

Extended Data

Functional Integration of 3D Printed Cerebral Cortical Tissue into a Brain Explant

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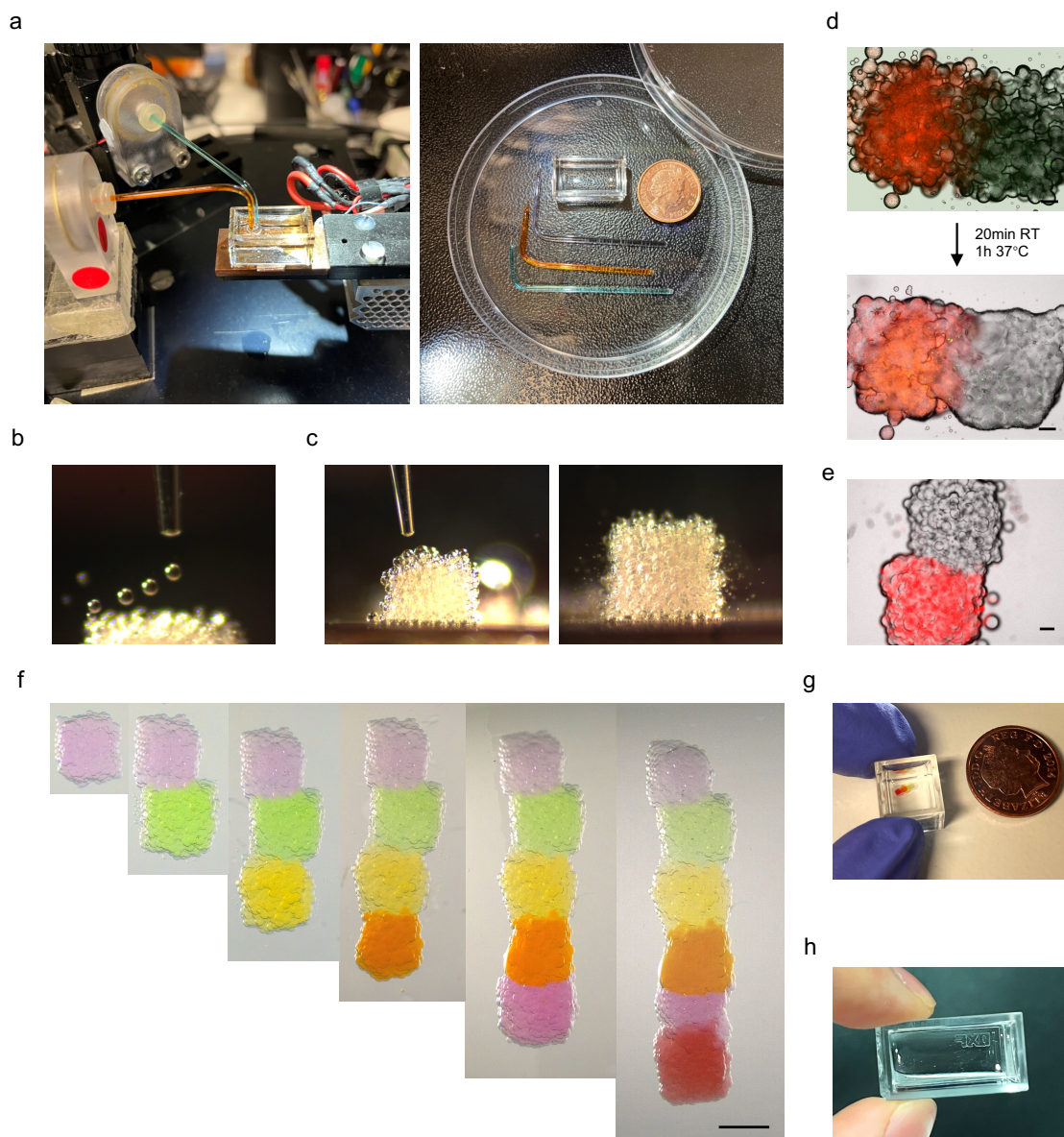
