Apr 21, 2022

Engineering brain assembloids to interrogate human neural circuits

Yuki Miura[1,2], Min-Yin Li[1,2], Omer Revah[1,2], Se-Jin Yoon[1,2], Genta Narazaki[1], Sergiu Pasca[1,2]

[1]Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA, USA;

[2]Stanford Brain Organogenesis, Wu Tsai Neurosciences Institute, Stanford University, Stanford, CA, USA.

dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Neurodegeneration Method Development Community
Tech. support email: ndcn-help@chanzuckerberg.com

Sergiu Pasca

The development of neural circuits involves wiring of neurons locally following their generation and migration, as well as establishing long-distance connections between brain regions. Studying these developmental processes in the human nervous system remains difficult because of limited access to the tissue that can be maintained as functional over time in vitro. We have previously developed a method to convert human pluripotent stem cells into regionalized neural organoids that can be fused and integrated to form assembloids and study neuronal migration. In this protocol, we describe approaches to model long-range neuronal connectivity in human brain assembloids. We present how to generate 3D spheroids resembling specific domains of the nervous system and then how to integrate them physically to allow axonal projections and synaptic assembly. In addition, we describe a series of assays including viral labeling and retrograde tracing, 3D live imaging of axon projection and optogenetics combined with calcium imaging and electrophysiological recordings to probe and manipulate the circuits in assembloids. We anticipate that these approaches will be useful in deciphering human-specific aspects of neural circuit assembly and in modeling neurodevelopmental disorders with patient-derived cells.

DOI

dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca 2022. Engineering brain assembloids to interrogate human neural circuits. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

protocol

https://www.nature.com/articles/s41596-021-00632-z

human neural circuits, brain assembloids, viral labeling, retrograde tracing, 3D live imaging of axon projection, optogenetics

protocol ,

Nov 01, 2021

Apr 21, 2022

Nov 01, 2021 Renuka Suresh

Nov 11, 2021 Sergiu Pasca

54690

Troubleshooting

A	B	C	D
Step	**Problem**	**Possible reason**	**Solution**
Throughout	Cultures were contaminated	Inadequate sterile technique	Improve sterile technique. Wear laboratory coat. Use 70% ethanol to spray your hands and any equipment or reagents that go into the safety cell culture cabinet. Autoclave all non-sterile equipment. Change HEPA filters in ncubators regularly. Autoclean incubators regularly. Identify sources of contamination in the incubator or cell culture room. Clean the water bath in the incubator and in the cell culture room on a weekly basis.
29	Individual spheroids do not form	Overfilled individual wells of the AggreWell plate	Count cell number carefully and add the required amount of cells to each well. Centrifuge the plate with optimal speed
31	Spheroids do not form	hPS cells are differentiating	Always check hPS cell quality before use
		hPS cell colonies are too small	Allow hPS cell colonies to grow larger. Do not use until the optimal day for splitting (80–85% confluency)
		Over-crowding of hPS cell Colonies	Start differentiation by using hPS cell colonies with the optimal confluency (80–85%)
		Mycoplasma or other contamination	Perform mycoplasma testing regularly (ideally, once per month) and discard the cells and use new hPS cell stock if mycoplasma positive
38	Difficulty in inducing forebrain fate (low FOXG1 expression)	hPS cells are challenging to differentiate into forebrain cells	Consider adding XAV-939 (1.25 µM) from days 1–6 of differentiation
	Spheroids stick to one another	Too many spheroids cultured per plate	Transfer some spheroids to a new or larger low-attachment plate. If already attached, separate them by gently pipetting up and down by using a 10-or 25-ml pipette (for spheroids up to day 25) or two P20 pipette tips at later stages
	Spheroids are too small	hPS cells are differentiating	See solutions for Step 31
		hPS cell colonies are too small	See solutions for Step 31
		Mycoplasma or other contamination	See solutions for Step 31
	Spheroids are disintegrated	Excessive pipetting	Be gentle when handling spheroids at all times

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

38 and 39	Growth of spheroids is fast	Abnormal genome integrity in the hPS cell line	Check the genome integrity of the hPS cell line by using SNP array, and confirm that there is no de novo large duplication or deletion
39 and 40	Medium turns acidic overnight	Too many spheroids cultured per plate	In the initial stages of differentiation, 12 ml of medium per 10-cm plate is sufficient. As spheroids grow, use 12–15 ml of medium per plate. Maintaining <30 spheroids per 10-cm plate is recommended
40A	Sparse or no viral labeling	The viral titer was too low	Make sure that viral titers are sufficiently high
		Insufficient time between infection and fluorescent protein expression	Wait ≥1–2 weeks for viral gene expression
		Incorrect serotype	Use optimal AAV serotypes
40B	Unable to assemble hCS and hStrS	hCS or hStrS at late stages of differentiation	Try to fuse smaller or early-stage spheroids (days 47–80). After day 80, the success rate is lower
40F	Unable to find GCaMP6+ cells inassembloids under a confocal microscope	The signal of GCaMP6 is weak	Check expression of GCaMP6 by using an epifluorescence microscope (e.g., EVOS or REVOLVE). Increase laser intensity
		Imaging only a few z-slices	Increase thickness of slices by changing the pinhole size and/or image more slices
40G	Cells are hard to find under bright field	Cell bodies are not always easy to identify by using bright field	Identify cells by using fluorescence imaging
	Cells are blown away by pressure	The pressure is too high	Apply minimal positive pressure
		Pipette tips are too large	Use a pipette with smaller tips
	It is dif ficult to form a tight seal. Cells cannot form a fully tight seal even though cells are partially sucked into the pipette	Pipette tips are too large	Use a pipette with smaller tips
	Photobleaching of fluorescence when checking cells	The LED power is too high	Lower the LED power and increase gain and/or exposure time in the camera software setting
	Cells are hard to break in	For some cells, it is hard to break in with just suction	Try 'zap' in the 700B panel for multiple trials; start from short durations such as 50 µs and then increase durations step by step; try strong suctions immediately after zaps. Use pipettes with slightly bigger tips
	Cells get significantly resealed after break-in	Pipette tips are too small	Use pipettes with slightly bigger tips

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

		Osmolarity of the external solution and/or the internal solution is not appropriate	Check osmolarity of both the external solution and the internal solution
		The relative position of the pipette tip and the cell changes in whole-cell mode	Slightly adjust the position of pipette tips

Media

CRITICAL: When ready for use, distribute aliquots of the required volume of medium into **50 mL** sterile tubes and pre-warm in a water bath at **37 °C** or at **Room temperature** . Avoid multiple cycles of refrigeration and warming. Add growth factors and small molecules before use and only after pre-warming aliquots of media.

Biological materials

hPS cells

REAGENT

DMEM/F-12, HEPES **Thermo Fisher Scientific Catalog #11330032**

Essential 8™ Medium **Thermo Fisher Catalog #A1517001**

Essential 6™ Medium **Thermo Fisher Scientific Catalog #A1516401**

Neurobasal™-A Medium **Thermo Fisher Catalog #10888022**

B-27™ Supplement (50X), minus vitamin A **Thermo Fisher Catalog #12587010**

CRITICAL The B-27 supplement must be without vitamin A.

B-27™ Plus Supplement (50X) **Thermo Fisher Catalog #A3582801**

GlutaMAX™ Supplement **Thermo Fisher Scientific Catalog #35050061**

Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #15140122**

CRITICAL: Differentiations can also be performed without penicillin-streptomycin, but the risk of contamination, especially in long-term cultures, will be higher.

Y-27632 ROCK inhibitor **Selleckchem Catalog #S1049**

Dorsomorphin SMAD inhibitor **Sigma Aldrich Catalog #P5499-5MG**

SB 431542 **Tocris Catalog #1614**

XAV 939 **Tocris Catalog #3748**

Recombinant Human EGF Protein CF **R&D Systems Catalog #236-EG**

Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein **R&D Systems Catalog #233-FB**

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

IWP-2 **Selleckchem Catalog #S7085**

Recombinant Human/Murine/Rat Activin A (CHO derived) **peprotech Catalog #120-14P**

SR 11237 **Tocris Catalog #3411**

Recombinant Human/Murine/Rat BDNF **peprotech Catalog #450-02**

Recombinant Human NT-3 **peprotech Catalog #450-03**

L-Ascorbic Acid 2-Phosphate Trisodium Salt **FUJIFILM Wako Pure Chemical Corporation Catalog # 323-44822**

Db-cAMP (dibutyryl-cyclic AMP) **Sigma Catalog #D0627**

cis-4710131619-Docosahexaenoic acid **Sigma Aldrich Catalog #D2534-25MG**

DAPT 5 mg **Stemcell Technologies Catalog #72082**

Cell culture reagents

Vitronectin (VTN-N) Recombinant Human Protein, Truncated **Thermo Fisher Catalog #A14700**

DPBS (no Ca, no Mg) **Thermofisher Catalog #14190144**

0.5M EDTA solution **Thermo Fisher Scientific Catalog #15575020**)

Dimethyl sulfoxide **Sigma Aldrich Catalog #D2650**

Anti-Adherence Rinsing Solution **Stemcell Technologies Catalog #07010**

Accutase (cell detachment solution) **Innovative Cell Technologies, Inc Catalog #AT-104**

Corning® 500 mL Cell Culture Grade Water Tested to USP Sterile Water for Injection Specifications **Corning Catalog #25-055-CV**

● Ethyl ethanol 200 proof (Gold Shield, cat. no. 412804)

Ethyl ethanol is flammable and should be stored in a flame-protective cabinet.

For cryosection and immunostaining only

16% Paraformaldehyde **Electron Microscopy Sciences Catalog #15710**

PFA should be stored in a safety cabinet and should be disposed of according to institutional guidelines after use.

