glycerol 100%	25%	250 µL
dH_2O	N/A	250 µL
Total		**1 mL**

PCR working solution (volume for 1 sample in single replicate)

Reagent	Final concentration	Amount
Master Mix 2×	1×	10 µL
Primers F and R 100 µM	10 µM	0,1 µL
DNAse Free Water	N/A	5,8 µL
Total		**16 µL**

RIPA Buffer (can be prepared and stored 2–3 weeks at 4°C; 2–3 months at −20°C)

Reagent	Final concentration	Amount
TrisHCl 1 M pH 7.4	50 mM	500 µL
NaCl 5 M	100 mM	200 µL
NP-40	1%	50 µL
Sodium deoxycholate (DOC) 10%	1%	1 mL
$MgCl_2$ 1 M	5 mM	50 µL
Glycerol 100%	3%	300 µL
dH_2O	N/A	7.9 mL
Total		**10 mL**

Note: The detergents in the RIPA buffer may precipitate over time. If this happens, heat the solution (37°C) and mix to dissolve the components.

Complete RIPA BUFFER (to be prepared fresh and used right away)

Reagent	Final concentration	Amount
Dithiothreitol (DTT) 0.5 M	5 mM	10 µL
PMSF (stock 200 mM in EtOH 100%)	5 mM	25 µL
Protease inhibitor 7×	1×	142.9 µL
Phosphatase inhibitor 10×	1×	100 µL
RIPA buffer	1×	722.1 µL
Total		**1 mL**

STEP-BY-STEP METHOD DETAILS

Mouse embryonic (E14.5) neural stem cells isolation from SGZ

Timing: 1 h

The starting point for the generation of mouse brain organoids is the isolation of neural stem cells (NSCs) from the subgranular zone (SGZ), a well-known stem cells niche.[2,3] The peak of neurogenesis of mouse brain is at embryonic day 14.5 (E14.5)[4,5] therefore to ensure an efficient isolation of NSCs we extract cells at this developmental stage.

⚠ CRITICAL: tissue harvesting, and cells isolation procedures should take no more than 1 h to ensure a high yield of viable stem cells.

Note: This protocol is based on the use of c57Bl/6J mouse colonies. Similar results could be obtained with other mouse colonies (e.g. CD1 mice).

1. Sacrifice pregnant adult female mouse by cervical dislocation.

   Note: to ensure the collection of embryos at E14.5 it is important to determine the day 0 of pregnancy by following specific procedure[6] or by relying on vendor information.

   ⚠ CRITICAL: cervical dislocation must be performed by trained and qualified personnel only. Cervical dislocation without prior anesthesia with CO_2 or isofluorane inhalation ensures high yield of healthy, viable embryos[7,8] however a fast CO_2 narcosis or isoflurane induction before the cervical dislocation can be performed.

2. Clean the mouse skin with Et-OH 70% and quickly harvest the embryos.
   a. Grab skin below the center of the belly with forceps and cut through the skin to expose the abdominal and thoracic cavities.
   b. Remove the sac containing the string of embryos.
   c. Remove the embryos one-by-one by gently cutting away the outer membrane (Figure 1A).
3. Collect the embryos' heads in HBSS 1× on ice.

   Note: hereafter work under sterile hood.

4. Under stereomicroscope isolate the brain, divide the hemispheres and process one-by-one (Figures 1B–1D, Methods video S1).

   ⚠ CRITICAL: keep the half hemispheres hydrated with HBSS 1× during the procedure to avoid tissue degradation.

5. Identify the SGZ region (Figures 1E and 1F), carefully isolate the zone by using forceps.

   Note: at this embryonic stage, the hippocampal region is not completely formed and thus the dentate gyrus and the SGZ is not clearly visible.

Refer to Methods video S1 for the procedure of SGZ isolation.

6. Collect the extracted tissues in a 15 mL falcon tube, previously filled with 6 mL of HBSS and keep it on ice (4°C).
7. Repeat step 4–6 for each embryos' brain and pool all the harvested SGZs together.
8. Centrifuge 1 min 300 g and discard the supernatant solution by using pipette.

   ⚠ CRITICAL: the tissue pellet is faint, do not turn the falcon tube to remove the supernatant.

9. Wash the tissue pellet with PBS 1×.
10. Repeat step 8.
11. Resuspend the tissue pellet in 3 mL of SGZ-NSCs extraction solution by gently mixing.

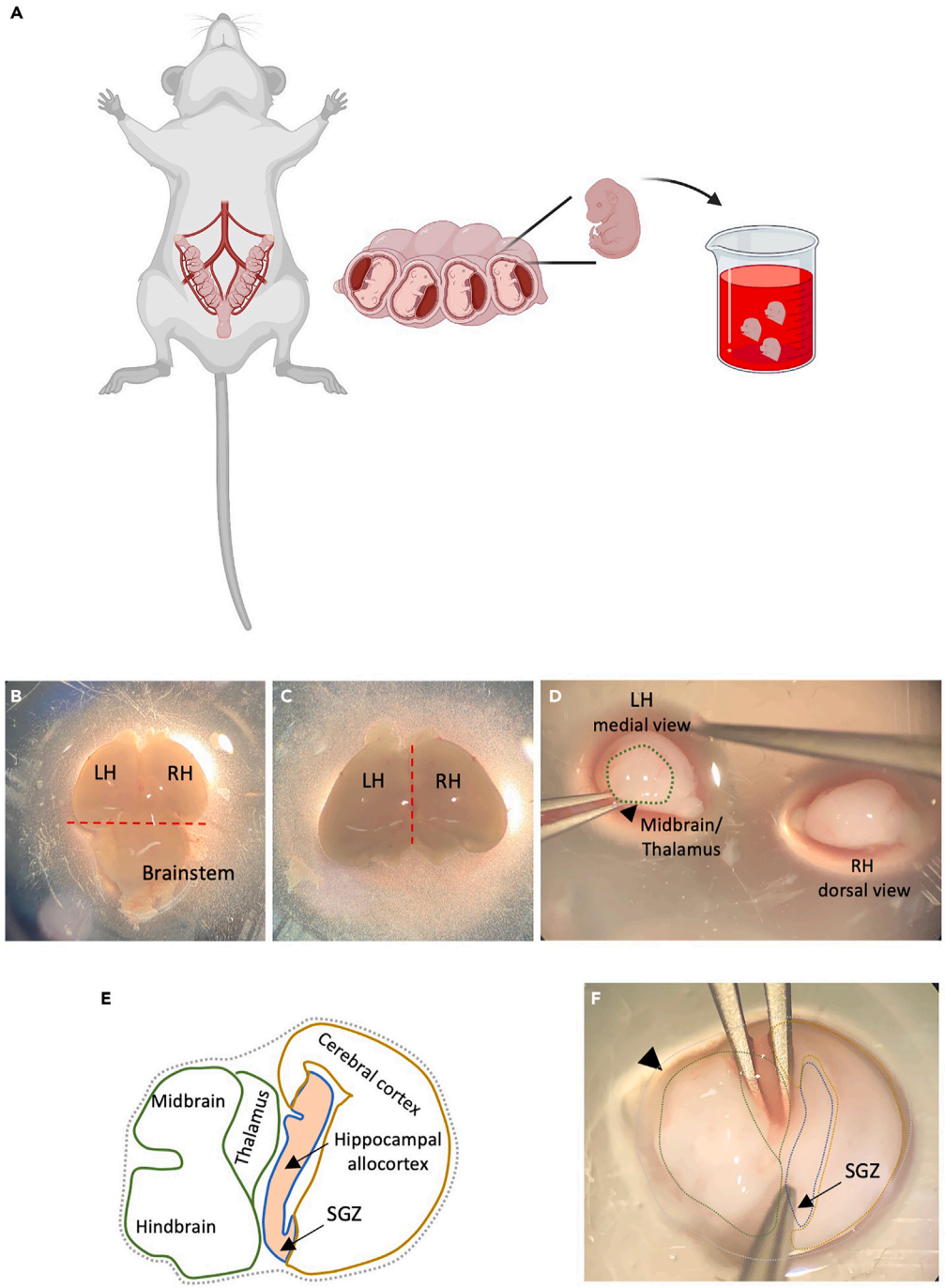

Figure 1. Representative images of embryonic (E14.5) mouse brain to facilitate SGZ recognition and dissection

(A) Procedure of embryos extraction.

(B) Coronal view of intact brain extracted from E14.5 mouse embryo. Both hemispheres and a portion of brainstem are visible. Cut off the brainstem to proceed only with the hemispheres.

(C) The hemispheres are separated by cutting at the middle. Red dashed lines in B-C indicate the cutting lines.

(D) One by one hemisphere is flipped 90° side down. The flipped hemisphere presents a tissue hump (delimitated by green dashed line and black head arrow) representing the midbrain and thalamus inner brain regions.

(E) Scheme of a sagittal section of E14.5 mouse brain highlighting the anatomical structures. The hippocampal allocortex (delimitated by blue dashed line) hosts the primordium of SGZ.

(F) The SGZ resides behind the midbrain and thalamus inner brain tissue; carefully lift it to harvest the SGZ. LH: left hemisphere, RH: right hemisphere; SGZ: sub granular zone.