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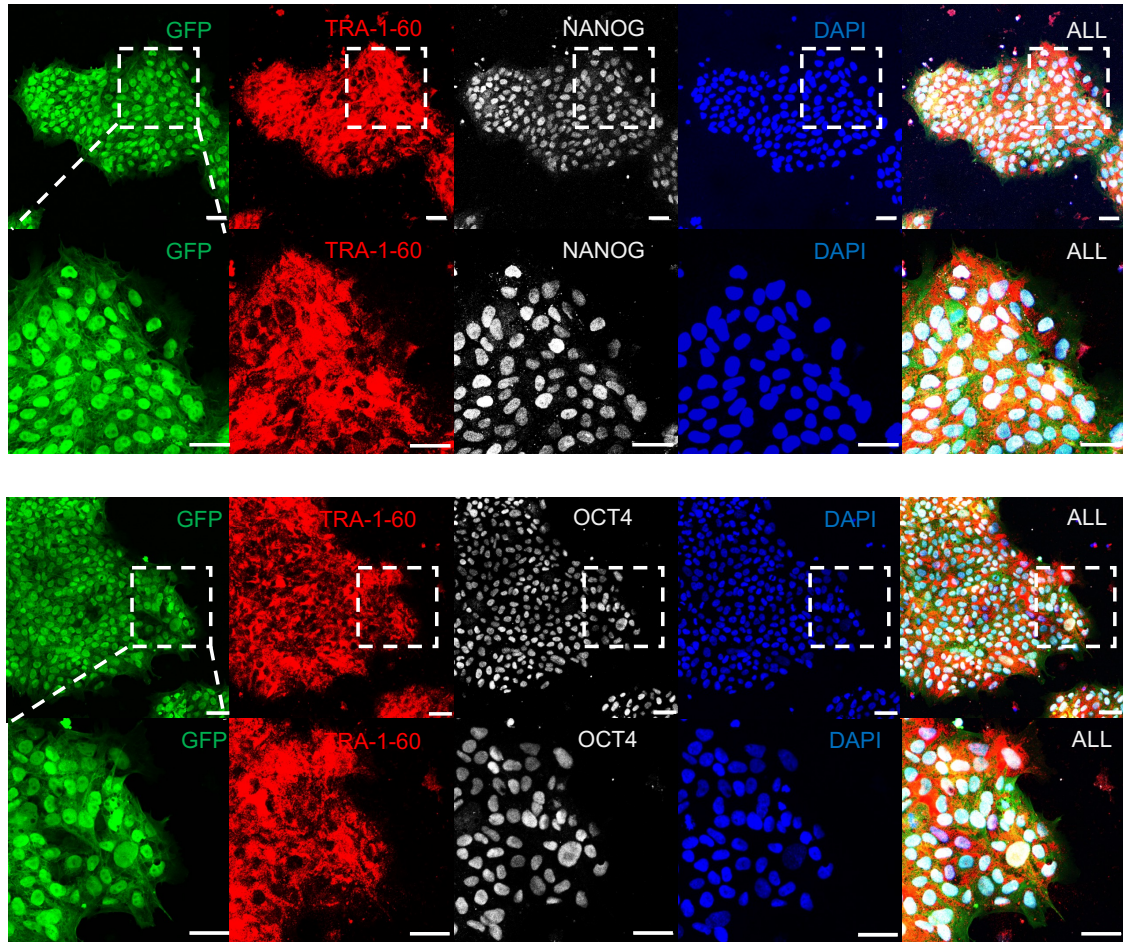
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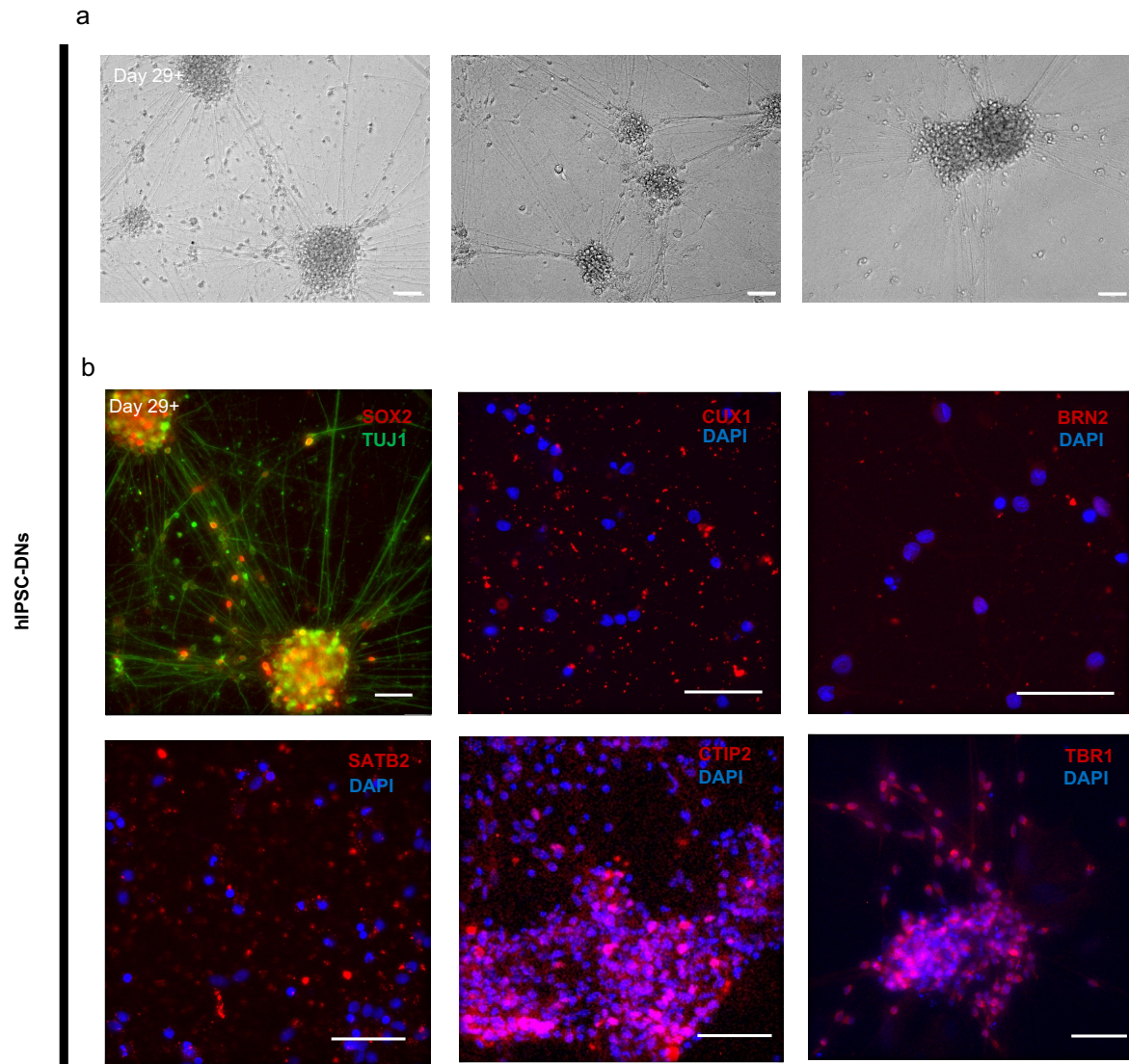