Sucrose **Sigma Aldrich Catalog #S9378**

Tissue-Plus™ O.C.T. Compound **Fisher Scientific Catalog #23-730-571**

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

☒Triton X-100 **Sigma Aldrich Catalog #T8787-50ML**

☒Donkey Serum **Emd Millipore Catalog #S30-100ML**

☒Recombinant Anti-DARPP32 antibody [EP720Y] (ab40801) **Abcam Catalog #ab40801**

☒Anti-GAD67 Antibody clone 1G10.2 **Merck Millipore Catalog #MAB5406**

☒Anti-Ctip2 antibody [25B6] (ab18465) **Abcam Catalog #ab18465**

☒Anti-NeuN antibody [1B7] - Neuronal Marker **Abcam Catalog #ab104224**

☒GFP antibody **Genetex Catalog #GTX13970**

☒mCherry antibody **Genetex Catalog #GTX128508**

☒MAP 2 antibody - 188 004 **Synaptic Systems Catalog #188 004**

☒Anti-SATB2 antibody [SATBA4B10] - C-terminal (ab51502) **Abcam Catalog #ab51502**

☒Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 **Thermo Fisher Scientific Catalog #A-21206**

☒Donkey anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 568 **Thermo Fisher Scientific Catalog #A10037**

☒Alexa Fluor® 647 AffiniPure Donkey Anti-Rat IgG (H L) **Jackson Immunoresearch Catalog #712-605-153**

☒Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H L) **Jackson Immunoresearch Catalog #703-545-155**

☒Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 568 **Thermo Fisher Scientific Catalog #A10042**

☒Alexa Fluor® 647 AffiniPure Donkey Anti-Guinea Pig IgG (H L) (min X Bov Ck Gt Sy Hms Hrs Hu Ms **Jackson Immunoresearch Catalog #706-605-148**

☒AF647 Donkey anti-mouse IgG **Thermofisher Catalog #A-31571**

☒Hoechst 33258 **Thermo Fisher Scientific Catalog #H3569**

☒Aqua-Poly/Mount **Polysciences Catalog #18606-100**

For clearing and 3D staining of assembloids only

☒Tissue-Clearing Reagent CUBIC-L [for Animals] **TCI Chemicals Catalog #T3740**

☒Tissue-Clearing Reagent CUBIC-R (M) [for Animals] **TCI Chemicals Catalog #T3741**

For viral labeling only (see Table 1 for full details)

- AAV-DJ-hSyn1::eYFP (Stanford University Neuroscience Gene Vector and Virus Core, cat. no. GVVC-AAV-16)
- AAV-DJ-hSyn1::mCherry (Stanford University Neuroscience Gene Vector and Virus Core, cat. no. GVVC-AAV-17)
- Rabies-ΔG-Cre-eGFP (Salk Institute Viral Vector Core)
  - ☒pAAV-EF1a-CVS-G-WPRE-pGHpA **addgene Catalog #67528**)
- AAV-DJ-EF1-DIO-mCherry (Stanford University Neuroscience Gene Vector and Virus Core, cat. no. GVVC-AAV-14)

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

- AAV1-Syn::ChrimsonR-tdT (Addgene, cat. no. 59171-AAV1)
- AAV-DJ-Ple94 (GPR88)-iCre (Addgene, cat. no. 49125)
- AAV-DJ-EF1a-DIO-GCaMP6s (Stanford University Neuroscience Gene Vector and Virus Core, cat. no. GVVC-AAV-91)

Table 1:

A	B	C	D	E	F
	Virus and serotype	**Gene of interest**	**Target cell type**	**Stock concentration**	**Working concentration**
AAV-DJ-Syn1::eYFP	AAV-DJ	eYFP	Neuron	4.56 × 10^12 Vg/ml	1.14 × 10^10 Vg/ml
AAV-DJ-Syn1::mCherry	AAV-DJ	mCherry	Neuron	1.50 × 10^13 Vg/ml	3.75 × 10^10 Vg/ml
AAV-DJ-EF1-DIO-mCherry	AAV-DJ	mCherry	Any	1.60 × 10^13 Vg/ml	4.00 × 10^10 Vg/ml
AAV-DJ-EF1a::G	AAV-DJ	Rabies glycoprotein	Any	3.00 × 10^12–2.08 × 10^13 Vg/ml	7.50 × 10^9-5.20 × 10^10 Vg/ml
AAV1-hSyn-ChrimsonR-tdT	AAV1	ChrimsonR-tdTomato	Neuron	2.10 × 10^13 Vg/ml	5.25 × 10^10 Vg/ml
AAV-DJ-Ple94-iCre	AAV-DJ	iCre	Neuron	1.09 × 10^12 Vg/ml	2.73 × 10^9 Vg/ml
AAV-DJ-EF1a-DIO-GCaMP6s	AAV-DJ	GCaMP6s	Any	1.60 × 10^13 Vg/ml	4.00 × 10^10 Vg/ml
ΔG-Rabies-Cre-eGFP	G-deletedrabies virus	Cre-eGFP	Any	6.52 × 10^8 TU/ml	1.63 × 10^5 TU/ml

For whole-cell patch-clamp recording only

☒LOW MELT AGAROSE **IBI**
Scientic Catalog #IB70051)

☒Sodium chloride **Sigma**
Aldrich Catalog #S7653

☒KCl **Millipore**
Sigma Catalog #P9333

☒Sodium phosphate monobasic monohydrate **Sigma**
Aldrich Catalog #S9638)

☒Magnesium sulfate heptahydrate (MgSO4) **Millipore**
Sigma Catalog #63138

☒CaCl2 solution **Sigma**
Aldrich Catalog #21115

☒Sodium Bicarbonate **Sigma**
Aldrich Catalog #S6297

☒D-( )-glucose **Sigma**
Aldrich Catalog #G7528)

☒Potassium gluconate **Sigma**
Aldrich Catalog #P1847

☒Adenosine 5'-triphosphate magnesium salt **Sigma**
Aldrich Catalog #A9187

☒Sodium GTP **Sigma**
Aldrich Catalog #G8877 (MilliporeSigma, cat. no. G8877)

☒HEPES **Sigma**
Aldrich Catalog #H3375

☒Ethylene glycol-bis(2-aminoethylether)-NNN'N'-tetraacetic acid **Sigma**
Aldrich Catalog #03777

☒Albumin from Bovine Serum (BSA) Alexa Fluor™ 488 conjugate **Thermo Fisher**
Scientific Catalog #A13100

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

BrainPhys™ Neuronal Medium **Stem Cell Technologies Catalog #05790**

BrainPhys without phenol red **Stemcell Technologies Catalog # 05791**

EQUIPMENT

AggreWell™800
Microwell Culture Plates
AggreWell **34815**

Corning® Primaria™ 100 mm x 20 mm Standard Cell Culture Dish
Cell Culture Dish
Corning® Primaria **353803**

Corning® 15 mL PP Centrifuge Tubes
Corning® 15 mL PP Centrifuge Tubes, Bulk Packed with CentriStar™ Cap, Sterile, 50/Sleeve, 500/Case
Corning® **430791**

Corning® 50 mL Centrifuge Tubes
Corning® 50 mL PP Centrifuge Tubes, Conical Bottom with CentriStar™ Cap, Bulk Packed, Sterile, 25/Pack, 500/Case
Corning® **430829**

Corning® 100 mm Ultra-Low Attachment Culture Dish
Culture Dish
Corning® **Discontinued: 3262; Suggested Replacement: 4615**

;

Corning® 60 mm Ultra-Low Attachment Culture Dish
Ultra-Low Attachment Culture Dish
Corning® **3261**

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Corning® Costar® Ultra-Low Attachment Multiple Well Plate
size 6 well, flat bottom clear Ultra-Low Attachment Multiple Well Plate

Corning® **CLS3471-24EA**

Costar® 24-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates
24-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates

Corning® **3473**

Fisherbrand™ Premium Microcentrifuge Tubes: 1.5mL
Microcentrifuge Tubes

Fisherbrand™ **05408129**

Autoclave tubes before use.

5 mL Serological Pipet, Polystyrene, 0.1 Increments, Individually Packed, Sterile, 50/Bag, 200/Case
Serological Pipet, Polystyrene

Falcon® **356543**

10 mL Serological Pipet, Polystyrene, 0.1 Increments
Serological Pipet, Polystyrene

Falcon® **356551**

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Falcon® 25 mL Serological Pipet, Polystyrene, Space Saver, 0.25 Increments, Individually Packed, Sterile, 50/Bag, 200/Case
Serological Pipet, Polystyrene
Falcon® **356525**

Falcon® 50 mL Serological Pipet, Polystyrene, 1.0 Increments, Individually Wrapped, Sterile, 25/Pack, 100/Case
50 mL Serological Pipet, Polystyrene, 1.0 Increments
Falcon® **356550**

Low Retention Aerosol Barrier Pipette Tip MBP® ART® SoftFit-L™ 1,000 µL Sterile
TIP, PIPETTE ART SOFTFIT-L HINGED RACK 1000UL
MBP® ART® SoftFit-L™ **2779-HR**

Aerosol Barrier Pipette Tip MBP® ART® SoftFit-L™ 200 µL Sterile
TIP, PIPETTE W/FILTER HINGED COVER 200UL-200L (5PK/CS)
MBP® ART® SoftFit-L™ **2769-HR**

Aerosol Barrier Pipette Tip MBP® ART® SoftFit-L™ 20 µL Sterile
TIP, PIPETTE W/FILTER HINGED COVER 200UL-20L (5PK/CS)
MBP® ART® SoftFit-L™ **2749-HR**

- Sterilized scissors

VWR® Air Jacketed CO2 Incubators
Air Jacketed CO2 Incubator
VWR® **10810-902**

- Biological safety cabinet (Labconco, cat no. 97000-862)
- Water bath

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

- Culture microscope (Olympus, cat. no. CKX41)
- EVOS FL cell imaging system (Life Technologies, model AMF4300)
- REVOLVE 4 upright, inverted, bright field, fluorescent microscope (Echo Laboratories, cat. no. FJSD2001)
- Confocal microscope (Leica, model TCS SP8)
- Stainless steel surgical blade, sterile, no. 10 (Medicon, cat. no. 01.22.10)
-

Falcon® 40 µm Cell Strainer, Blue, Sterile, Individually Packaged, 50/Case
Cell Strainer
Falcon® **352340**

-

Corning® non-treated culture dishes
culture dishes
Corning® **CLS430589-500EA**

- Hemocytometer

Nylon Syringe Filter, 0.22µm, 30mm, Bulk Packed, Non-sterile
Syringe Filter
Nylon **229775**

- Centrifuge 5810 R (Eppendorf, cat. no. 022628102)

For viral labeling and assembloid formation only
- Sterile 1.5-ml microcentrifuge
- Scissors and/or razor blades

For cryosection and immunostaining only

VWR® Superfrost® Plus Micro Slide, Premium
Superfrost slides
VWR® Superfrost® **48311-703**

Fisherbrand™ Disposable Base Molds
Disposable Base Molds
Fisherbrand™ **22363553**

- Humidified chamber

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Elite PAP Pen
PAP Pen
Elite **K039**

● Cover glasses, rectangles (Fisherbrand, cat. no. 22-266882)
●

Leica CM1860 - Routine Histopathology Applications
Cryostats
Leica **CM1860**

Red Sable Brushes
Sable Brushes
Sable **11812**

For optogenetics experiments only

M625F2 - 625 nm, 13.2 mW (Min) Fiber-Coupled LED, 1000 mA, SMA
Fiber-Coupled LED
M625F2 **M625F2**

● CYCLOPS LED driver 3.6 (Open Ephys)

For whole-cell patch-clamp recording only

Leica VT1200 - Semi-Automatic Vibrating Blade Microtome
Semi-Automatic Vibrating Blade Microtome
Leica VT1200 **VT1200**

● Microwave
● Upright SliceScope microscope (Scientifica)
● MultiClamp 700B amplifier (Molecular Devices)
● Digidata 1550B digitizer (Molecular Devices)

P-1000 Next Generation Micropipette Puller
Micropipette Puller
P-1000 **P-1000**

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

● Thick/standard wall borosilicate glass with filament (Sutter Instrument, cat. no. BF150-86-10)

pE-4000
universal Illumination System for fluorescence microscopy
CoolLED pE-4000

● Infinity2 CCD camera (Teledyne Lumenera)

SOFTWARE
● Fiji (ImageJ, version 2.1.0, National Institutes of Health)
● MATLAB (version R2018a, 9.4.0, MathWorks)
● pCLAMP software (version 10.6, Molecular Devices)
● Mini Analysis software (version 6.0.3, Synaptosoft)
● Infinity Capture software (Teledyne Lumenera) compatible with the CCD camera