12. Transfer the suspension in a gentleMACS tube (Purple-cap C Tubes – for tissue dissociation) (Miltenyi Biotec).
13. Insert the tube into the gentleMACS Dissociator instrument, running the following pre-set gentleMACS Program: BRAIN_01_01′.
14. Incubate the tube at 37°C water bath for 10 min.
15. Insert the tube into the gentleMACS Dissociator instrument.
    a. Running the pre-set gentleMACS Program BRAIN_02_01′.
    b. Running the pre-set gentleMACS Program BRAIN_03_01′.

⚠ CRITICAL: if the tissue looks not completely homogenate, repeat once again step 15a-b.

Alternatives: if the gentleMACS Dissociator instrument is not available, tissue dissociation can be performed by manual mechanical dissociation: vigorously pipette up and down (at least 20 times) the solution by using a p1000 and then 10 times by using a Pasteur pipette. Note that this procedure could result in a high number of dead cells.

16. Filter the solution with a 40 μm cell strainer in a 50 mL falcon tube.
17. Centrifuge 10 min 300 g, gently remove the supernatant by using a pipette.
18. Resuspend the pellet in 1 mL of Expansion medium to obtain the cell suspension.
19. Count cells:
    a. Mix 90 μL of Trypan Blue with 10 μL of the cell suspension (step 18) in a 500 μL Eppendorf.
    b. Put 10 μL of the suspension prepared in a. into the Burker counting chamber.
    c. Count viable cells by using a bright-field inverted microscope.

Note: cell count can be performed with the preferred method; automatic cell counter or different chambers e.g., Neubauer chamber can be used.

20. Seed 500′000 viable cells in a 75 cm^2 flask filled with 12 mL of Expansion medium.

⚠ CRITICAL: low yield of cells could be obtained from isolation due to few starting embryos or to an incomplete tissue dissociation. Seed the cells in 25 cm^2 flask or 6-wells plate accordingly to the number of cells obtained.

21. Culture the NSCs in incubator 37°C until the formation of neurospheres.

⚠ CRITICAL: daily examinate the NSCs culture under microscope to check potential contamination.

22. Refill the cells medium with 1 mL of fresh Expansion medium every other day.

Note: at least 1–2 weeks are necessary for neural stem cells neurospheres to reach the right density before splitting them (see steps 23–34).

Neurospheres splitting and expansion

Timing: 20 min

This section describes the procedure to enzymatically dissociate neurospheres to obtain single cells necessary to generate organoids. Single cells can be expanded and maintained in culture or even frozen.

23. Collect the medium containing neurospheres into a 15 mL falcon tubes.

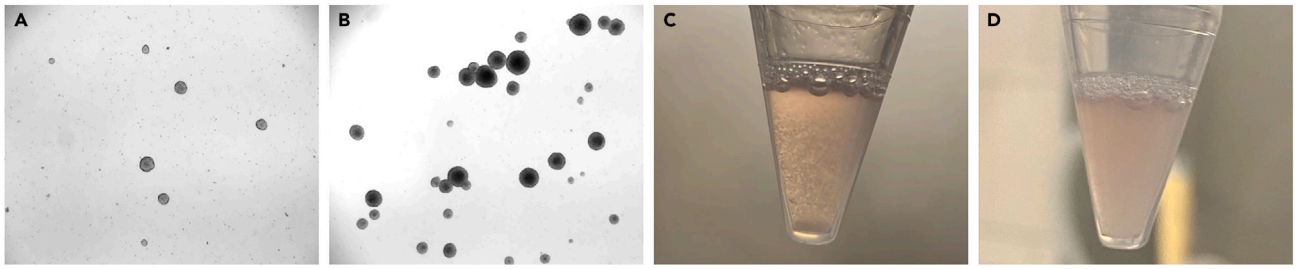

Figure 2. Representative images of neurospheres to be processed for Accutase treatment

The majority of cells inside the Flask should be as neurospheres before splitting. Once the Accutase is added to cell pellet, gently pipetting up and down the cellular suspension until the solution became milky and neurospheres are no longer visible.

(A) Neurospheres not ready to be splitted.

(B) Neurospheres ready to be splitted.

(C) Cell suspension before Accutase treatment.

(D) Cell suspension after Accutase treatment.

⚠ CRITICAL: check under the microscope if any neurospheres are still present in the flask to ensure to collect most of the cells.

Note: if a lot of neurospheres are still visible in the flask, wash it with PBS 1× ensuring to harvest almost all of them.

24. Centrifuge 5 min at 300 g.
25. Carefully discard the supernatant by using a 10 mL serological pipette.
    a. Use p1000/p200 tip to completely remove any liquid residual.

⚠ CRITICAL: excess of liquid will affect the Accutase dissociation.

26. Add 200 µL of Accutase to the pellet.

Note: 200 µL of Accutase is used for a cell pellet derived from 2 T75 flasks.

27. Gently homogenize the pellet with Accutase for max 3–5 min avoiding bubbles formation.

Note: stop the Accutase process as soon as the solution become opaque and neurosphere aggregates are no longer visible to unaided eye (see Figure 2).

⚠ CRITICAL: long Accutase treatment or aggressive pipetting may cause cell death.

28. Add PBS 1× up to 6 mL to dilute the Accutase.
29. Centrifuge 5 min at 300 g.
    a. Carefully discard the supernatant by using a 10 mL serological pipette.
    b. Use p1000/p200 tip to completely remove any liquid residual.
30. Resuspend the single cells pellet in 1 mL of Expansion medium to obtain cell suspension.
31. Cell count:
    a. Mix 90 µL of Trypan Blue with 10 µL of the cell suspension (step 30) in a 500 µL Eppendorf.
    b. Put 10 µL of the suspension prepared in a. into the Burker counting chamber.
    c. Count viable cells by using a bright-field inverted microscope.

Note: 3 millions of cells are generally obtained from a single T75 flask. If high number of cells is obtained, NSCs can be frozen (step 33) or used to further expansion (step 34).

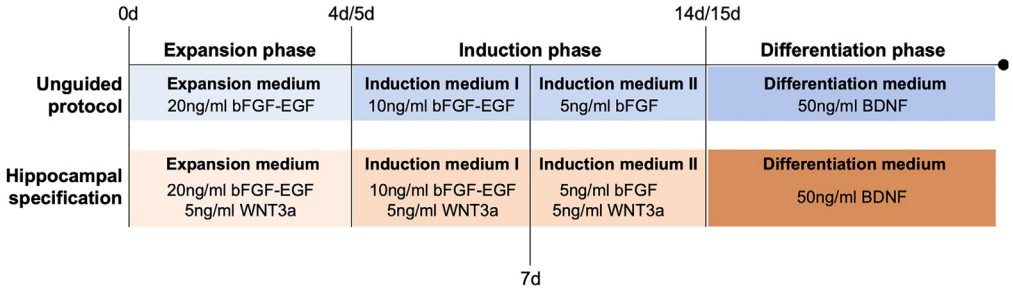

Figure 3. Culturing steps of mouse brain organoids protocol and related media and factors used

The protocol consists of three steps: expansion (days 0 to 4), induction (days 5 to 14) and differentiation (days 15 to 32) phase. In each phase, the organoids are provided with media enriched with defined growth (bFGF and/or EGF) or differentiation (BDNF) factors. For hippocampal phenotype induction the morphogen WNT3a is added to culture media during the expansion and induction phase.

32. At this step cells can be directly used in step 35 to generate organoids following unguided protocol or in step 38 to generate organoids with for hippocampal specification.
33. (Optional) Resuspend the cells to be frozen in at least 1 mL of 50% expansion medium and 50% of freezing solution (20% DMSO plus 80% FBS).
    a. Quick store the vial at −20°C for few hours to let the cells freeze.
    b. Move the frozen cells at −80°C.

Note: frozen cells can be stored at −80°C for several months.

    c. Move the frozen cells in liquid azote for long (years) storage.
34. (Optional) Plate 500′000 viable cells or 250′000 viable cells in T75 or T25 flask, respectively.
    a. Supplement the T75 or T25 flask with 12 mL or 6 mL of Expansion medium, respectively.
    b. Refill with 1 mL or 500 μL fresh Expansion medium every other day, until neurospheres forming.

⚠ CRITICAL: avoid repeated splitting and expansion of cells as neurospheres to prevent NSCs differentiation and attachment to the plate. The maximum number of splitting recommended is 4–5.

Brain organoid culture (unguided protocol)

Timing: 1 month

This section describes the three phases (see Figure 3) that lead to the generation of mouse brain organoids with consistent size, organized neural network and functional neurons within 32 days. The organoid generation method described in this section relies on spontaneous neuronal differentiation without any extrinsic patterning to guide the development into a specific brain region. However, due to the neural stem cell source used, the organoids may have an intrinsic patterning toward dorsal, rather than ventral, brain region specification. To generate organoids with hippocampal-specific phenotype directly go to step 38.

Note: Keep brain organoids in dynamic culture throughout all the phases (ORBi shaker at 75rpm).

35. Expansion phase (days 0–4):
    a. Day 0: seed 20′000 single viable cells/well (day 0) on 24 well plates in 500 μL of culture medium Expansion medium.