Extended Data Fig. 1: Droplet-based 3D printing. **a.** The droplet-based 3D bioprinter (left) and various components (right) including the glass printing cuvette and printing nozzles in comparison to a ten-pence coin. **b.** Side-view image of ongoing printing. **c.** Side-views of printed droplet networks containing Matrigel only. **d.** Fluorescence images of a two-layered droplet network containing RFP-labelled UNPs and unlabelled DNPs. Raising the temperature, from room to physiological, facilitated gelation and annealing of printed two-layer networks. **e.** Fluorescence image of two-layered droplet network with fluorescent microbeads in one layer. **f.** Sequential generation of six-layered network by the droplets containing food dye coloured DPBS. Scale bar, 1000 μm . **g.** View of the six-layered network in 'f', in comparison to a ten-pence coin. **h.** View of the droplet network in Fig. 1l. The network was printed as a mirror image of 'OXF' for imaging with an inverted microscope. For 'd' and 'e': scale bar, 200 μm .

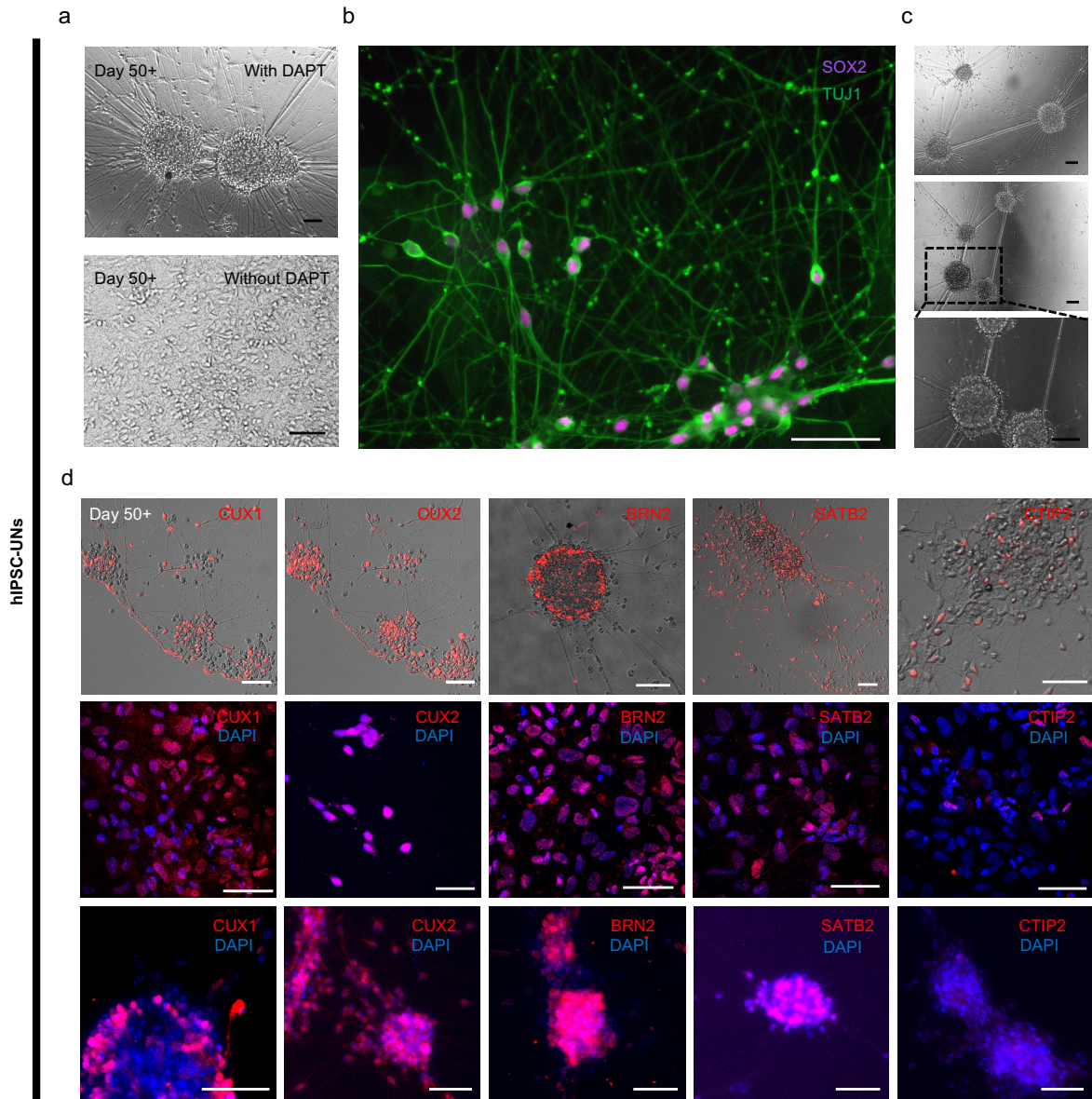
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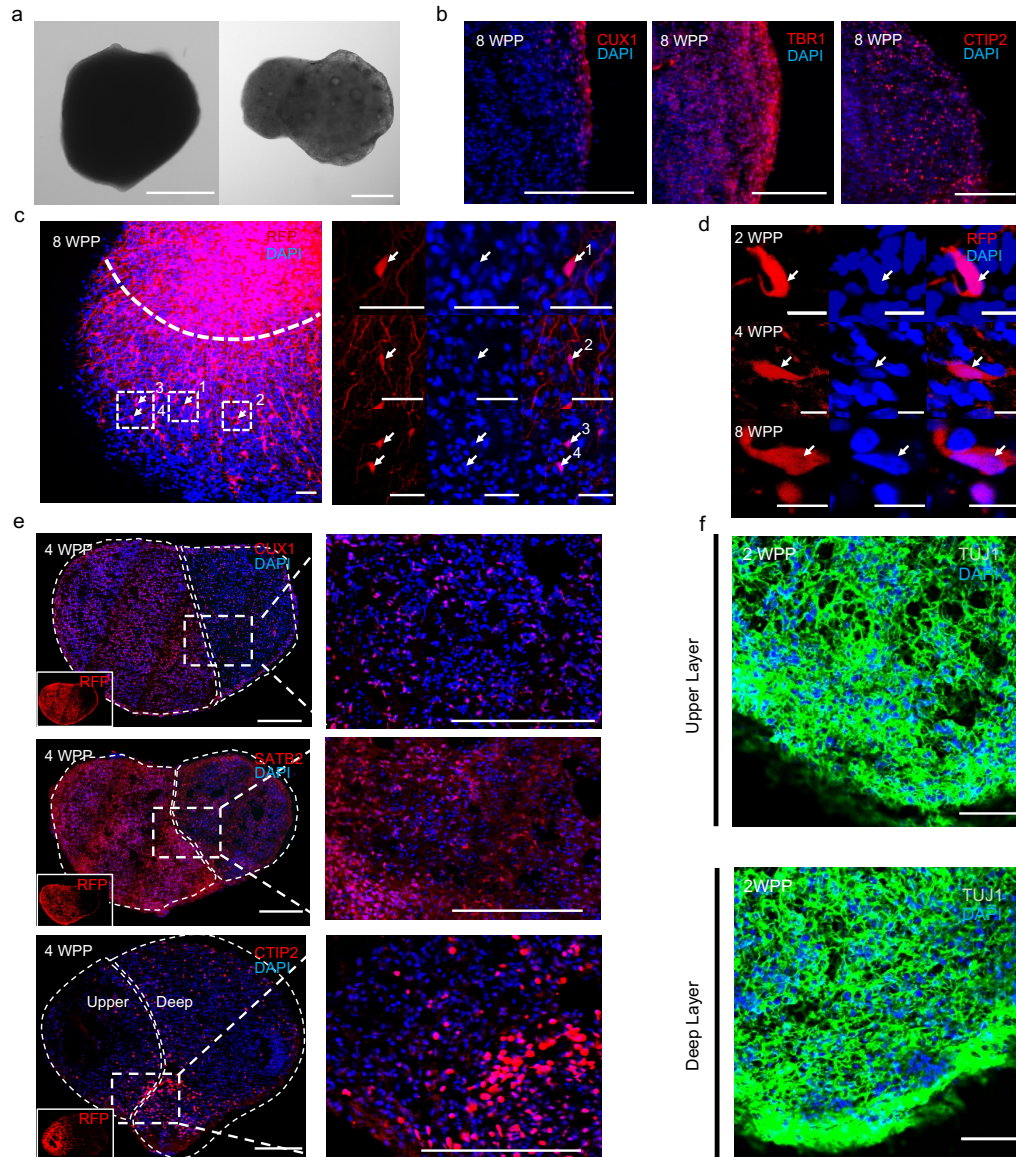
Extended Data Fig. 2: Characterisation of human induced pluripotent stem cells (hiPSCs). a. Confocal fluorescence images of immunostained hiPSCs showing the expression of pluripotent stem-cell markers TRA-1-60, NANOG and OCT4 in majority of the cells. Images at higher magnification of the regions indicated by the dashed boxes are shown in the second row. Scale bar, 50 μ m.