REAGENT SETUP
Growth factors and chemical stock solutions
BiologyResuspend **25 mg** of dorsomorphin in **12.52 mL** of sterile DMSO to obtain a **5 millimolar (mM)** stock solution and use at a final concentration of **2.5 micromolar (µM)** (1:2,000). Resuspend **10 mg** of SB-431542 in **2.60 mL** of 100% ethanol to obtain a **10 millimolar (mM)** stock solution and use at a final concentration of 10 µM (1:1,000). Resuspend EGF, FGF2, BDNF and NT-3 growth factors in sterile 0.1% (wt/vol) BSA/PBS to 20 µg/ml stock concentration and use at **20 ng/ml** (1:1,000). Resuspend **10 mg** of IWP-2 in **8.58 mL** of sterile DMSO to obtain a **2.5 millimolar (mM)** stock solution; use at a final concentration of **2.5 micromolar (µM)** (1:1,000). Resuspend **100 µg** of activin A in **2 mL** of cell culture-grade water to obtain a **50 µg/ml** stock solution; use at a final concentration of **50 ng/ml** (1:1,000). Resuspend **10 mg** of SR11237 in **5.26 mL** of sterile DMSO to obtain a **5 millimolar (mM)** stock solution and further dilute the stock solution to obtain a **5 micromolar (µM)** solution and use at a final concentration of **5 nanomolar (nM)** (1:1,000). Resuspend **322 mg** of AA in **5 mL** of cell culture-grade water to obtain a **200 millimolar (mM)** stock solution; use at a final concentration of **200 micromolar (µM)** (1:1,000). Resuspend **250 mg** of dibutyryl cAMP sodium salt in **5.088 mL** of cell culture–grade water to obtain a **100 millimolar (mM)** stock solution and use at a final concentration of **50 micromolar (µM)** (1:2,000). Resuspend **5 mg** of DAPT in **2.31 mL** of sterile DMSO to obtain a **5 millimolar (mM)** stock solution and use at a final concentration of **2.5 Milimolar (mM)** (1:2,000). Aliquots of stock solutions are stored at either **-20 °C** or **-80 °C** for 2 months. Once thawed, stocks can be kept at **4 °C** for ≤ **336:02:00** .

Essential 8 medium (for use in Steps 1 –30). Prepare the medium as shown in the table below. 314 This medium can be stored for 2 weeks at 4 °C.

A	B	C
Composition	**Volume (500 ml total) (ml)**	**Final concentration**
Essential 8 medium	490	–
Essential 8 supplement	10	2% (vol/vol)

Essential 6 medium (for use in Steps 31–38). Prepare the medium as shown in the table below. This medium can be stored for 2 weeks at **4 °C** .

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

A	B	C	D
Composition	**Volume (500 ml total) (ml)**	**Final concentration**	**Comments**
Essential 6 medium	495	–	
Penicillin-streptomycin	5	1% (vol/vol)	Optional
Dorsomorphin	–	2.5 µM (1:2,000)	Add just before use
SB-431542	–	10 µM (1:1,000)	Add just before use
XAV-939 (optional)	–	1.25 µM (1:2,000)	Add just before use

Neural differentiation medium (NM) for hCS (for use in Steps 39 and 40, on days 6 and onward of differentiation). Prepare the medium as detailed in the table below. Store stock for ≤ **168:02:00** at **4 °C** and add growth factors just before use if required for the culture.

A	B	C	D
Composition	**Volume (500 ml total) (ml)**	**Final concentration**	**Comments**
Neurobasal A	480		–
B-27 supplement	10	2% (vol/vol)	Needed from day 6 to day 46
B-27 Plus supplement	10	2% (vol/vol)	Needed from day 46 onward
GlutaMax	5	1% (vol/vol)	–
Penicillin-streptomycin	5	1% (vol/vol)	–
EGF (20-µg/ml 411 Q25 stock)	–	20 ng/ml (1:1,000)	Needed from day 6 to day 22. Add just before use
FGF2 (20-µg/ml stock)	–	20 ng/ml (1:1,000)	Needed from day 6 to day 22. Add just before use
BDNF (20-µg/ml stock)	–	20 ng/ml (1:1,000)	Needed from day 22 to day 46. Add just before use
NT-3 (20-µg/ml stock)	–	20 ng/ml (1:1,000)	Needed from day 22 to day 46. Add just before use
AA (200 mM stock)	–	200 µM (1:1,000)	Needed from day 22 to day 46. Add just before use
Dibutyryl cAMP sodium salt (100 mM stock)	–	50 µM (1:2,000)	Needed from day 22 to day 46. Add just before use
DHA (10 mM stock)	–	10 µM (1:1,000)	Needed from day 22 to day 46. Add just before use

NM for hStrSs (for use in Steps 39 and40, on days 6 and onward of differentiation). Prepare the medium as detailed in the table below. Store the stock for ≤1 week at 4 °C and add growth factors just before use if required for the culture.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

A	B	C	D
Composition	**Volume (~500 ml total) (ml)**	**Final concentration**	**Comments**
Neurobasal A	480	–	–
B-27 supplement	10	2% (vol/vol)	Needed from day 6 to day 46
B-27 Plus supplement	10	2% (vol/vol)	Needed from day 46 onward
GlutaMax	5	1% (vol/vol)	–
Penicillin-streptomycin	5	1% (vol/vol)	Needed from day 6 to day 22. Add just before use
IWP-2 (2.5 mM stock)	–	2.5 µM (1:1,000)	Needed from day 6 to day 22. Add just before use
Activin A (50-µg/ml stock)	–	50 ng/ml (1:1,000)	Needed from day 6 to day 22. Add just before use
SR11237 (100 µM stock)	–	100 nM (1:1,000)	Needed from day 22 to day 46. Add just before use
BDNF (20-µg/ml stock)	–	20 ng/ml (1:1,000)	Needed from day 22 to day 46. Add just before use
NT-3 (20-µg/ml stock)	–	20 ng/ml (1:1,000)	Needed from day 22 to day 46. Add just before use
AA (200 mM stock)	–	200 µM (1:1,000)	Needed from day 22 to day 46. Add just before use
Dibutyryl cAMP sodium salt (100 mM stock)	–	50 µM (1:2,000)	Needed from day 22 to day 46. Add just before use
DHA (10 mM stock)	–	10 µM (1:1,000)	Needed from day 22 to day 46. Add just before use
DAPT (5 mM stock)	–	2.5 µM (1:2,000)	Needed from days 42 to 46. Add just before use

PFA

Prepare 4% (vol/vol) PFA by mixing **10 mL** of 16% PFA with **30 mL** of DPBS and store at **4 °C** for ≤1 week.

Embedding solution

Prepare a 30% (wt/vol) sucrose solution by mixing **15 g** of sucrose with PBS to a final volume of **50 mL** . Store the 30% (wt/vol) sucrose/PBS solution at **4 °C** for ≤2 weeks. Add **25 mL** of OCT compound to **25 mL** of 30% (wt/vol) sucrose/PBS to make a 1:1 embedding solution. Shake the solution at **Room temperature** until the OCT compound and sucrose are completely mixed. Leave the mixed solution overnight at **4 °C** to remove bubbles formed from the mixing process. This solution can be stored at **4 °C** for ≤2 weeks.

Blocking solution for immunostaining

Prepare the blocking solution for immunostaining by mixing 10% (vol/vol) normal donkey serum with 0.1% (wt/vol) BSA and 0.3% (vol/vol) Triton-X100 in PBS. Keep this solution at **4 °C** for ≤1 week.

Antibody solution for immunostaining

Prepare the antibody solution for immunostaining by mixing 2% (vol/vol) normal donkey serum with 0.1% (vol/vol) Triton-X100 in PBS. Keep this solution at **4 °C** for ≤1 week.

Artificial cerebrospinal fluid (aCSF)

Prepare concentrated (10×) aCSF stock solution containing **1.26 Molarity (M)** NaCl, **25 millimolar (mM)** KCl, **12.5 millimolar (mM)** NaH2PO4, **260 millimolar (mM)** NaHCO3 and **100 millimolar (mM)** D-(+)-glucose. Keep this solution at **4 °C** for ≤1 month. Dilute this solution and add MgSO4 and CaCl2 to prepare aCSF working solution containing **126 millimolar (mM)** NaCl, **2.5 millimolar (mM)** KCl, **1.25 millimolar (mM)** NaH2PO4, **1 millimolar (mM)** MgSO4, **2 millimolar (mM)** CaCl2, **26 millimolar (mM)** NaHCO3 and **10 millimolar (mM)** D-(+)-glucose before use.

Internal solution for whole-cell recording

Prepare internal solution containing **127 millimolar (mM)** K-gluconate, **8 millimolar (mM)** NaCl,

[M]**4 millimolar (mM)** MgATP, [M]**0.3 millimolar (mM)** Na2GTP, [M]**10 millimolar (mM)** HEPES and [M]**0.6 millimolar (mM)** EGTA, pH 7.2, adjusted with KOH (290 mOsm). Keep this solution at **-20 °C** for ≤1 year.

:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

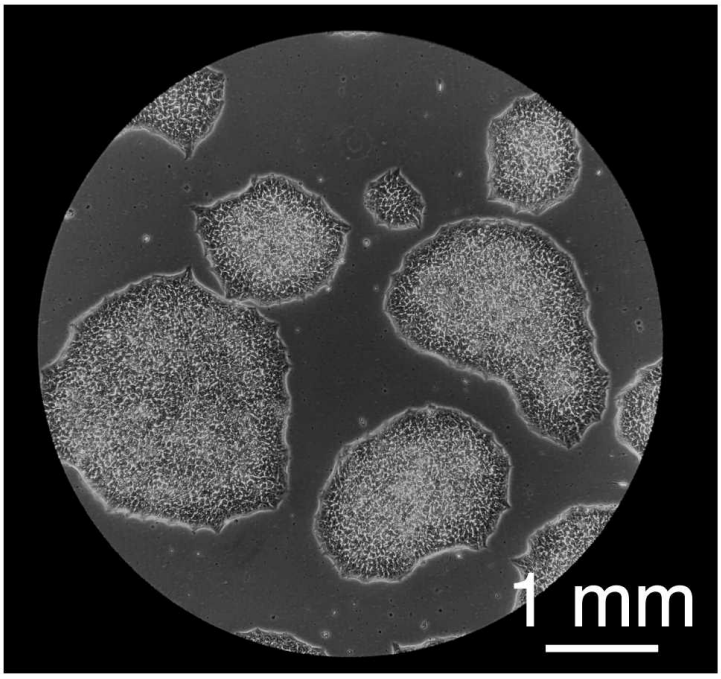
The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

CRITICAL: Before starting hPS cells and the long-term organoid/assembloid cultures, carefully cleaning the incubator is important to avoid potential contamination. Because most culture plates will be in the incubator for several hundred days, the surface of the incubator and culture dishes should be regularly cleaned. The amount of water in the incubator should be monitored frequently to maintain the required humidity. All cell culture-related procedures should be performed in a sterile environment with biological safety cabinets and sterilized materials. The biological safety cabinets should be regularly certified.