⚠ CRITICAL: seeding must be homogeneous for the organoids to form in a proper way (see Figure 4). Ensure pipetting before each seed. Troubleshooting 1

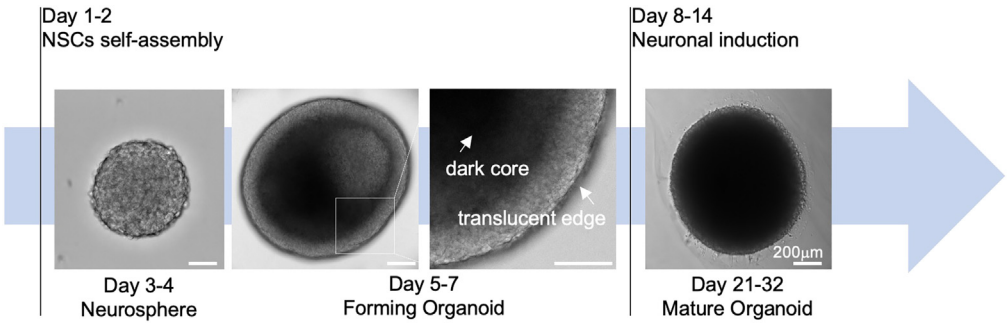

Figure 5. Organoids morphology at different stages of the culture protocol. Scale bars: 200μm

Note: seed the cells only in 18 wells of the 24 well plate can help to maintain the humidity of the plate and better allow the growth of the brain organoids. Putting sterile PBS 1× into the last row of the plate.

b. Day 1: check cells assembly under a microscope. Troubleshooting 2.
c. Day 2: discard 80 μL of the culture medium and refill with 100 μL of fresh Expansion medium.
d. Day 4: discard 80 μL of the culture medium and refill with 100 μL of fresh Expansion medium.

36. **Induction phase (days 5–14):** to promote spontaneous NSCs differentiation, gradually lower growth factors' concentration.
    a. Day 5: completely replace Expansion medium with 500 μL of Induction medium I.

⚠ CRITICAL: at this stage, brain organoids are fragile, and it is recommended to avoid touching them during the medium change. To ease the medium change, put a black sheet under the plate to better visualize the samples and tilt the plate to let the brain organoids lay on one side of the well.

    b. Day 7: completely replace Induction medium I with 500 μL of Induction medium II.

⚠ CRITICAL: To ease the medium change, put a black sheet under the plate to better visualize the samples and tilt the plate to let the brain organoids lay on a side.

    c. Day 9: discard 80 μL of the medium and refill with 100 μL of fresh Induction medium II every other day (on day 11 and day 13).

37. **Differentiation phase (days 15–32)**: boost brain organoids' neural differentiation by supplementing the culture medium with BDNF.
    a. Day 15: completely replace Induction medium II with 500 μL of Differentiation medium.

⚠ CRITICAL: at this stage, brain organoids are small and they could be accidentally taken up. To ease the medium change, put a black sheet under the plate to better visualize the samples and tilt the plate to let the brain organoids lay on a side.

    b. Day 17: discard 80 μL of the medium and refill with 100 μL of fresh Differentiation medium every other day (on days 19, 21, 23, 25, 27, 29, 31).

Note: Brain organoids characterization can be performed at different time points (i.e., day 7, day 14, day 21 and day 32) to follow the cellular organization and the differentiation progress. To do that, some of the organoids kept in culture can be appropriately stored at the desired time for further analysis (see step 41 for immunofluorescence analysis; step 79 for calcium imaging; step 85 for mtDNA analysis; step 107 for protein extraction).

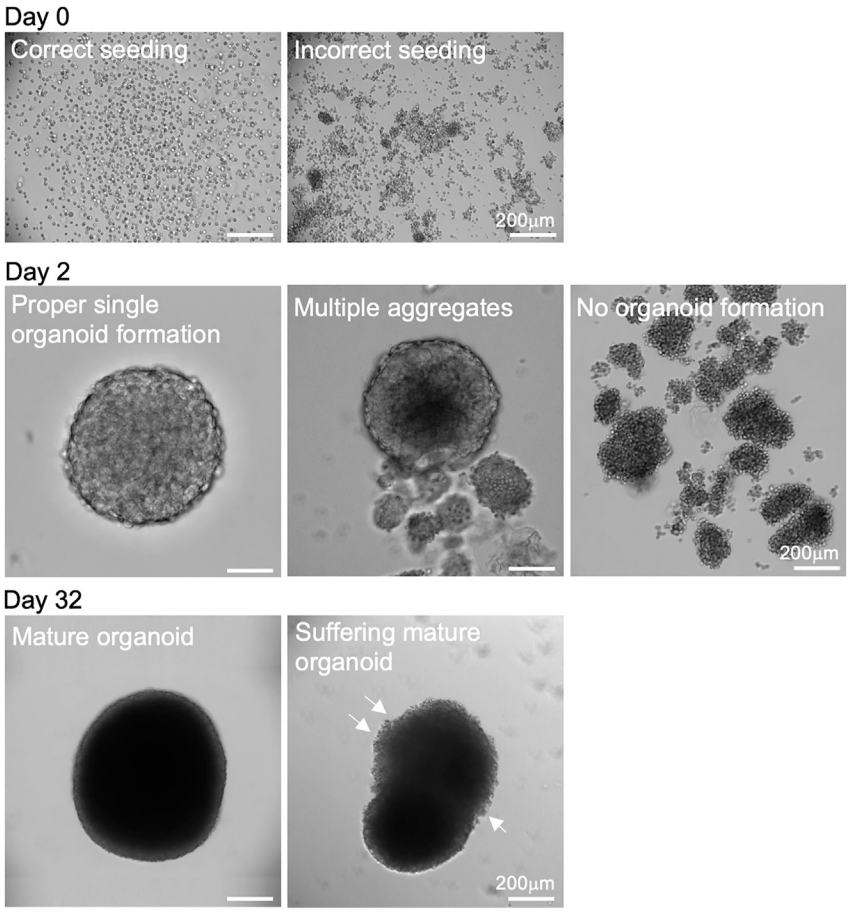

Figure 4. Representative organoids images at critical step of the protocol. Scale bars: 200μm

Representative images of brain organoids morphology at different stages of the culture protocol are reported in Figure 5.

Hippocampal phenotype specification in brain organoids

Timing: 1 month

This section describes the generation of mouse brain organoids with hippocampal-specific phenotype.

The hippocampus is one of the brain regions mostly involved in the pathology progression of many neurodegenerative disorders including Alzheimer's disease and epilepsy.[9,10] By adding the WNT3a factor, crucial for the hippocampal fate determination,[11] it is possible to generate mouse brain organoids that specifically own hippocampal signature. To this end, the three steps of the brain organoid culture are maintained (steps 35–37) (see Figure 3), with slight modification on the media provided during the expansion and induction phase. Specifically, 5 ng/mL WNT3a is added to Expansion medium, Induction medium I and Induction medium II. Key passages are reported below.

Note: Keep brain organoids in dynamic culture throughout all the phases (ORBi shaker at 75 rpm).

38. **Expansion phase (days 0–4)**:
    a. Day 0: seed 20'000 single viable cells/well (day 0) on 24 well plates in 500 μL of Expansion medium plus 5 ng/mL WNT3a. Troubleshooting 1.
    b. Day 1: check cells assembly under microscope. Troubleshooting 2.
    c. Day 2: discard 80 μL of the culture medium and refill with 100 μL of fresh Expansion medium plus 5 ng/mL WNT3a.
    d. Day 4: discard 80 μL of the culture medium and refill with 100 μL of fresh Expansion medium plus 5 ng/mL WNT3a.
39. **Induction phase (days 5–14):** to promote spontaneous NSCs differentiation, gradually lower growth factors' concentration.
    a. Day 5: completely replace Expansion medium with 500 μL of Induction medium I plus 5 ng/mL WNT3a.
    b. Day 7: completely replace Induction medium I with 500 μL of Induction medium II plus 5 ng/mL WNT3a.
    c. Day 9: discard 80 μL of the medium and refill with 100 μL of fresh Induction medium II plus 5 ng/mL WNT3a every other day (on day 11 and day 13).
40. **Differentiation phase (days 15–32)**: boost brain organoids neural differentiation by supplementing the culture medium with BDNF.

    Note: hereafter, addition of Wnt3a is not necessary anymore.

    a. Day 15: completely replace Induction medium II with 500 μL of Differentiation medium.
    b. Day 17: discard 80 μL of the medium and refill with 100 μL of fresh Differentiation medium every other day (on day 19, 21, 23, 25, 27, 29, 31).
    We did not report any statistically significant differences in the morphology of brain organoids with hippocampal signature and the ones generated with the unguided protocol when observed in brightfield microscopy (see Figure 6). Likewise, we didn't find any significant differences in size between the two set of brain organoids (see Figure 6). However, by adding WNT3a at low concentration (5 ng/mL) you will be able to obtain hippocampal specific neurons, expressing both the pan-hippocampal ZBTB20 marker, typical of all neurons committed to hippocampal identity, and the KA1 marker, primarily expressed by pyramidal neurons in the CA3 subfield[1] (see also Figure 9). Conversely, by using a high concentration of WNT3a (20 ng/mL) it is reported the specific induction of the DG hippocampal neurons phenotype.[12,13]

Characterization of brain organoids by immunofluorescence:

Brain organoids' fixing for immunofluorescence

Timing: 20 min

Fixing the brain organoids at critical time points allow to perform IF analysis and compare their development at different stages. Early organoids (before the differentiation phase) are very fragile so must be handled with care. On the contrary, late organoids, although being more compact, are smaller. Fixing brain organoids at different stage of the protocol, e.g., at 7, 14, 21 and 32 days, allows us to have a snap of the cellular organization at each phase of the culture as well as to follow the neuronal maturation. Any other desired time point can be selected to perform the analysis.