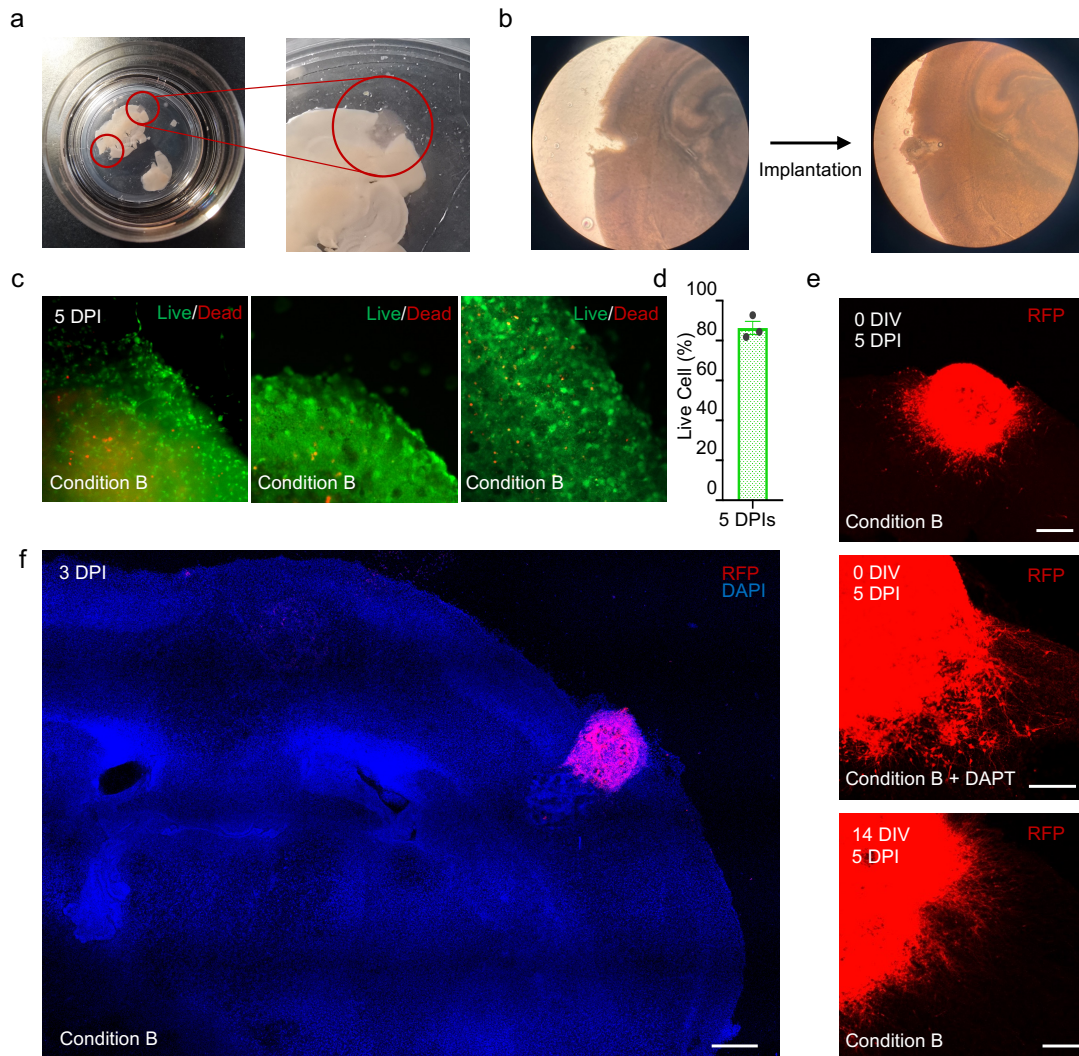
Extended Data Fig. 3: Characterisation of hiPSCs derived deep-layer neurons (hiPSCs-DNs). **a.** Bright-field images of DIV29+ hiPSCs-DNs showing mature neural morphology. **b.** Immunostaining of DNs showing expression of the neural stem cell marker SOX2, the general young neuron markers TUJ1 and the deep-layer markers (CTIP2 and TBR1). Expression of upper-layer markers (CUX1 and BRN2) and the middle-upper-layer marker (SATB2) are not detected despite high fluorescence intensity was used to reveal the background. For all panels: scale bar, 50 μ m.