Maintenance of hPS cells in feeder-free conditions Timing 4–6 d

1 Culture hPS cells on human recombinant vitronectin with Essential 8 medium. When hPS cell cultures reach ~80% confluency, passage hPS cultures by using [M]**0.5 millimolar (mM)** EDTA in PBS.

CRITICAL: To ensure consistency and obtain high-quality differentiation, the hPS cell culture should not grow denser than 90%. Ideally, passages can be performed at 80% confluency



Representative image of hiPS cell culture.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

2

Regularly check hPS cell cultures for mycoplasma (e.g., PCR) and chromosomal abnormalities (e.g., SNP or comparative genomic hybridization arrays).

Preparing vitronectin-coated dishes 1h 30m

3 Prepare the vitronectin-coated plates at **Room temperature** and pre-warm the required volume of Essential 8 medium at **Room temperature** .

CRITICAL: According to the manufacturer's recommendation, Essential 8 medium should not be warmed in a **37 °C** water bath.

4 1h

To coat a well of a six-well plate, dilute **60 µL** of vitronectin in **6 mL** (1:100) of DPBS (without calcium and magnesium), add **1 mL** of diluted vitronectin solution to the well and incubate at **Room temperature** for **01:00:00** .

Vitronectin can be aspirated just before transferring the cell suspension to the well.

Passaging hPS cells 30m

5

Aspirate culture medium from the hPS cells plate and rinse with **2 mL** of DPBS (without calcium and magnesium).

6 7m

Add **1 mL** of **0.5 millimolar (mM)** EDTA in DPBS to the well of a six-well plate and incubate at **Room temperature** for **00:07:00** .

Cells are ready to be removed when colonies start to separate.

7

Aspirate the EDTA solution and add **1 mL** of pre-warmed complete Essential 8 medium per well of a six-well plate.

8

Remove cells from the well by gently squirting medium and pipetting the colonies with either a **5 mL** serological pipette or P1000 tips with a pipette.

CRITICAL: Do not pipette too many times: three to five times should be enough. Difficulty with detaching cells

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

suggests differentiation of hPS cells.

9 Aspirate the residual vitronectin solution from the pre-coated plate.

It is not necessary to wash culture plates after the removal of vitronectin.

10 

Add **2 mL** of pre-warmed Essential 8 medium to each well of a coated six-well plate. Mix the cell suspensions by pipetting and transfer the appropriate volume of cell suspension into each well containing pre-warmed complete Essential 8 medium according to the desired split ratio (1:6 to 1:20 ratio, depending on the confluency and growth rate of individual hPS cell lines).

11 Move the plate in several quick, figure-of-eight-like movements to disperse cells.

12 

Gently place the plate in a **37 °C** , 5% CO_2 incubator.

13 Perform a medium change after every passage (every **18:00:00** to **24:00:00**), except for the day after passage, when confluency of cells is low. 1d 18h

Preparation of hPS cells for 3D neural differentiation 3d

14 Passage hPS cells from a six-well plate to 10-cm plates and grow to ~80–85% confluency before the day of aggregation.

15  3d

48:00:00 and **24:00:00** before the aggregation, pre-treat cells with 1% DMSO (**100 µL** per **10 mL** of Essential 8 medium for a 10-cm dish) to improve differentiation of hPS cell lines that are more challenging to differentiate.

Generation of 3D spheroids by using an AggreWell plate 3d

16  10m

Add **500 µL** of anti-adherence rinsing solution in a well of a new AggreWell plate and incubate the plate for **00:05:00** at **Room temperature** If you notice (under the microscope) bubbles in any of the wells of the plate, centrifuge the AggreWell plate at **1300 x g, 00:05:00** in a swinging bucket rotor. Aspirate the rinsing solution and wash with **1 mL** of PBS.

17 Pre-warm Essential 8, accutase and DMEM/F12 medium at **Room temperature** . Supplement Essential 8 medium with ROCK inhibitor (Y-27631; 1:1,000) to a final concentration of **10 micromolar (µM)** .

18  5m

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Add **0.5 mL** of Essential 8 medium supplemented with Y-27632 to each well of an Aggrewell plate. Before starting aggregation, an Aggrewell plate can be centrifuged at **2000 x g, 00:05:00** in a swinging bucket rotor that is fitted with a plate holder to remove any air bubbles in the microwells.

CRITICAL: Remaining air bubbles in the Aggrewell plate interrupt the aggregation of hPS cells.

19 Set the plate aside in an incubator while preparing a single-cell suspension of hPS cells for aggregation.

20 

Aspirate the maintenance medium from the hPS cell culture plates and rinse cells twice with DPBS (without calcium and magnesium).

21  7m

Add **4 mL** of accutase per 10-cm plate and incubate at **37 °C** for **00:07:00** in a 5% CO_2 incubator until cells detach by gentle shaking from the plate.

22 

Gently pipette the cell suspension two to three times with a serological pipette to ensure that any remaining clumps are fully dissociated.

23 

Transfer the cells to a **50 mL** conical tube.

CRITICAL: The use of **50 mL** conical tubes for cell culture (instead of **15 mL** conical tubes) is highly recommended to avoid the risk of contamination by accidentally attaching the side of the pipette to a wall of the conical tube when pipetting.

24 

Add **6 mL** of DMEM/F12 to rinse any remaining cells off the plate, transfer to a conical tube, and mix the well with the cell suspension in a total volume of **10 mL** .

25  4m

Count cells and centrifuge the conical tube containing the cell suspension at **200 x g, 00:04:00** .

26 

Resuspend the cells in Essential 8 medium supplemented with Y-27632 by adjusting the concentration of the single-cell suspension to 2.5–3 million in **1 mL** , and transfer the **1 mL** of cell suspension into a well of an AggreWell plate to achieve a final volume of **1.5 mL** per well

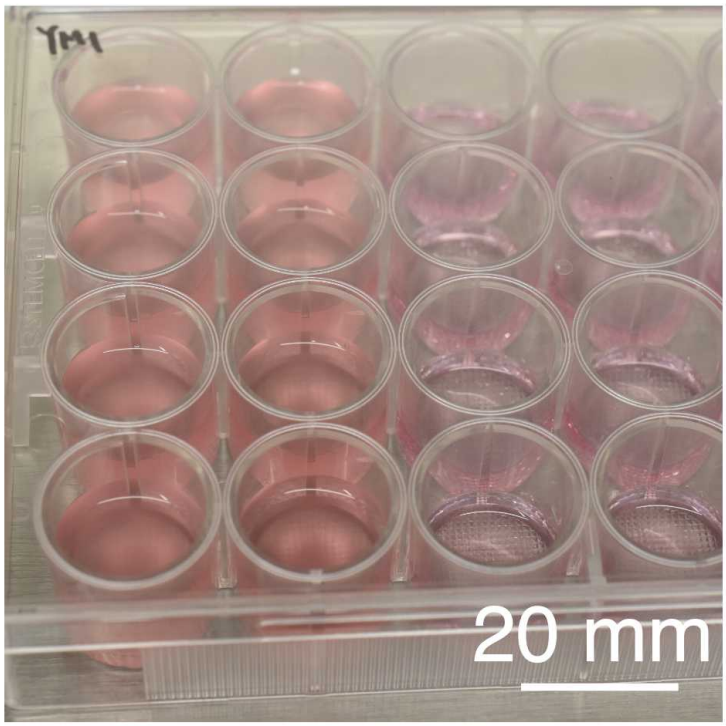


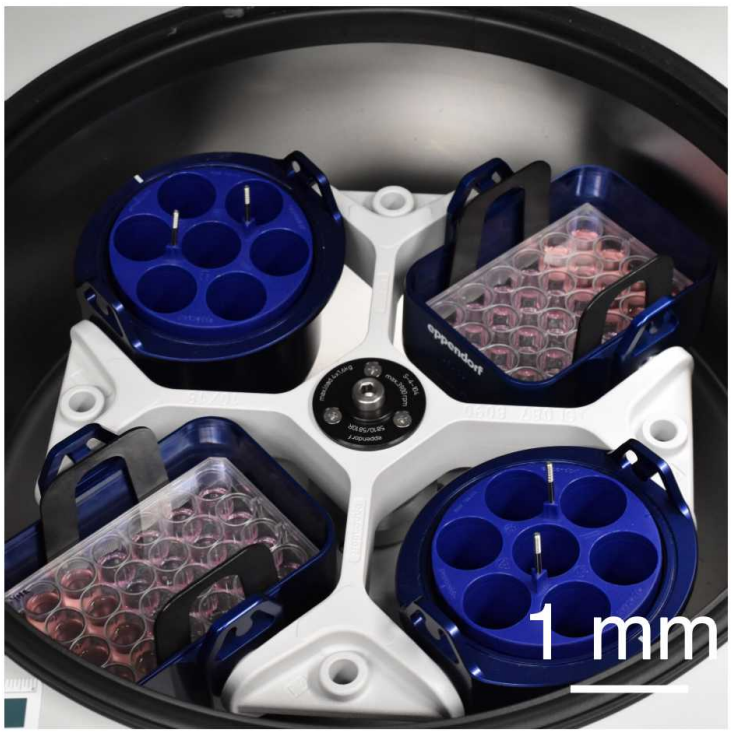
Image of detached cells in an Aggrewell plate during the aggregation process.

27 Pipette gently several times to re-establish an even distribution of cells throughout the well.

28 3m

Centrifuge the AggreWell plate at **100 x g, 00:03:00** to distribute the cells in the micro-wells.

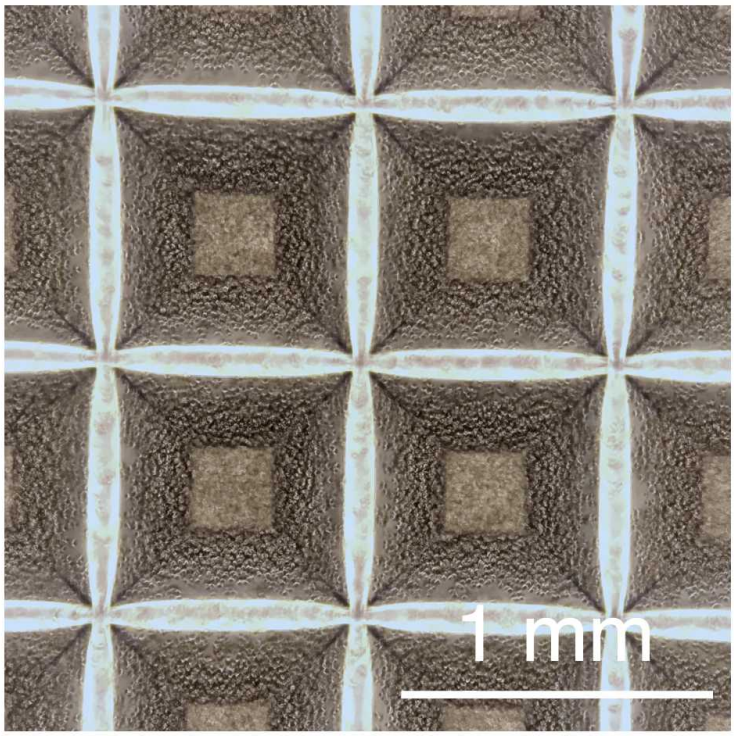
Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



Centrifugation of an Aggrewell plate.