41. Collect the brain organoid by using a p1000 with cut tip, at any desirable time points.
    a. Put the organoid in a well of a 24-well plate.
    b. Aspirate and discard any medium residual.
    c. Add 500 μL of fixing solution (see "materials and equipment" section for fixing solution recipe).
42. Incubate at 20°C–22°C for 15 min.

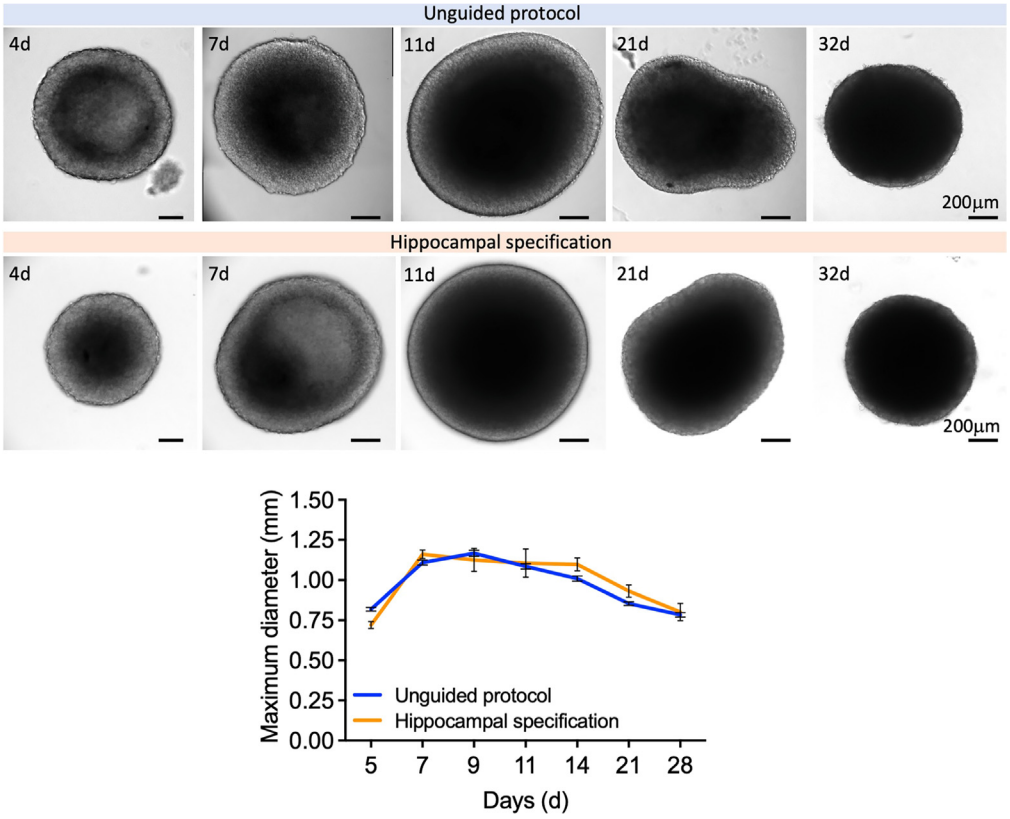

Figure 6. Representative brain organoids brightfield images and size at different stages of the culture protocols. Scale bars: 200μm

⚠ CRITICAL: longer periods of incubation will determine the deterioration of the 3D structures.

43. Aspirate and remove the fixing solution and replace with 500 μL of PBS 1×.
44. Aspirate and remove the PBS 1× with any residual of fixing solution and replace with 500 μL of PBS 1×.

⚠ CRITICAL: To ease this passage, put a black sheet under the plate in order to better visualize the samples and tilt the plate to let the brain organoids lay on a side.

45. Repeat step 44.
46. Store the fixed brain organoids in PBS 1× at 4°C for no longer than 3 weeks.

Note: for best results proceed to step 47 (for immunofluorescence on sliced brain organoids) or step 68 (for immunofluorescence on whole mount brain organoids) within a week.

Cryosectioning of brain organoids for immunofluorescence

Timing: 30 min

In this section we describe the steps to obtain 30 μm organoid's slices for further immunostainings. Thinner slices are very likely to break during the cutting. Given their smaller dimensions compared to human brain organoids, be careful not to lose the organoids during the various steps. Early

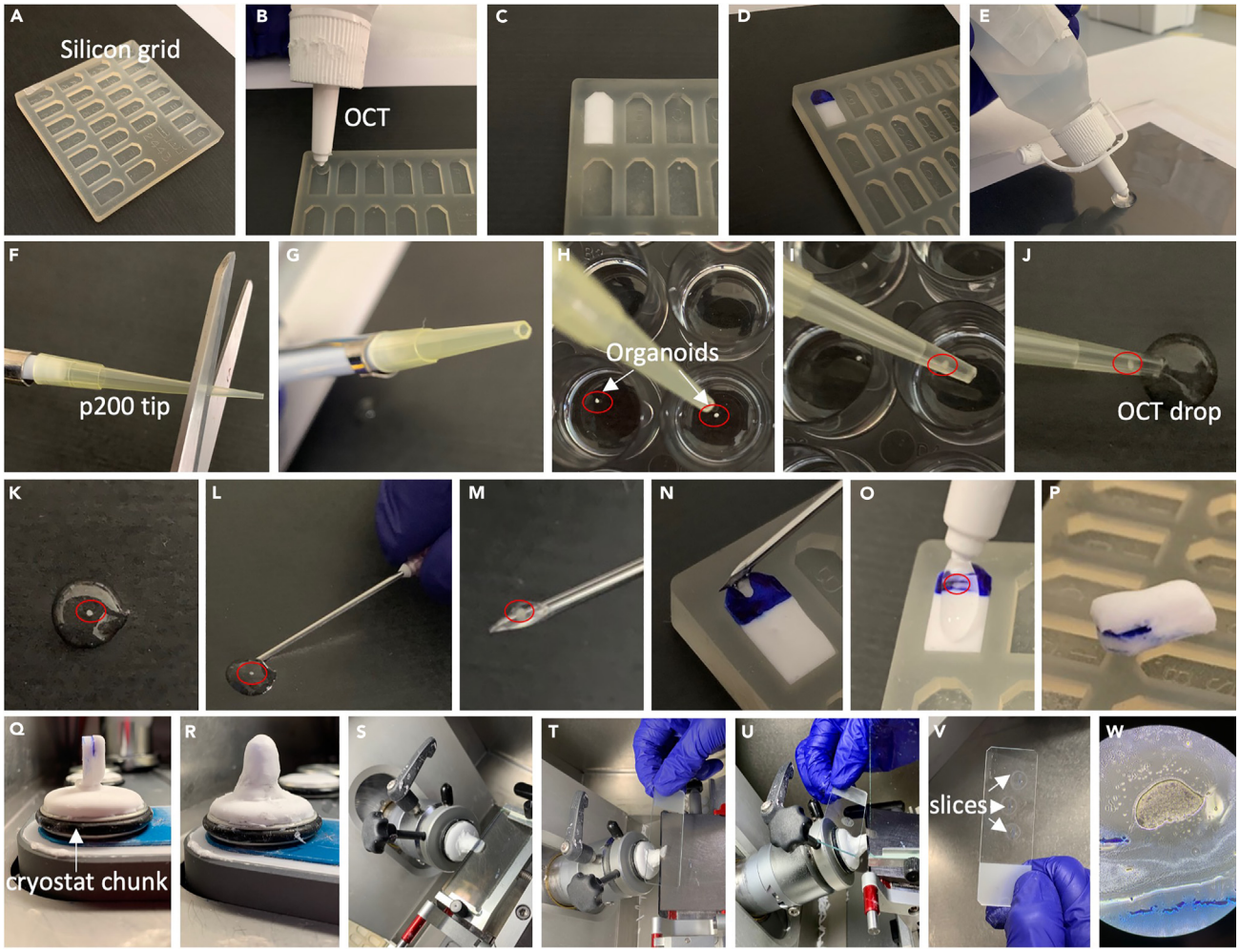

Figure 7. Steps of organoids' cutting for immunofluorescence analysis

organoids (before differentiation phase), although having bigger size, are more fragile compared to the late ones. Refer to Figure 7 to visualize all the passages of the brain organoids' cutting procedure.

⚠ CRITICAL: carefully avoid air bubbles throughout all the steps, since they will determine the breaking/loss of the brain organoid's slices.

Note: for best results, it is important to maintain constant the working temperature of the cryostat (−20°C/−25°C) during all the steps. Keeping the room at cool temperature may help avoiding unwanted increased temperature of the instrument.

47. Create OCT stamps.
    a. Cooling the OCT solution in the pre-formed mold (Figures 7A–7C).

Note: We use transmission electron microscopy (TEM) silicon grids (Figure 7A) but any other suitable mold can be used to create OCT stamps.

    b. Keep the OCT stamps at −20°C.

Note: OCT stamps can be prepared in advance.

48. Once the OCT is solidified, define the zone where the brain organoid will be placed with a dark marker (Figure 7D). This step will ease the cutting process by making the organoid visible when it will be placed on the stamp.
49. Put a drop of OCT on a black support (Figure 7E) in order to make the brain organoid visible while transferring it.
    a. Pick up the organoid from the 24 well with a cut p1000/p200 pipette tip.
    b. Place the organoid into the OCT drop (Figures 7F–7J).

Note: Work at 20°C–22°C.

⚠ CRITICAL: minimize the volume of PBS transferred with the organoid. Troubleshooting 3.