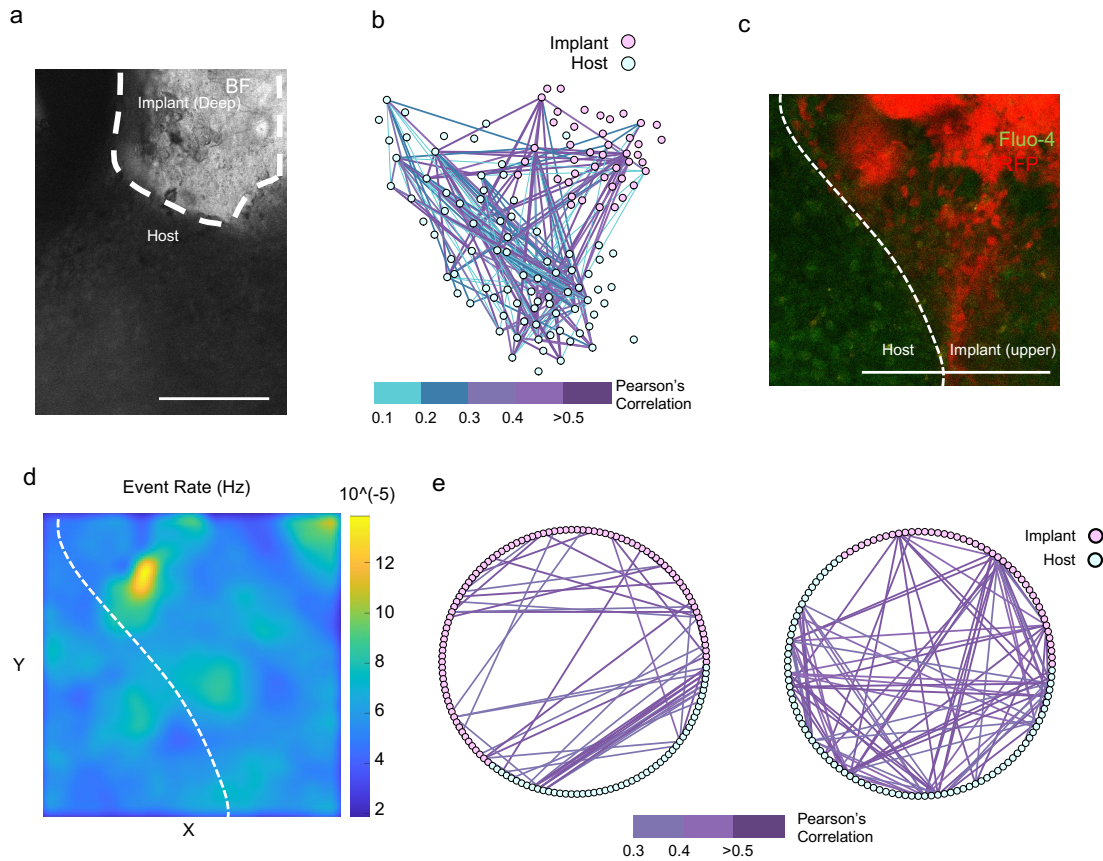
Extended Data Fig. 4: Characterisation of hiPSC-derived upper-layer neurons (hiPSCs-UNs). **a.** Bright-field images of DIV 50+ hiPSCs-UNs demonstrating the neuronal morphologies with (top) and without (bottom) DAPT treatment during maturation. UNs matured in NTM with DAPT are shown in **b-d**. **b.** DIV 50+ UNs immunostained with the neural stem cell marker SOX2 and the general young neuron marker TUJ1. **c.** Bright-field images hiPSCs-UNs showing mature morphology as indicated by extensive process outgrowth. **d.** Fluorescence images of DIV 50+ UNs from three independent experiments on each row showing expression of upper-layer markers (CUX1, CUX2 and BRN2) and the middle-upper-layer marker (SATB2), but low expression of the deep-layer marker (CTIP2). For all panels: scale bar, 50 μ m.