29

Check under a microscope to verify that cells are evenly distributed among the microwells.



hiPS cells in the Aggrewell plate after centrifugation.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

CRITICAL: Overfilled individual wells of the AggreWell plate after centrifugation will facilitate aggregation across wells and prevent the formation of individual spheroids.

30 1d

Incubate the cells at **37 °C** , 5% CO_2 for **24:00:00** .

TROUBLESHOOTING

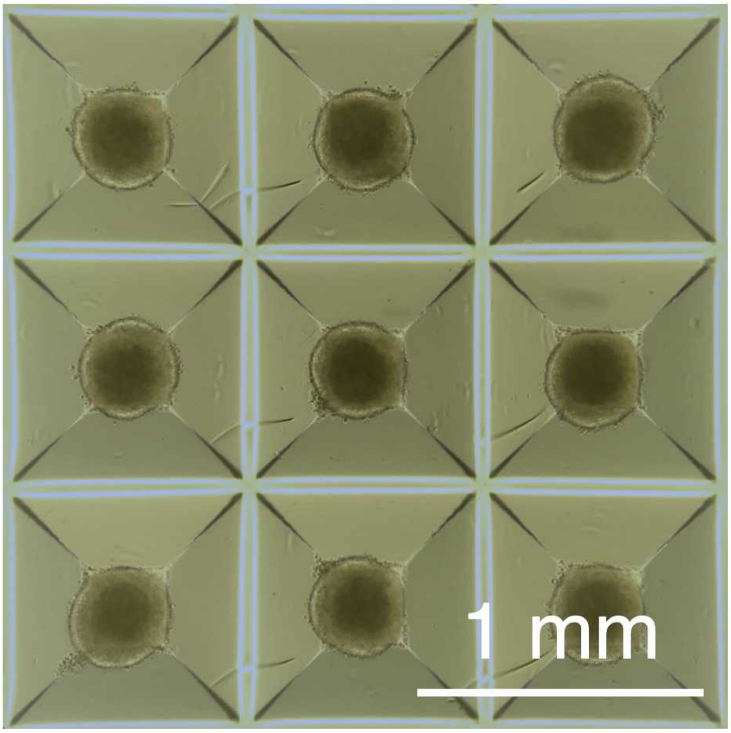
Dislodging 3D spheroids from the AggreWell plate ● Timing 1 h 1w 0d 1h 30m

31 1h 30m

Pre-warm DMEM/F12 and Essential 6 medium at **Room temperature** for **00:30:00** to **01:00:00** before dislodging.

32

Collect the spheroids from the microwells by gently pipetting the medium in the well up and down two to three times with a micropipetter with a **1000 µL** tip that was cut with scissors to allow a wider opening.



Aggregated hiPS cells days after aggregation in the Aggrewell plate.

33

Place a **40 µm** cell strainer on top of a **50 mL** conical tube and pass the suspension of spheroids through the strainer. The aggregates will remain on the filter of the strainer.

34

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Pipette 1 mL of DMEM/F-12 medium across the entire surface of the well to dislodge any remaining spheroids. Collect washes and pass over the strainer used in the previous step.

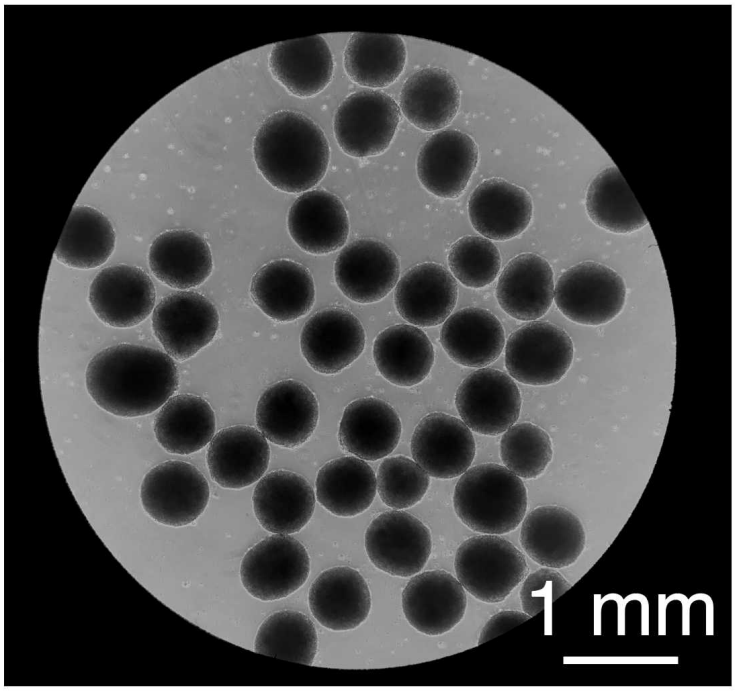
35

Invert the strainer and place it over a new 50 mL conical tube. Collect the spheroids by adding 12 mL of Essential 6 medium on top of the inverted strainer. Observe the AggreWell plate under the microscope to ensure that all aggregates have been removed from the wells. Repeat the wash if necessary.

CRITICAL: Difficulty with detaching cells from the Aggrewell plate suggests differentiation of hPS cells.

36 Place spheroids in ultra-low-attachment 10-cm plates in Essential 6 medium supplemented with dorsomorphine ([M]5 micromolar (µM)), SB-431542 ([M]10 micromolar (µM)).

If forebrain induction (e.g., low FOXG1 expression) is challenging with spheroids derived from a specific hPS cell line, we recommend testing whether addition of XAV-939 ([M]1.25 micromolar (µM)) from days 24:00:00 to 144:00:00 of differentiation may facilitate this process.



Representative images of spheroids.

Neural induction 1w 3d

37 1w 3d

Add dorsomorphine and SB-431542 to Essential 6 medium for the first 120:00:00 . XAV-939 ([M]1.25 micromolar (µM)) can also be added for the first 120:00:00 of differentiation if forebrain differentiation is not highly efficient.

38

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Perform a medium change daily (but not on day 1) at approximately the same time of the day by gently transferring the neural spheroids to a **50 mL** Falcon tube (if they are too small) and aspirating the medium once they sit at the bottom of the tube. When spheroids are large, perform the medium change by gently tilting the plate (until spheroids settle at the bottom) and removing the remaining medium. This can minimize the transferring time of the spheroids in and out of the plate at this critical differentiation period.

CRITICAL: The time that spheroids are kept outside of the incubator should be minimized at all stages of differentiation. After they reach 4 mm in diameter, keep no more than 30 spheroids per 10-cm dish.
TROUBLESHOOTING

Patterning and differentiation

39 From day 6, replace the medium daily with NM with supplements listed in option A for hCSs and option B for hStrSs.
TROUBLESHOOTING

40 **(A) Generation of hCSs**

40.1 From day 6, replace the medium daily with NM supplemented with **20 ng/ml** FGF2 and **20 ng/ml** EGF for generating hCSs. Change the medium daily from day 6 to day 15.

40.2 From day 16 onward, change the medium every other day until day 22. Continue to use NM supplemented with **20 ng/ml** FGF2 and **20 ng/ml** Q29 EGF.

40.3 From day 22 to day 46, replace FGF-2 and EGF with **20 ng/ml** BDNF, **20 ng/ml** NT-3, **200 micromolar (µM)** AA, **50 micromolar (µM)** dibutyryl cAMP sodium salt and **10 micromolar (µM)** DHA. Perform medium changes with **12 mL** to **15 mL** of supplemented NM per 10-cm plate every 2 d.

41 **(B) Generation of hStrSs**

41.1 From day 6, replace the medium daily with NM supplemented with **50 ng/ml** activin A and 704 **2.5 micromolar (µM)** IWP-2. Change the medium daily from day 6 to day 11.

41.2 From day 12 to day 15, supplement NM with **50 ng/ml** activin A, **2.5 µm** IWP-2 and **100 nanomolar (nM)** SR11237. Perform medium changes with **12 mL** to **15 mL** of NM per 10-cm plate every day.

41.3 From day 16 onward, change the medium every other day until day 22. Continue to use NM supplemented with **50 ng/ml** activin A, **2.5 µm** IWP-2 and **100 nanomolar (nM)** SR11237. Perform medium changes with **12 mL** to **15 mL** of NM per 10-cm plate every 2 d.

41.4 From day day 22 to day 41, supplement NM with **20 ng/ml** BDNF, **20 ng/ml** NT-3, **200 micromolar (µM)** AA, **50 micromolar (µM)** dibutyryl cAMP sodium salt and **10 micromolar (µM)** DHA. Perform medium changes with **12 mL** to **15 mL** of NM per 10-cm plate every 2 d.

41.5 From day 42 to day 46, supplement NM with **20 ng/ml** BDNF, **20 ng/ml** NT-3,

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

200 micromolar (µM) AA, **50 micromolar (µM)** dibutyryl cAMP sodium salt, **10 micromolar (µM)** DHA and **2.5 micromolar (µM)** DAPT. Perform medium changes with 12 mL to15 mL of NM per 10-cm plate every 2 d.

Long-term culture and phenotyping ● Timing indefinite 18h 40m

42 After day 46, perform medium changes with 12–15 ml of NM without growth factors every 4–5 d.

Examples of additional experiments that can be carried out by using these spheroids include viral labeling of spheroids (option A), generation of cortico-striatal assembloids (option B), 3D clearing with CUBIC (option C), axon projection imaging (option D), retrograde labeling with ΔG rabies virus (option E), optogenetics coupled with calcium imaging (option F) and electrophysiological recording (option G)

43 **(A) Viral labeling of neural spheroids ● Timing 3 d**

43.1 For labeling neural spheroids, dilute **0.5 µL** of virus solution (recommended virus particle volumes are listed in Table 1) in **200 µL** of NM and add mixed NM to a **1.5 mL** Eppendorf tube. Then, transfer one to three spheroids in the tube containing NM and viruses. The tube can be placed overnight in an incubator at **37 °C** , 5% CO_2.

We note that transduction efficiency depends on the target cell types, the promoter, stage of differentiation and brain region. **CRITICAL** The optimal serotype and titer of each virus depends on the gene construct of interest, as well as the target cell type. AAV-1, AAV-8, and AAV-DJ serotypes have been more extensively tested in hCSs and hStrSs. Virus at a titer higher than 1.0 × 10^12–1.0 × 10^13 genome copies/ml was obtained from the Stanford Neurosciences Institute Gene Vector and Virus Core or from Addgene and stored at **-80 °C** . ΔG-rabies virus at a titer higher than 1.0 × 100.8 transduction units/ml was obtained from the Salk Institute, diluted to 1:10 by using NM and stored at **-80 °C** .

43.2 1d

The next day, add an extra **800 µL** of fresh NM to the tube and incubate for **24:00:00** .

43.3

On day 3, wash the spheroids with **1 mL** of fresh NM (two to three times) and then transfer the spheroids back to their culture plate.