50. Pick up the brain organoid from the OCT drop using a needle and place it on the marked zone of the OCT stamp (Figure 7N).

⚠ CRITICAL: carefully pick up the organoid with the back part of the needle (Figures 7K–7M), since the organoid gets stuck into the gauge. Alternatively, use a cut pipette tip but be careful not to get the organoid stuck in it.

51. Place the stamp containing the brain organoid into the cryostat to allow the sample freezing.
52. Prepare cryostat chucks, covering them with a thin layer of OCT (Figures 7O and 7P).
53. When the sample is frozen, cover the brain organoid into the stamp with additional OCT. Let it freeze.
54. Once the brain organoid is frozen, place the stamp vertically on the cryostat chunk and use some liquid OCT to allow it to stick to the chunk (Figure 7Q).
55. Cover the structure with additional OCT to obtain a resistant structure that will not detach from the chunk while cutting (Figure 7R).
56. When the structure is frozen, start cutting at 30μm (Figures 7S–7V).
57. Check the integrity of the brain organoids slices under microscope (Figure 7W) and dark mark the glass slice in correspondence of organoid's slices to ease their visualization.

Pause point: slices can be eventually stored at −20°C until use or directly used (step 59).

Immunofluorescence on sliced brain organoids

Timing: 8 h

In this section we report the protocol for immunofluorescence analysis on sliced brain organoids, that allow to visualize the spatial distribution of the cells composing the organoids and to characterize their phenotype, as well as their developmental stage. By immunofluorescence analysis at defined time point i.e., 7, 14, 21 and 32 days it is possible to follow the progression of cellular organization and maturation within the brain organoids. The list of antibodies used to characterize the mouse brain organoids is reported in Table 1. To characterize the brain organoid development, we use Vimentin and SOX2 antibodies for neural stem cells[14–16]; Ki67 marker for proliferative cells[17,18]; DCX, B3Tubulin and MAP2 markers for neuronal precursors cells, immature and mature neurons,[19–23] respectively; GFAP marker is used for glial cell identification.[24,25] Representative images of immunofluorescence analysis performed on sliced brain organoids at different time point are reported in Figure 8.

Immunofluorescence analysis on sliced organoids can also give information about specific brain regional identity. Indeed, specific brain regional markers can be used to highlight the presence of

Table 1. List of primary antibodies and related target used to perform immunofluorescence analysis on brain organoids

Antibody	Target
Brain organoid's developmental characterization	
SOX2 (R&D System, # AF2018)	Neural stem cells
VIMENTIN (Millipore, # AB5733)	Neural stem cells
KI67 (Abcam, # ab16667)	Proliferative cells
DCX (Cell signaling technology, #4604)	Neuronal precursors cells
B3 TUBULIN (Promega, #G7121)	Immature neurons
MAP2 (Sigma-Aldrich, #M1406)	Mature neurons
GFAP (Abcam, #ab53554)	Astrocytes
SYNAPTOPHYSIN (Synaptic System, #101 004)	Synaptic puntae
ZBTB20 (Genetex, #GTX121616)	Pan-hippocampal cells
KA1 (Abcam, #ab67404)	CA3-hippocampal cells
PSD95 (Millipore, #MAB1596)	Post-synaptic terminals of the excitatory synapses
GEPHYRIN (Synaptic System, #147 011)	Post-synaptic terminals of the inhibitory synapses
VGAT (Synaptic System, #131 004)	Pre-synaptic terminals of the inhibitory synapses
VGLUT (Synaptic System, #135 303)	Pre-synaptic terminals of the excitatory synapses
GAD65/67 (Santa Cruz biotechnology, #sc-365180)	Inhibitory neurons
NMDA (Santa Cruz biotechnology, #sc-365597)	Excitatory neurons
GLSYN (Santa Cruz biotechnology, #sc-74430)	Glutamine synthetase produced by astrocytes
Brain organoid's regional identity characterization	
GSX2 (Genetex, #GTX129390)	Ganglionic eminences
FOXG1 (Abcam, #ab18259)	Cortical neurons
NKX2.1 (Genetex, #GTX34907)	Ganglionic eminences
PREALBUMIN (TTR) (Genetex, #GTX85112)	Choroid plexus
FRIZZLED 9 (FZD9) (Genetex, #GTX71581)	CA2-hippocampal cells
OCT6 (Abcam, #ab272925)	CA1-hippocampal cells
PROX1 (Abcam, #ab101851)	Dentate gyrus cells

hippocampal (i.e., ZBTB20, Ka1, OCT6 and FZD9)[12,13,26] rather than cortical (i.e., FOXG1) cells within brain organoids. Pre-albumin (TTR) and NKX2.1 or GSX2 can be used to detect cells with choroid plexus and ganglionic eminences signature,[26] respectively. Representative images of sliced organoids stained with brain regional identity markers are reported in Figure 9.

Note: Check the integrity of the brain organoids' slices at the microscope. Perform immunofluorescence staining only on well-preserved slices.

⚠ CRITICAL: be careful throughout all the steps, brain organoids' slices may detach from the slide. To prevent this, we recommend to gently apply drop by drop each solution near the slices avoiding the direct contact with the organoid's slice.

58. Thaw the cryosectioned slices at 20°C–22°C.
59. Add 200 μL of PBS 1× per slice to hydrate it.
60. Remove PBS 1× by gently drain the solution.
61. Incubate brain organoids' slices at 20°C–22°C for 1 h with 200 μL of blocking solution per slice.

Note: create a humidified chamber to prevent the evaporation of solutions.

⚠ CRITICAL: use 0.25% Triton-X for cytosolic markers and 0.5% Triton-X for nuclear markers.

62. Drain the blocking solution and incubate with appropriate concentration of primary antibodies in 200 μL of blocking solution per slice for 2 h at 20°C–22°C.

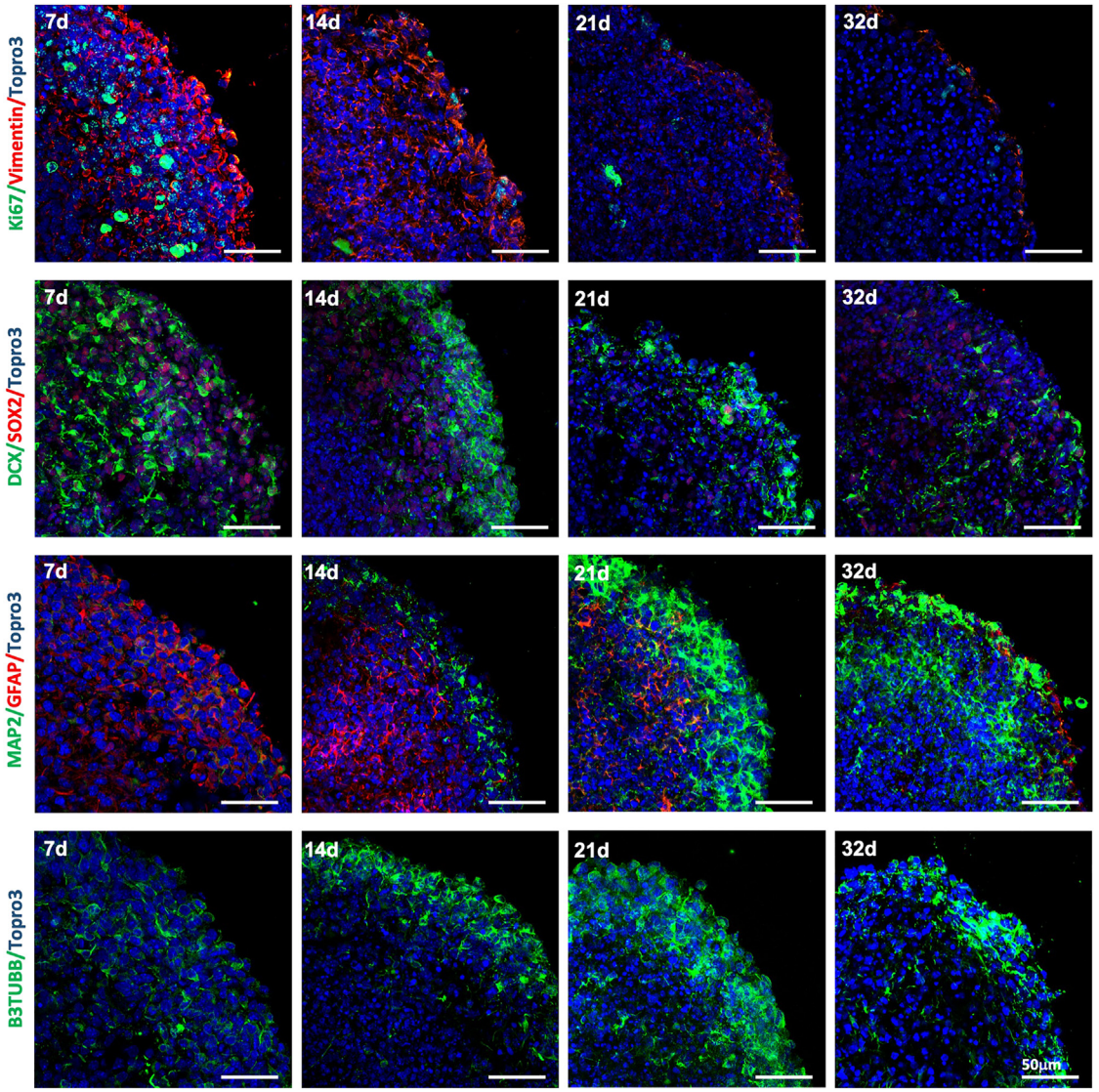

Figure 8. Representative confocal images of immunofluorescence analysis performed on sliced organoids generated with the unguided protocol at different stages of the culture protocol to evaluate the cellular organization and maturation. Scale bars: 50µm

Note: the primary antibodies validated on mouse brain organoids and used to characterize the organoids during development are reported in the key resources table and in Table 1. Please refer to Ciarpella et al.[1] for further details. Any other antibodies against protein of interest could be used. Refer to related datasheet to check the applicability.