Extended Data Fig. 5: Further characterisation of droplet-printed cerebral cortical tissues. **a.** Bright-field images of one 2 WPP single-layer (with DNs, top) and one two-layer (with DNs and UNs, bottom) cortical tissues. **b.** Fluorescence images of sectioned 8 WPP deep-layer cortical tissues showing the abundant expression of the deep layer markers (CTIP2 & TBR1), and the sparse expression of upper-layer marker (CUX1). **c.** Confocal z-projection image (left) and high magnification images (right) showing cross-layer neuron migration in printed two-layer tissue at 8 WPP, visualized by RFP expression in UNs and DAPI nuclear staining in both UN and DNs. Dashed boxes indicate the magnified regions. Arrows and numbers indicate migrating neurons. Scale bar, 50 μm . **d.** Confocal images of 30 μm -thickness sections of 2, 4 and 8 WPP two-layer tissues showing cross-layer neuron migration, visualized by RFP and DAPI co-localisation. Scale bar, 10 μm . **e.** Immunofluorescence images at 4WPP of sectioned two-layer tissues showing expression of the layer-specific markers (CUX1, SATB2 and CTIP2). Bottom left small shows RFP expression of the tissue. Dashed lines outline the layers and dashed boxes indicate the magnified areas. **f.** Confocal images of sectioned two-layer tissues showing the expression of young neuronal marker TUJ1. Scale bar, 50 μm . For panels 'a', 'b' & 'e': scale bar, 200 μm .



Extended Data Fig. 6: Characterisation of implanted mouse brain explants. **a.** 0 DPI explant with a lesion in the left cerebral hemisphere and a lesion implanted with printed deep-layer tissue in the right cerebral hemisphere. Right, a magnified image of lesion on the right hemisphere implanted with printed deep-layer cortical tissue. **b.** A bright-field image of a 0 DPI explant with a lesion implanted with a printed deep-layer cortical tissue. **c.** Fluorescence images of a live/dead assay of deep-layer cortical tissue implanted explant cultured under condition B at 5 DPIs. **d.** Quantitative live/dead analysis of host cells of 5 DPIs at condition B (n = 3). **e.** Further examples of implanted RFP-labelled deep-layer tissues under different nutrient conditions and with different pre-implantation culture times. **f.** Tiled fluorescence confocal image of an explant implanted with a two-layer printed tissue in the right hemisphere. Cells were visualized by RFP (UNs) and DAPI nuclear staining in UNs, DNs and the host. For all panels: scale bar, 200µm.



Extended Data Fig. 7. Functional analysis of implanted mouse brain explants.
a. Bright-field image at 5 DPIs of an explant implanted with deep-layer cortical tissue (as indicated in '**Fig. 5h-j**'). The contrast difference between the implant and host marks the border between them. **b.** Network analysis of firing-correlated neurons between the implant and the host in '**Extended Data Fig. 7a**' on 5 DPI. Circles correspond to neurons and lines indicate correlated firings. **c.** Fluorescence image of an explant implanted with upper-layer tissue, as also indicated on '**Fig. 5k-n**'. The RFP-labelled UNs and Fluo-4 labelled implant and host tissue mark the implant-host interface. **d.** Heatmap of neuron firing rate showing comparable neuron activity between explant and the implanted tissue. **e.** Network analysis of firing-correlated neurons for the 5 DPI implanted explants found in '**Fig. 5h**' (Left) and '**Fig. 5k**' (Right) in a circular layout. For all panels: scale bar, 200µm.