Expression of the gene of interest is usually observed 7–14 d after AAV infection and 3–5 d after ΔG-rabies virus infection. ? **TROUBLESHOOTING**

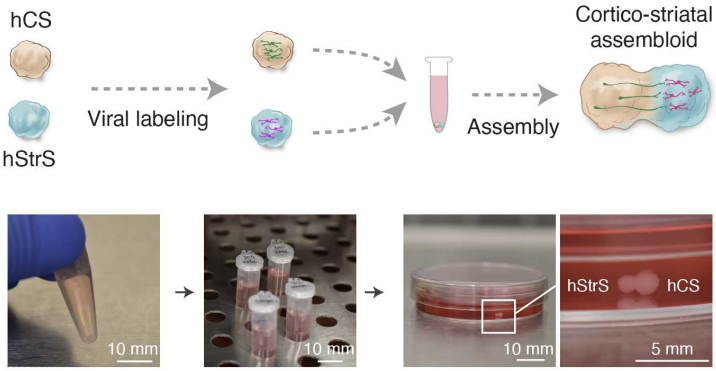
44 **(B) Generating cortico-striatal assembloids ● Timing 3–4 d**

44.1

To generate cortico-striatal assembloids, transfer one hCS and one hStrS into a **1.5 mL**

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

Eppendorf tube containing **1 mL** of NM.



Scheme illustrating viral labeling and assembly of an hCS and an hStrS, and images showing the process of assembly.

CRITICAL The size of the spheroids for assembly should be <3 mm. Once in the tube, each spheroid should be placed horizontally next to each other at the bottom of the tube (but not stacked vertically) (Fig. 2m).

44.2

Incubate the spheroids at **37 °C** for 3–4 d, completely replacing the medium on day 2.

CRITICAL The medium change should be performed gently to avoid breaking the fusion.

44.3

After spheroids are assembled, transfer them into an ultra-low-attachment plate by using a P1000 pipette with the tip cut for a larger bore opening. Assembly of hCS and hStrS between days 46 and 80 is more efficient.

Assembly is usually complete after 3–4 d. ? TROUBLESHOOTING

45 (C) Cryosection and immunostaining ● Timing 6–7 d

45.1 1d

When ready to perform immunocytochemistry, transfer spheroids to a **1.5 mL** microcentrifuge tube by using a cut P1000 tip. Gently remove the medium and add **500 µL** to **1 mL** of cold 4% PFA/PBS (or warm 4% PFA/4% sucrose/PBS). Leave spheroids at **4 °C** **Overnight** .

45.2

After fixation, gently remove PFA and wash with PBS at **Room temperature** .

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

45.3

Add **1 mL** of 30% (wt/vol) sucrose/PBS for cryopreservation. Leave spheroids or assembloids at **4 °C** until they sink to the bottom of tube (≥24–48 h).

45.4 Prepare square disposable molds that fit with the sample size and number and fill the molds with embedding solution (1:1, OCT/30% sucrose; see Reagent setup).

CRITICAL To avoid creating bubbles while filling the mold, use a P1000 pipette in which the tip has been slightly cut to widen the opening.

45.5

Transfer spheroids from tubes into the mold by using a P1000 cut pipette tip. Place 4–10 spheroids in the mold. If spheroids move while embedding, use a P20 tip to gently place them in the embedding solution.

CRITICAL Avoid carrying sucrose when transferring spheroids.

45.6 Place the spheroids in a cryomold **On ice** for **00:20:00** and allow the spheroids to sink to the bottom of the mold. 20m

45.7

Snap-freeze the spheroids by placing the mold directly on dry ice. Once completely frozen, store molds at **-80 °C** .

Frozen molds can be stored indefinitely at **-80 °C** until ready to cryo-section.

45.8 When ready for sectioning, transfer the mold from **-80 °C** to the cryostat chamber at **-20 °C** for ~ **00:30:00** before sectioning. 30m

45.9 Remove the block from the mold, paste the specimen on the stage by using OCT and section by using standard techniques. Use a brush to prevent crumbling of the sections. Spheroids and assembloids can be cryo-sectioned at **10 µm** – **30 µm** thickness, depending on the purpose of the experiments.

45.10 Collect sections on Superfrost Plus slides. Slides can be stored at **-20 °C** to **-80 °C** until ready to immunostain.

45.11 For immunostaining, remove the slide from the freezer and leave at **Room temperature** to thaw for **00:05:00** – **00:10:00** 15m

45.12

Wash the slide three times with PBS to remove excess OCT/sucrose on the slides.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

45.13 Remove PBS and block for **01:00:00** at **Room temperature** with 10% normal donkey serum (NDS), 0.3% Triton- X and 0.1% BSA in PBS. 1h

Parafilm can be placed on top of the slide glass to avoid drying of the antibody solution. Alternatively, a hydrophobic PAP pen can be used to draw circles around the sections, and staining can be performed inside these circles.

45.14 Perform the immunostaining steps in a humidified chamber to avoid drying of sections.

45.15  1h

Add the primary antibodies diluted in antibody solution containing 2% NDS and 0.1% Triton-X in PBS and keep at **4 °C** **Overnight** .

45.16  5m

The next day, perform three **00:05:00** PBS washes at **Room temperature** (1/3).

45.17  5m

The next day, perform three **00:05:00** PBS washes at **Room temperature** (2/3).

45.18  5m

The next day, perform three **00:05:00** PBS washes at **Room temperature** (3/3).

45.19  1h

Add secondary antibodies diluted in antibody solution for **01:00:00** at **Room temperature** .

Parafilm can be placed on top of the slide glass to avoid drying.

45.20  5m

Perform three **00:05:00** washes at **Room temperature** with PBS (1/3).

45.21  5m

Perform three **00:05:00** washes at **Room temperature** with PBS (2/3).

45.22  5m

Perform three **00:05:00** washes at **Room temperature** with PBS (3/3).

45.23  12m

Add Hoechst in PBS (1:10,000 dilution) for **00:05:00** – **00:07:00** at **Room temperature** .

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

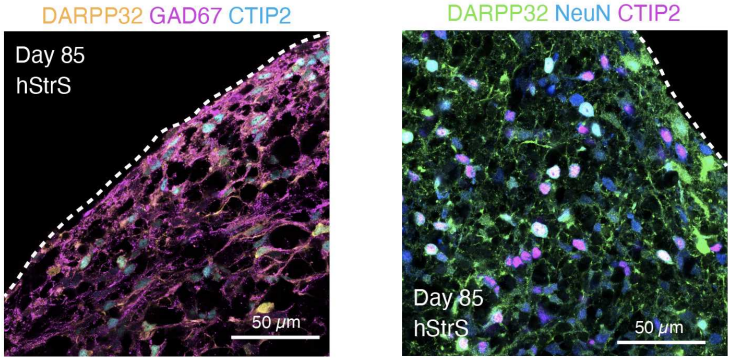
45.24

Aspirate the Hoechst solution and wash twice with PBS.

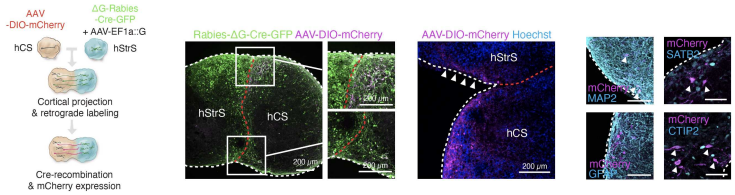
45.25 Aspirate PBS and mount the sections by adding one drop of Aquamount solution on top of sections and by placing a coverglass on top.

45.26

Use a fluorescent microscope to image.



Left: Image showing immunostaining for DARPP32 (yellow), GAD67 (magenta) and CTIP2 (cyan) in an hStrS at day 85. Right: Image showing immunostaining for DARPP32 (green), NeuN (cyan) and CTIP2 (magenta) in an hStrS at day 85.



Scheme illustrating retrograde viral labeling of cortical projecting neurons in cortico-striatal assembloids, and representative retrograde viral images of Daf21 cortico-striatal assembloids.

CRITICAL Ideally, cryosections should be sampled from several layers of the spheroids or assembloids

46 (D) CUBIC clearing and imaging ● Timing 4–7 d

46.1 40m

Transfer spheroids to a **1.5 mL** microcentrifuge tube by using a cut P1000 tip. Gently remove the medium and add **500 µL** to **1 mL** of cold 4% PFA/PBS (or add to **1 mL** of warm 4% PFA/4% sucrose/PBS and leave at **Room temperature** for **00:20:00**). Leave spheroids at **4 °C** **Overnight** .

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

46.2

After fixation, wash samples with PBS twice at **Room temperature** .

46.3 2d

Add **500 µL** to **1 mL** of CUBIC-L for de-lipidation and incubate at **37 °C** for **48:00:00** .

46.4 2h

Wash with PBS three times at **Room temperature** for **02:00:00** (1/3).

46.5 2h

Wash with PBS three times at **Room temperature** for **02:00:00** (2/3).

46.6 2h

Wash with PBS three times at **Room temperature** for **02:00:00** (3/3).

46.7 2h

Add a combination of primary antibodies diluted in solution containing 0.2% Triton-X100, 3% NDS in PBS and incubate assembloids at **37 °C** for **02:00:00** .

46.8 2h

Wash with PBS three times at **37 °C** for **02:00:00** (1/3).

46.9 2h

Wash with PBS three times at **37 °C** for **02:00:00** (2/3).

46.10 2h

Wash with PBS three times at **37 °C** for **02:00:00** (3/3).

46.11 2h

Add secondary antibodies diluted in solution containing 0.2% Triton-X100 and 3% NDS in PBS and incubate assembloids at **37 °C** for **02:00:00** .

46.12 2h

Wash with PBS three times at **Room temperature** for **02:00:00** (1/3).

46.13 2h

Wash with PBS three times at **Room temperature** for **02:00:00** (2/3).

2h

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1

46.14

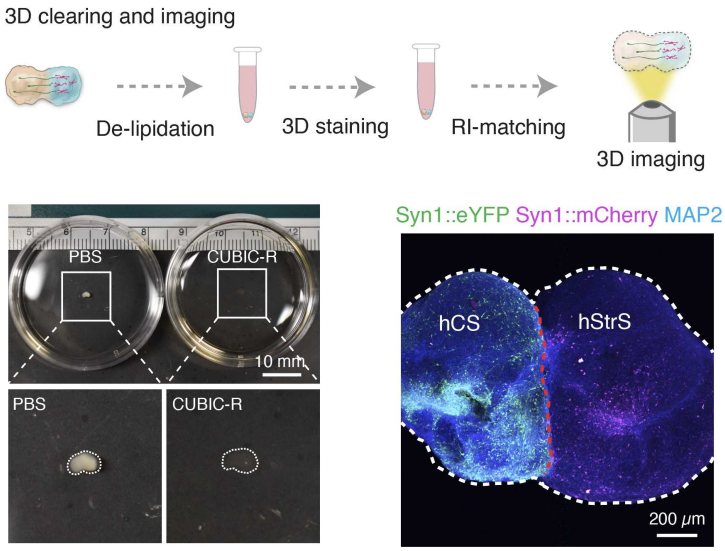
Wash with PBS three times at **Room temperature** for **02:00:00** (3/3).

46.15 2h

For refractive index–matching, remove PBS from the **1.5 mL** tubes, add **1 mL** of CUBIC-R in the **1.5 mL** tube and leave for **02:00:00** at **Room temperature** .