63. Drain the primary antibodies solution and wash 6 times 5 min with 200 µL of blocking solution
64. Drain the last blocking solution used for washing and incubate with secondary antibodies in 200 µL of blocking solution per slice for 1.5 h at 20°C–22°C in dark.

Note: the secondary antibodies validated on mouse brain organoids are reported in the key resources table. Please refer to Ciarpella et al.[1] for further details. Any other secondary antibodies could be used. Refer to related datasheet to check the applicability.

65. Drain the secondary antibodies solution and wash 3 times 5 min with 200 µL of blocking solution.

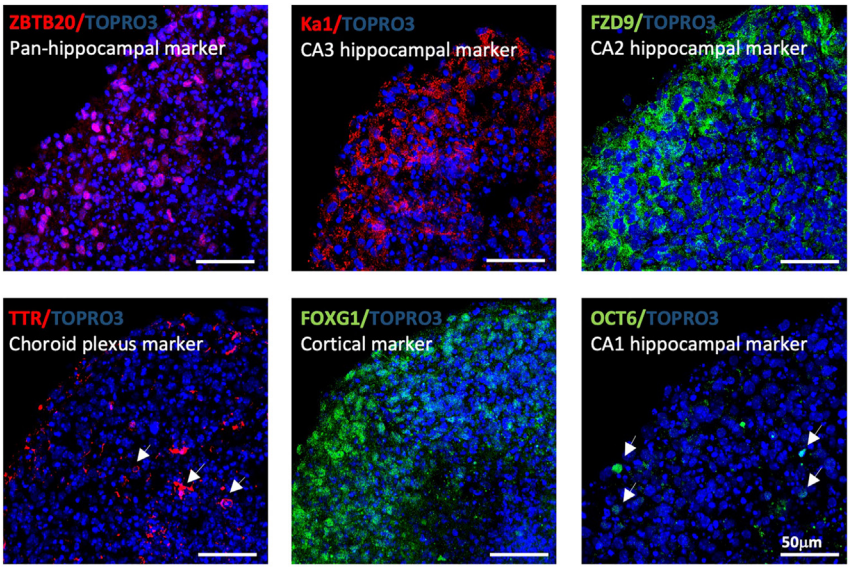

Figure 9. Brain organoid's regional identity characterization

Representative confocal immunofluorescence images of sliced organoids at 32 days with hippocampal specification stained for pan-hippocampal (ZBTB20), CA3- (KA1) and CA2- (FZD9) hippocampal markers and sliced organoids at 32 days generated with the unguided protocol stained for choroid plexus (TTR, positive cells indicated by white arrows), cortical (FOXG1) and CA1- hippocampal (OCT6, positive cells indicated by white arrows) markers. Scale bars: 50μm.

66. Drain the last blocking solution used for washing and wash 3 times 5 min with 200 μL of PBS 1×.
67. At this step, nuclear staining is performed:
    a. drain the PBS 1× and incubate each slice with TOPRO-3 in 200 μL of PBS 1× per slice for 10 min at 20°C–22°C in dark.

Note: Any other nuclear staining could be performed, also accordingly to the wavelength of the laser's microscope used.

    b. Drain the nuclear staining solution and wash once with 200 μL of PBS 1×.
    c. Drain the PBS 1× and close slides with 2 drops of DABCO and the glass coverslip, avoiding the formation of DABCO bubbles. Seal the glass coverslip on the slide with transparent nail polish.

Alternatives: ready-to-use commercial Mountant reagent could be used rather than DABCO.

Note: to ensure the optimal imaging, we usually acquire by confocal microscope the stained slices the following day.

Immunofluorescence on whole mount brain organoids

Timing: 48 h

In this section we report the protocol to perform immunofluorescence on whole mount brain organoids. Preserving the 3D structure, this protocol allows us to analyze the network formation and gives information about the 3D spatial architecture and morphology of the organoids. To perform whole mount immunofluorescence, brain organoids must be previously fixed in Fixing solution (see steps 41–46).

68. Put the fixed organoid in a well of a 96-well plate and incubate it with 80 μL of 0.5% blocking solution at 20°C–22°C for 1 h.

⚠ CRITICAL: always use 0.5% Triton-X when performing the whole mount IF.

69. Carefully remove the blocking solution avoiding withdrawing the organoid and incubate with appropriate concentration of primary antibodies (see Table 1 and key resources table) in 80 μL of blocking solution per organoid at 4°C 12 h, on an orbital shaker (60 rpm).
70. Remove the primary antibodies solution:
    a. wash 6 times 5 min with 80 μL of blocking solution.
71. Remove the last blocking solution used for washing and incubate with secondary antibodies in 80 μL of blocking solution per organoid for 6 h, at 20°C–22°C, static, in dark.
72. Remove the secondary antibodies solution:
    a. wash 3 times 5 min with 80 μL of blocking solution.
73. Replace the last blocking solution used for washing with PBS 1×, wash 3 times 5 min.
74. Perform the nuclear staining:
    a. remove the PBS 1×.
    b. incubate each organoid with TOPRO-3 in 80 μL of PBS 1× for 10 min at 20°C–22°C in dark.

Alternatives: Any other nuclear staining could be performed, also accordingly to the wavelength of the laser's microscope used.

75. Remove the nuclear staining solution and wash once with 80 μL of PBS 1×.
76. Create a parafilm chamber on the slide (see Figure 10).

⚠ CRITICAL: this step is important to preserve the 3D morphology of the brain organoids. The parafilm chamber should be as thick as the sample to prevent its mashing and to avoid any extra space between the organoid and coverslip.

77. Place the organoid inside the chamber and close it with 1 drop of DABCO using a round coverslip.

⚠ CRITICAL: Do not break the organoid while moving it. Make sure the organoid does not move into the corners of the chamber while closing with DABCO. Avoid the formation of DABCO bubbles.

Alternatives: ready-to-use commercial Mountant reagent could be used rather than DABCO.

78. Seal the glass coverslip above the chamber by applying transparent nail polish at the edge.

Note: Images acquisition should be performed as soon as possible, since organoids will either move inside the chamber or become auto-fluorescent as time passes by.

Functional assessment of brain organoids' by using Fluo-4 calcium assay

Timing: 5 h

Functional assessment of the brain organoids' spontaneous activity provides information about the maturation of the forming organoids.[27,28] Fluo-4 assay on whole mount organoids allows detecting cellular activity preserving the organoids' structure, rather than dissociating or slicing them as performed in several published protocols.[29–32] This is a rapid assay that could be easily used to discriminate the effect of specific drugs on the cellular maturation and functionality.

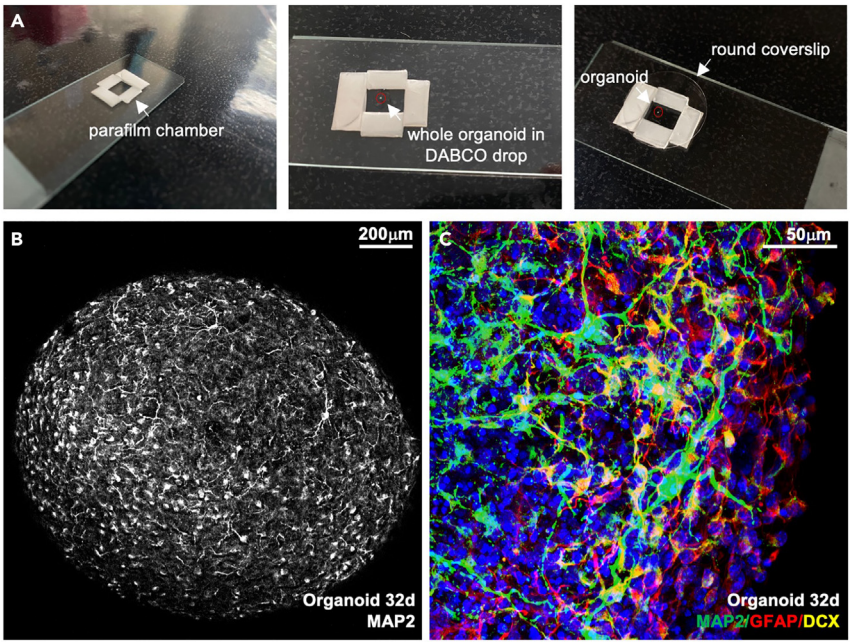

Figure 10. Immunofluorescence on whole mount brain organoids

(A) Parafilm chamber for whole mount organoid immunostaining confocal microscope acquisition.

(B) Representative maximum intensity projections of the z-stack confocal images of the entire mouse brain organoid stained for the mature neuronal marker MAP2 at 32 days. Scale bar: 200µm.

(C) Representative maximum intensity projections of the z-stack confocal images of a region of the entire mouse brain organoid stained for the mature neuronal marker MAP2, the astrocytes marker GFAP and the neuronal progenitors' cells marker DCX. Scale bar: 50µm.