46.16

Once assembloids become transparent, transfer them to a well of a 96-well imaging- compatible plate filled with **150 µL** of CUBIR-R+ solution and image them with a confocal microscope



Scheme illustrating 3D clearing and imaging, and images of cleared assembloids and 3D stained cortico-striatal assembloids (eYFP: green; mCherry: magenta; MAP2: blue). RI, refractive index.

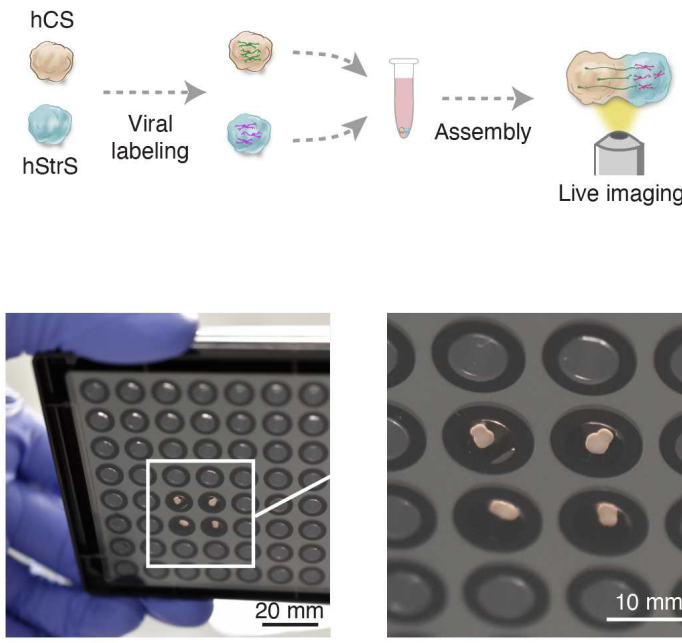
.

47 (E) Live imaging of axon growth ● Timing 2–8 h

CRITICAL For live imaging of axon growth in assembloids, viral labeling and fusion are performed at days 60–65, and assembloids are imaged at 8, 14 and 21 d after fusion.

47.1 Using a cut P1000 tip, transfer assembloids to a well of a 96-well imaging-compatible plate in **150 µL** – **200 µL** of NM.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



Scheme and images illustrating viral labeling, assembly and live imaging of cortico-striatal assembloids after their transfer into 96-well plates.

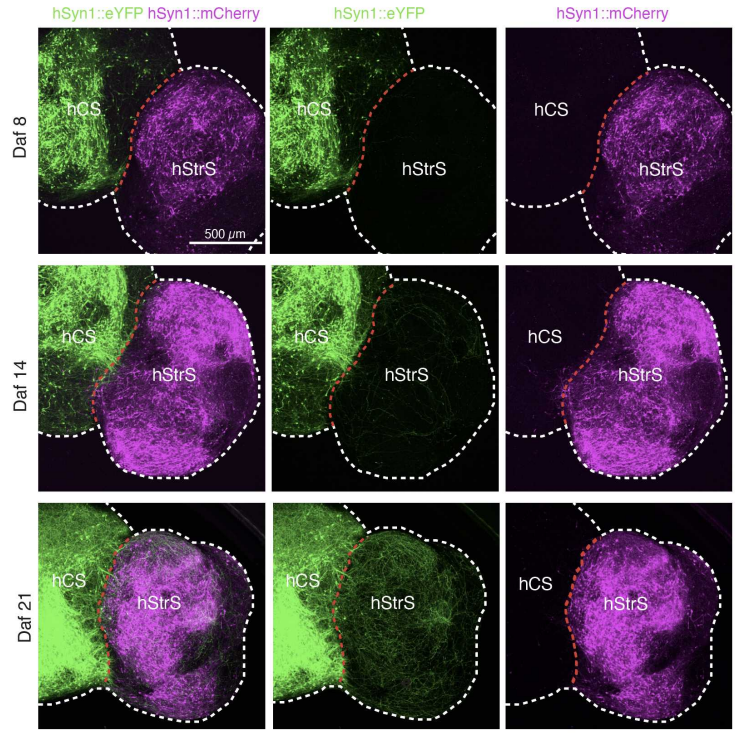
CRITICAL Avoid adding bubbles at the bottom of the wells. The distance from the lens to the sample is critical for imaging.

47.2 Place the 96-well plate into an inverted microscope with a motorized stage under environmentally controlled conditions (**37 °C** , 5% CO2), and keep the assembloids for **00:15:00** – **00:30:00** before experiments to let assembloids settle at the bottom of the plate. 45m

47.3 Set up imaging positions with z-stacks and take images by using an ×10 objective lens at a depth of 0–500 µm.

47.4 Process fluorescent images with Fiji and quantify the percentage of eYFP coverage in hStrSs and the percentage of mCherry coverage in hCSs.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



Representative images of Daf8–21 cortico-striatal assembloids labeled with eYFP and mCherry.

48 **(F) Optogenetic stimulation and calcium imaging** ● Timing 2–6 h

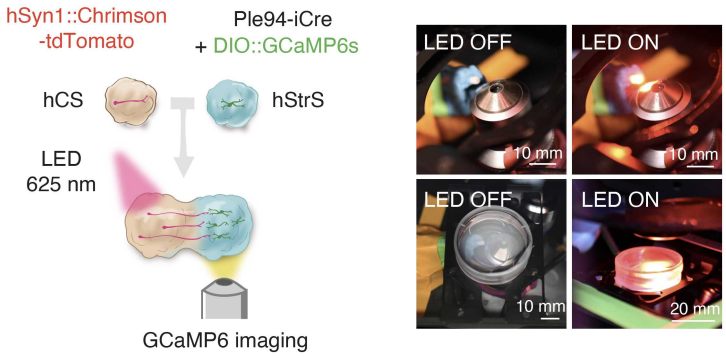
CRITICAL: For optogenetic stimulation and calcium imaging in assembloids, viral labeling and fusion are performed at days 60–65, and assembloids are imaged at 30–90 d after fusion.

48.1

Transfer an assembloid onto a **200 mm** glass coverslip in a **35 mm** plate in NM and image by using an ×10 objective on a Leica TCS SP8 confocal microscope.

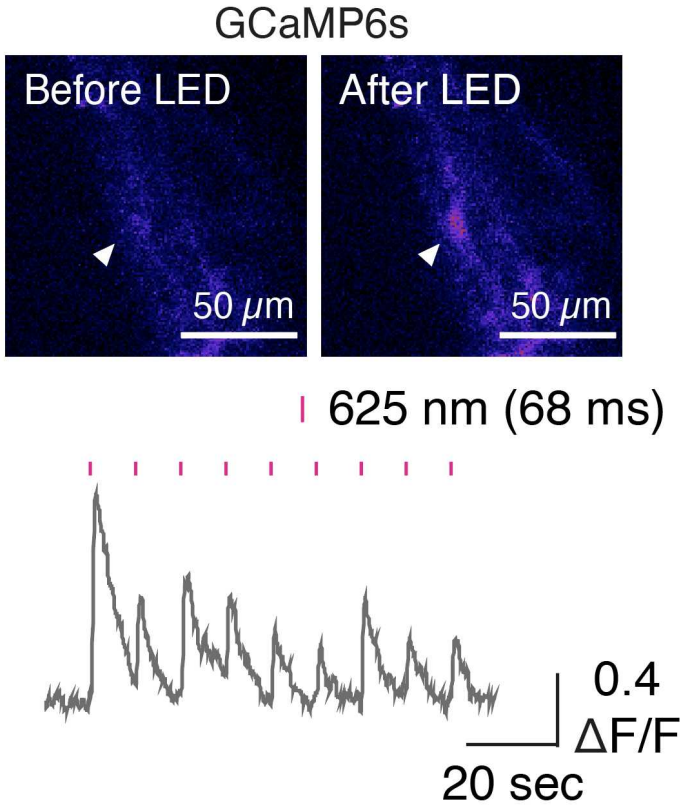
48.2 For optogenetic stimulation, activate ChrimsonR-tdTomato+ cells in hCSs with **625 nm** light by using an optical fiber-coupled LED. Our stimulation experiments included 1,500 frames, and one **625 nm** pulse of LED light (68 ms) was applied every 150 frames as generated by a Cyclops LED connected to the LEICA TCS SP8 microscope.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



Scheme illustrating viral labeling and assembly for optogenetics coupled with calcium imaging, representative images of imaging setup during optogenetic stimulation using a 625-nm LED.

48.3 Image GCaMP6 at a frame rate of 14.7 frames/s.



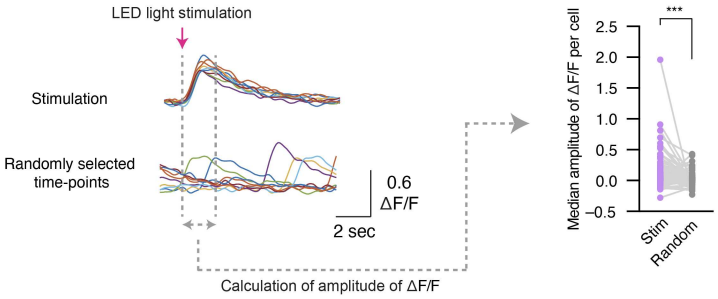
Representative image and trace of GCaMP6s signal in response to LED stimulation.

48.4 Process raw GCaMP6 signals with Fiji.

48.5 After registration of ROIs, transform raw time series to relative changes in fluorescence by using the following formula: $\Delta F/F = (F_t - F_0)/F_0$.

48.6 To verify whether responses are time-locked to LED stimulation, compare ΔF/F responses to ΔF/F values obtained at randomly selected time points.

48.7 For quantification of time-locked ΔF/F values, calculate the amplitude of ΔF/F values from each cell as the maximum ΔF/F values within 20–30 frames (1,360–2,040-ms window) after LED stimulation (minus the mean of the baseline 1 s before stimulation).



Quantification of stimulation-dependent response.n=180 cells from 10 assembloids with three hiPS cell lines; two-tailed Wilcoxon test. ***P=0.0002. Stim, stimulation.

TROUBLESHOOTING

49 (G) **Whole-cell electrophysiological recording** ● Timing 2–6 h

CRITICAL For whole-cell electrophysiological recording in assembloids, viral labeling and fusion are performed at days 60–65, and recordings are performed at 30–60 d after fusion.