Note: Fluo-4 is not exclusive for neuronal activity, but also for glial one. To exclusively examinate the neuronal activity, GcAMP6 lentiviral construct under the synaptophysin promoter can be used.[33]

79. Wash the brain organoids with HEPES 0.3 M pH 7.3 for 10 min.
80. Incubate organoids for 3 h at 37°C with a solution of 50% Fluo4 + 50% PSS (100 µL for each organoid).

⚠ CRITICAL: work in sterile conditions, protected from light.

81. Wash organoids three times with culture medium.
82. Incubate organoids for 30 min at 37°C with appropriate culture medium.

Note: this step ensures that all the AM-group of the Fluo4 are de-esterification so that the fluorescence signal is constant during acquisition.

83. Perform time-lapse analysis using the Nikon microscope, at 37°C and 5% CO_2. Troubleshooting 4.

Alternatives: other fluorescence, confocal, two-photon microscopes equipped with laser and/or lamp with the desired wavelength can be used to visualize the Fluo4. A microscope equipped with a temperature and gas controller is highly recommended. Keeping the temperature and CO_2 constant during acquisition help to avoid Ca^{2+} fluctuation artefacts.

84. Analyze the generated videos, for a qualitative indication of the Ca^{2+} flux, using ImageJ software:
    a. Open ImageJ software, then load the time-lapse images sequence: File > open.

Note: an "Import image sequence" tab will be open. Be sure to select the "use virtual stack" option. ImageJ also supports file .avi. An "AVI reader" tab will be open and "use virtual stack" should be clicked. We recommend working in greyscale. (Figure 11A)

    b. Draw a sufficient number of ROI for each organoid, related to a single cell or small group of cells: Oval/Rectangular selection > Analyze > Tools > ROI manager > add (Figures 11B–11D).

Note: draw a ROI in a background zone for further quantitative analysis.

    c. Select one ROI by one from the ROI manager: Image > Stacks > Plot Z-axis Profile (Figure 11E).
    d. A graph showing the Ca^{2+} fluctuations is now open (Figure 11F) and, if needed, it could be saved.
    e. Save the values related to the graph for further analysis: Data > Save data > (.xls/.csv).

Note: it is also possible to save the list of values, to be processed with any other analysis software: List > Plot Values (Figures 11F and 11G).

Alternatives: different software or custom-made calcium analyzer could be used to quantify Ca^{2+} flux and cellular activity. We report here a fast analysis to qualitatively analyze the Fluo4 assay output.

DNA extraction from brain organoids

Timing: 4 h

Fully mature neurons display high energy consumption to sustain their active metabolism and thus, neuronal differentiation implies a switch from glycolytic to oxidative metabolism.[34–39] Characterizing the metabolic profile of organoids allows us to acquire information about brain organoids' development: early organoids rely mainly on glycolysis, while late organoids activate oxidative phosphorylation hence, lower mtDNA copy number and mitochondrial subunits amount are expected in early differentiated compared to fully mature organoids. The DNA extracted with this optimized protocol can be used to evaluate the mtDNA copy number, a good indicator of the amount of mitochondrial mass, by following the method reported in.[40]

85. Put single organoids in a 1.5 mL Eppendorf, at any time point considered.
86. Wash organoids with sterile PBS 1×.
87. Spin quickly.

Note: step 87 can be skipped for early organoids.

88. Carefully remove the supernatant.

⚠ CRITICAL: organoids must be dry after this step.

Pause point: organoids can be eventually stored at −20°C or directly used (step 90).

89. On the day of the extraction thaw your samples and keep them on ice.

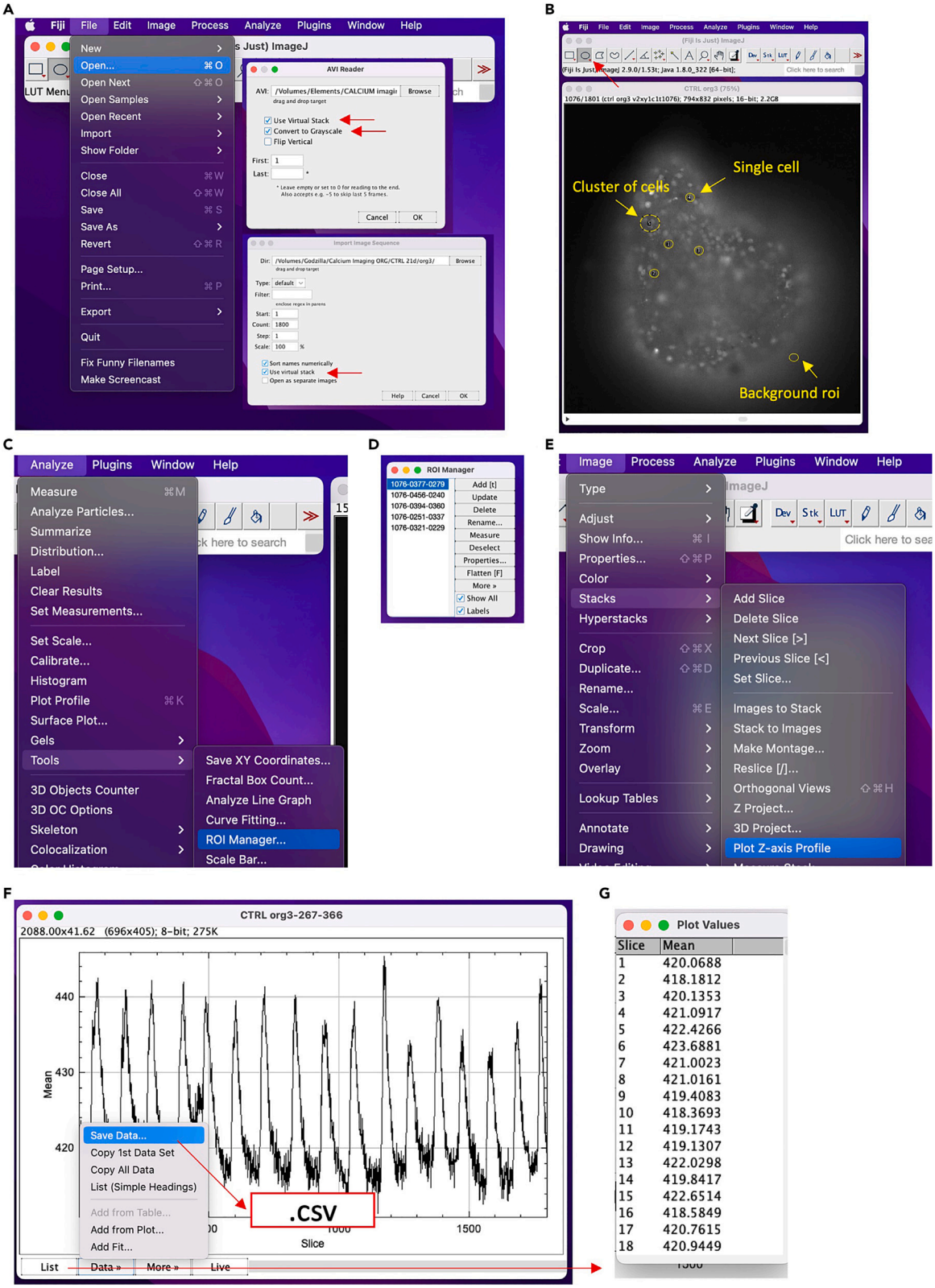

Figure 11. Step-by-step of qualitative calcium imaging analysis using ImageJ software

90. Add 250 μL of lysis buffer plus 2.5 μL of Proteinase K (10 mg/mL) inside the Eppendorf with the organoid and lysate the sample, avoiding bubbles.
91. Incubate at 55°C in a thermoblock for 2–2.5 h and vortex occasionally.
92. At the end of the incubation time, vortex the Eppendorf and centrifuge for 10 min at maximum speed, 20°C–22°C.
93. The pellet contains cellular debris. Take the supernatant and put it in a new Eppendorf.
94. Add 250 μL (1 volume) of isopropyl alcohol and 1 μL of glycogen (it helps visualizing the DNA).

⚠ CRITICAL: glycogen concentration cannot be over 4 μg/μL, otherwise it may inhibit PCR reaction.

Note: if DNA clump is not visible, put the Eppendorf at −80°C for about 30 min, it helps the glycogen precipitate the DNA.

95. Centrifuge at 17.530 g for 30 min at 20°C–22°C.
96. Wash the DNA pellet with 50 μL of EtOH 70%.
97. Air-dry the pellet.
98. Dissolve the pellet into 30 or 50 μL for late or early organoids, respectively of TE buffer pH 7.5.
99. Quantify the DNA concentration using the Nanodrop.

Pause point: DNA may be stored at −20°C until use or directly used (step 100).

Real-time PCR for mitochondrial DNA

Timing: 4 h

Evaluation of the mtDNA copy number can be a good indicator of the amount of mitochondrial mass. We report here the steps to perform the PCR reaction for the analysis of mtDNA/nDNA ratio in mouse cells using a SYBR green assay. It will allow to estimate the mtDNA copy number, by comparing the amount of mitochondrial versus nuclear DNA.[40]

Note: In a single 96 well plate, two different amplification reactions will be run: one for the genomic DNA, and one for the mitochondrial DNA; so ideally the plate will be divided in two sections. Each sample will run in three technical replicates for each amplification reaction. In the following steps, solutions amount is calculated for one sample in three technical replicates.

100. Dilute the DNA samples at a concentration of 5 ng/μL.
101. Prepare two separate PCR working solution (48 μL total volume each; see "materials and equipment" section for PCR working solution receipt):
    a. Genomic PCR working solution containing forward and reverse primers for the nuclear gene HK2 (hexokinase 2).
    b. Mitochondrial PCR working solution containing forward and reverse primers for the mitochondrial-encoded gene ND1.
102. Add 12 μL of DNA sample to each PCR working solution (genomic and mitochondrial).
103. Load the plate with 20 μL/well of the solutions obtained in step 102.
104. Seal the plate with the appropriate coverslip.
105. Set the software and run the PCR with the following settings for 45 cycles:

Hold Stage: 50°C for 2 min followed by 95°C for 5 min.

PCR Stage: 95°C for 5 s followed by 60°C for 1 min.

Melt curve Stage: 95°C for 5 s, 66°C for 1 min, 97°C for 1 s.

Note: we use the Real Time QS3 system (Thermofisher) equipped with QuantStudio™ Design & Analysis Software v1.5.1.

106. Calculate the number of copies of mtDNA with the following formulas[40] :

$$\Delta CT = Ct(nDNA\ gene) - Ct(mtDNA\ gene)$$

$$Copies\ of\ mtDNA = 2 \times 2^{\Delta Ct}$$

Protein isolation and analysis of brain organoids

Timing: 2 h

These steps allow the extraction of the protein content of the brain organoids. Organoids change in the morphology, dimension and cellular density during the *in vitro* culturing protocol. We optimized the number of organoids to be pooled together at each time point and the volume of solutions in order to have a reasonable yield of material. This resulted in pools of different number of organoids depending on the stage of maturation.

107. Collect pools of 3 organoids (for early organoids: day 7 and day 14) or 5 organoids (late organoids: day 21 and day 32) in 500 µL Eppendorf.
108. Wash organoids with sterile PBS 1×.
109. Spin quickly.

Note: for early organoids you can avoid this step.

110. Remove the supernatant.

⚠ CRITICAL: organoids must be dry after this step.

Pause point: organoids may be stored at −80°C until use or directly used (step 111).

111. Resuspend the pools of organoids in 25 or 13 µL of Complete RIPA buffer for early and late organoid respectively, dissociate by pipetting until the organoids are not visible.

Note: early organoids are quite easy to dissociate, since they are not very compact; the late ones may require a bit more effort to completely dissociate.

112. Vortex and keep on ice for 30 min. Vortex every 10 min.
113. Add 1 µL of Benzonase per sample and incubate at 20°C–22°C for 30 min.
114. Centrifuge at 10000 g for 15 min at 4°C.
115. Transfer the supernatant, that contains the extracted proteins, to a new Eppendorf.
116. Quantify extracted proteins with BCA assay kit following manufacture instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf). Troubleshooting 5.

Neuronal differentiation requires a switch from glycolytic to oxidative metabolism[35] and an increase of OXPHOS subunits and mitochondrial proteins is expected during organoid maturation. Extracted proteins are used to perform Western Blot analysis to detect and quantify the mitochondrial proteins and OXPHOS system quantity. The voltage-dependent anion-selective channel 1 (VDAC 1) protein, a mitochondrial channel of the outer mitochondrial membrane, is used as an indicator of mitochondrial mass while the amount of mitochondrial respiratory chain complexes I-V is given by the

OXPHOS cocktail antibody. We usually express the mitochondrial markers as fold-change of the nuclear marker Lamin B, rather than to the total protein content per lane. Indeed, given the heterogeneity of cell composition of brain organoids across the developmental stages, equal amounts of protein/organoid pool at different maturation phases may not reflect the cell number. Specific antibodies can be used for any other proteins of interest.

EXPECTED OUTCOMES

We established a reliable and reproducible three-phase protocol to generate functional brain organoids from mouse embryonic (E14.5) neural stem cells (NSCs) in 32 days, which displayed consistent size and functional features. The steps include a fine-tuning of media composition and application time, allowing the progressive neuronal cells maturation and organization into a 3D-brain like tissue. By using this step-by-step protocol, a 95% rate of successful brain organoid formation and maintenance along the *in vitro* culture timing (32 days) with a 5% rate of brain organoid disintegration after 14 days or no aggregation within the first 48 h is expected. The cellular organization and the progressive neuronal differentiation of the generate murine brain organoids can be observed by several techniques such as immunofluorescence analysis, western blot and calcium imaging. Extensive data about brain organoids characterization resulting from these methodologies are reported in Ciarpella et al.[1]

Mouse brain organoid generated by using this protocol allows for insights into neuronal development as well as disease modeling and drug screening. It represents a more cost-effective and less-time consuming alternative to human brain organoid models. Indeed, even if human brain organoids are a powerful technology for translational potential and precision medicine, mouse derived organoids represent a useful supportive tool to optimize protocols for human-derived brain organoids and to perform large-scale drug discovery studies before validation on human-derived models. Due to the possibility of obtaining genetically mutant phenotype samples, mouse organoids can also serve as systems to study the pathophysiology of different brain disorders, reducing the usage of animals.

LIMITATIONS

This work reports the generation of functional brain organoids starting from mouse embryonic neural stem cells in a time- and cost- efficient manner. However, there are some technical limitations to the protocol. Due to the small dimension of mouse brain organoids, a functional characterization of the neuronal network by technique such as multielectrode array (MEA) systems is hard to be addressed. Small organoids require small and high-density electrodes in order to detect neuronal activity. Unless the small dimension, the lack of vascular systems that ensure proper supplies of nutrition and gas exchange could lead to the formation of a necrotic core in the later stage of differentiation, which may affect the final readouts. To the best of our knowledge, mouse brain organoid model is populated by different cellular type such as neuronal precursors cells, immature and mature neurons, astrocytes and, at least in part during the mature stage, stem cells. Adding cellular components such as microglia or oligodendrocytes may represent an improvement of the proposed protocol, which could benefit in terms of functional maturation and cellular complexity.

TROUBLESHOOTING

Problem 1

More than one brain organoid is forming in a single well (related to Step 35a and 38a).

Potential solution

Following Accutase treatment ensure that each neurosphere is correctly dissociated in single cells. If some cellular aggregates persist after splitting, the cell seeding at day 0 will be not homogeneous. The coexistence of single cells and cellular aggregates will result in the formation of multiple cellular

aggregates that could led to an incorrect organoid formation. Check the 24-well plate at the microscope after seeding and exclude from the analysis the wells containing cellular aggregate.

Problem 2

Brain organoids are not forming within 48 h (related to Step 35b and 38b).

Potential solution

We recommend seeding the cells in the center of the well and to not spread them around the well to facilitate cells aggregation and organoid formation. To do this, place the pipette's tip at the center of the well and keep the position while gently pipetting up and down the cell suspension solution. Seed the precise number of cells in each well of a 24-wells plate by counting them accurately and ensuring homogeneous pipetting by mixing up and down the cells in the falcon tube before each seeding in 24-wells. Brain organoid's formation could also be impacted by cells splitting. Accutase treatment should be performed as gently as possible. Ensure to not proceed for more than 5 min in order to prevent cell death. Avoid seeding cellular suspension if the viability is less than 90%.

Problem 3

Brain organoids collapse or break during cryostat sectioning and the resulted slices cannot be further use for IF analysis (related to Step 49).

Potential solution

When the organoid is transferred from PBS to the OCT drop, ensure to pick up the organoid with the less PBS 1× solution possible. To favor the removal of PBS 1× excess, subsequently transfer the organoid from an OCT drop to another one until a small amount of PBS 1× is present. Reducing the cutting speed could also help to keep the slice intact during the procedure. If the problem persists, it is possible to increment the slice thickness.

Problem 4

Brain organoids doesn't reveal any calcium activity (related to Step 83).

Potential solution

Prolongated Fluo4-am dye incubation could result in saturated signal. When the signal is too high, it is difficult to detect intensity variation in the fluorescence. The incubation time could depend on organoid dimension: small organoids could benefit from an incubation of 2 h. Prolongated Fluo4-am dye incubation could also lead to cell dead: brightest cells are probably suffering. Avoid including these cells in the subsequent analysis. The best activity signal is expected to be at 21/32 days in culture. Little activity is observed at day 7, probably coming from glial cells, while an increment is observed starting from day 14.

Problem 5

Low or insufficient yield of protein amount is obtained after protein extraction procedure (related to Step 116).

Potential solution

Incomplete or not-efficient organoid lysis could lead to low protein amount. Ensure to completely dry the organoid before adding the Complete RIPA buffer. Due to the small volume of buffer used, any residual of PBS will dilute it and reduce the efficiency of the organoid lysis. Avoiding bubble formation during lysis could favor the tissue-like visualization and thus can help to evaluate when to stop the lysis process. If the amount of protein obtained is still not sufficient for further desired analysis, the number of organoids to be pooled together could be incremented.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ilaria Decimo (ilaria.decimo@univr.it)

Materials availability

This study did not generate any unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102413.

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AUTHOR CONTRIBUTIONS

F.C. designed, implemented, and wrote the protocol; R.G.Z., A.C., and G.P. designed, implemented the protocol and revised the manuscript; M.D.C. and E.B. participated in method development and revised the manuscript. I.D. conceived, designed, supervised the study, and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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