49.1

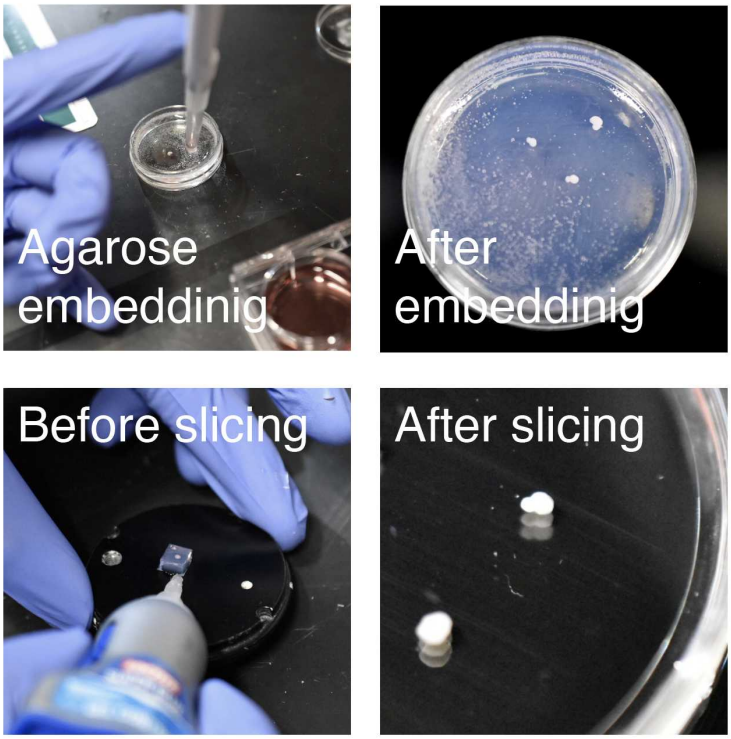
Add **0.2 g** of low-melt agarose into a **35 mm** plate containing **5 mL** of aCSF and mix well (4% agarose). Dissolve low-melt agarose by using a microwave (avoid spillover due to overheating).

CRITICAL:
- Usually 10 s is sufficient.
- STEP BrainPhys can be used in place of aCSF.

49.2

Wait for a few minutes for the agarose to cool down to **37 °C** and transfer spheroids or assembloids from the medium into agarose with a cut P1000 pipette tip.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



Images showing the process of agarose embedding and slicing of cortico-striatal assembloids.

CRITICAL: Avoid transferring the medium to prevent changes in the local concentration of agarose. Cool down agarose at **4 °C** for **00:10:00** until it becomes solid.

49.3 Cut a block of agarose out of the culture plate by using a blade and glue the block on the plate of the vibratome. Slice spheroids at a **200 µm** to **250 µm** thickness with cold aCSF in a vibratome. Transfer slices into aCSF (at **Room temperature** bubbled with 95% O_2/5% CO_2).

49.4 Patch-clamping on spheroid slices is usually performed on fluorescently labeled neurons. Record labeled neurons with glass pipettes with small tips (8–10 MΩ with K-gluconate internal solution, 7–9 MΩ with Cs internal solution)

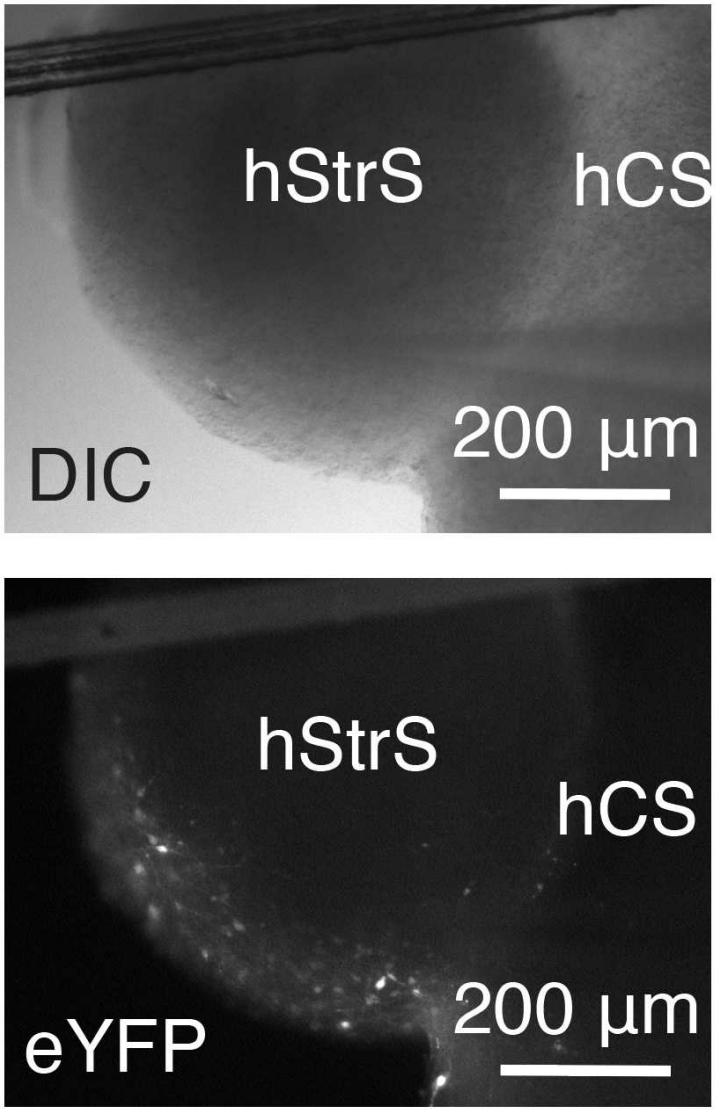
CRITICAL: Use glass pipettes with small tips instead of large tips.

.

49.5 10m 15s Once cells are identified through fluorescence, patch under differential interference contrast (DIC) or fluorescence. For DIC, the microscope is switched to a bright field. For patching under fluorescence, there are at least two approaches:

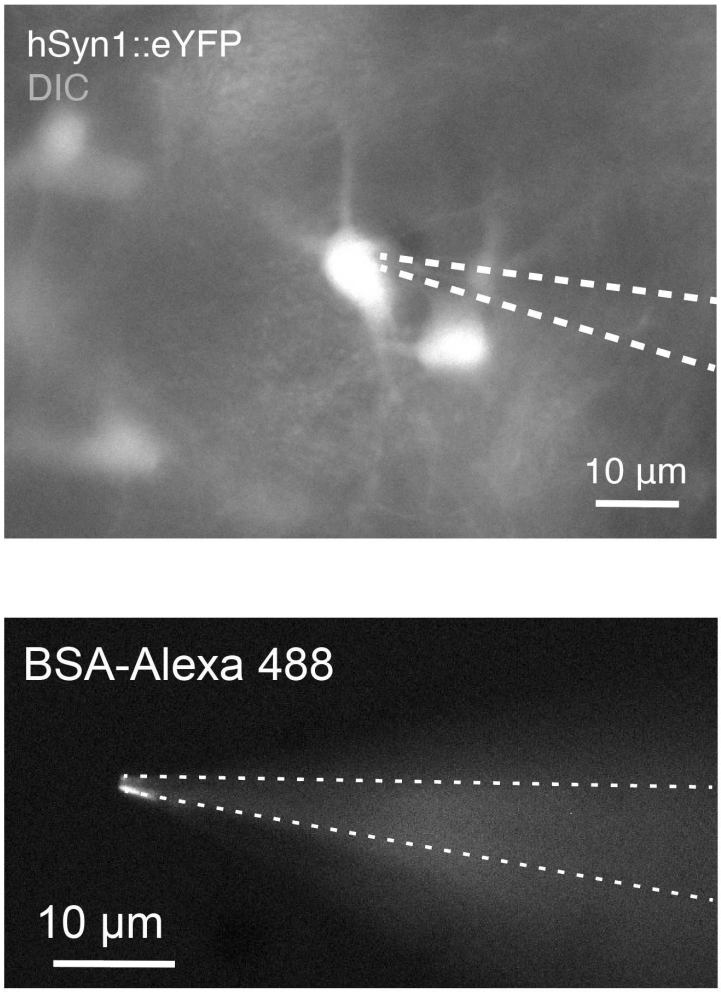
- Increase the bright field exposure under epifluorescence so that both fluorescence (to visualize the cell) and bright field (to visualize the pipette tip) can be seen simultaneously. Once a 'dimple' is seen under fluorescence or increased pipette resistance is achieved, release positive pressure and form a tight seal.
- Use fluorophore-coated pipettes, patching under epifluorescence. Back-fill glass pipettes by dipping the tips in the BSA-Alexa Fluor (0.02% in PBS) in a 1.5-ml Eppendorf tube for **00:00:05** to **00:00:10** and, after drying for **00:10:00**, fill pipettes with internal solution and patch.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



DIC (top) andfluorescent (bottom) images of sliced cortico-striatal assembloids expressing eYFP in an hStrS.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



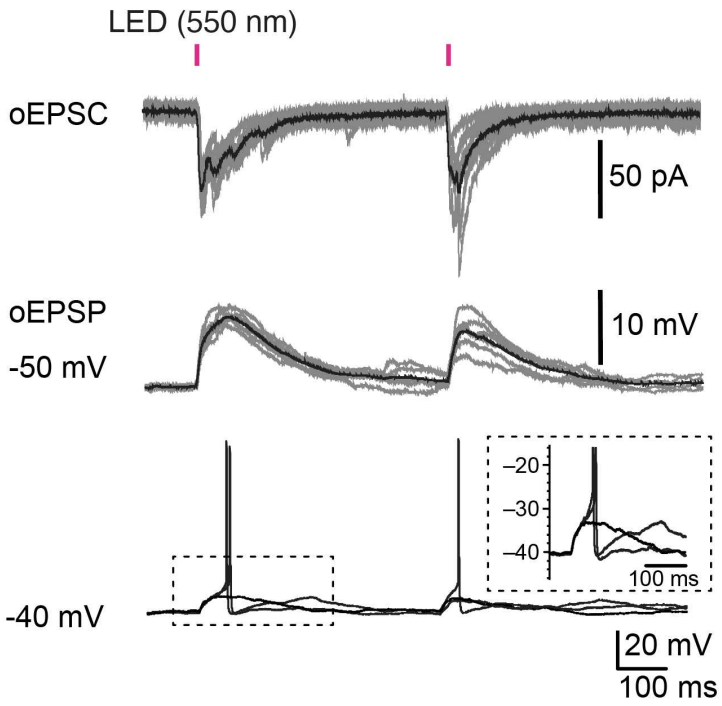
Representative image of patching from hSyn::eYFP+neurons with a non-fluorescently labeled pipette, and image showing afluorophore-coated patch pipette.

49.6 Once the pipette is approaching the target cell, provide minimal positive pressure. Once the tight seal is formed, strong suction is required to break cells.

CRITICAL: It is not always necessary to form a 'dimple' to get a Giga-seal; in most cases, an increase in the pipette resistance is suggestive of contact of the pipette tip with the cell membrane.

49.7 For recording optically evoked responses in assembloids, apply light at the maximal power through the 40× objective by using a CoolLED (**480 nm** for ChR2 and **550 nm** for Chrimson). CoolLED is controlled by a protocol in pClamp.

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1



Representative traces of oEPSC, oEPSP and spikes from striatal neurons in cortico-striatal assembloids. oEPSC, optically evoked excitatory postsynaptic current; oEPSP, optically evoked excitatory postsynaptic potential.

CRITICAL: Always check opsin expression before recording.

49.8 Make recordings at **30 °C** to **37 °C** or **Room temperature** within **05:00:00** to **06:00:00** after sectioning. 11h

TROUBLESHOOTING

Citation: Yuki Miura, Min-Yin Li, Omer Revah, Se-Jin Yoon, Genta Narazaki, Sergiu Pasca Engineering brain assembloids to interrogate human neural circuits https://dx.doi.org/10.17504/protocols.io.36wgq4xxkvk5/v1