Supplementary Information

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Supplementary Table 1. Culture Medium Formula

Neural Induction Medium (NIM) 100mL					
Item	Volume	Final Conc	Stock Conc	Supplier	Cat no
DMEM/F12 Medium	~49 mL	NA	1X	Life Technologies	21331020
Neurobasal Medium	~49 mL	NA	1X	Life Technologies	21103-049
B27 supplement	1 mL	NA	NA	Life Technologies	17504044
N2 supplement	0.5 mL	NA	NA	Life Technologies	17502-048
GlutaMax	1 mL	NA	100X	Life Technologies	35050-038
LDN193189	10 µL	100nM	1 mM	Sigma	SML0559
SB431542	100 µL	10 µM	10 mM	Cambridge Bioscience	ZRD-SB-50
Puromycin (opt)	50 µL	2.5µg/ml	5 mg/ml	MP Biomedicals UK	210055225
Neural Maintenance Medium (NMM) 100mL					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
DMEM/F12 Medium	~49 mL	NA	1X	Life Technologies	21331020
Neurobasal Medium	~49 mL	NA	1X	Life Technologies	21103-049
B27 supplement	1 mL	NA	NA	Life Technologies	17504044
N2 supplement	0.5 mL	NA	NA	Life Technologies	17502-048
GlutaMax	1 mL	NA	100X	Life Technologies	35050-038
Puromycin (opt)	50 µL	2.5µg/ml	5 mg/ml	MP Biomedicals UK	210055225
Neural Maintenance Medium + Growth Factors (NMM + GFs) 100mL					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
DMEM/F12 Medium	~49 mL	NA	1X	Life Technologies	21331020
Neurobasal Medium	~49 mL	NA	1X	Life Technologies	21103-049
B27 supplement	1 mL	NA	NA	Life Technologies	17504044
N2 supplement	0.5 mL	NA	NA	Life Technologies	17502-048
GlutaMax	1 mL	NA	100X	Life Technologies	35050-038
Fibroblast Growth Factor-2 (FGF-2)	10 µL	10 ng/mL	100µg/mL	R&D Systems	4114-TC-01M
Epidermal Growth Factor (EGF)	10 µL	10 ng/mL	100µg/mL	Life Technologies	PHG0311
Brain-derived Neurotrophic Factor (BDNF)	10 µL	10 ng/mL	100µg/mL	Life Technologies	PHC7074
Freezing Medium 10 mL					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
ESC-qualified FBS	9 mL	NA	NA	Gibco	16141061
DMSO	1 mL	NA	NA	Merck	D2650-100ML
Neural Terminal Medium (NTM) 100 mL					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
Neurobasal Medium	97 mL	NA	1X	Life Technologies	21103-049
B27 supplement	2 mL	NA	NA	Life Technologies	17504044
GlutaMax	1 mL	NA	100X	Life Technologies	35050-038
DAPT	10 µL	10 µM	100 mM	Tocris	Oct-34
Puromycin (opt)	50 µL	2.5µg/ml	5 mg/ml	MP Biomedicals UK	210055225
Brain Explant Culture Medium Condition A 100mL					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
DMEM/F12 Medium	~36 mL	NA	1X	Life Technologies	21331020
Neurobasal Medium	~36 mL	NA	1X	Life Technologies	21103-049
B27 supplement	1 mL	NA	NA	Life Technologies	17504044
N2 supplement	0.5 mL	NA	NA	Life Technologies	17502-048
GlutaMax	1 mL	NA	100X	Life Technologies	35050-038
Pen/Strep	1 mL	NA	10,000 U/mL	Gibco	15140122
Horse Serum	25 mL	NA	NA	Life Technologies	16050130
Brain Explant Medium Condition B 100mL					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
Brainphys Neural Medium	72.5 mL	NA	NA	Stemcell Technologies	5792
SM1 supplement	1.5 mL	NA	NA	Stemcell Technologies	5792
Pen/Strep	1 mL	NA	10,000 U/mL	Gibco	15140122
Horse Serum	25 mL	NA	NA	Life Technologies	16050130

Supplementary Table 2. Consumables

Consumables (Cell Culture)					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
mTeSR Plus Medium	NA	NA	NA	Stemcell Technologies	100-0276
Geltrex	NA	NA	NA	Gibco	A1413302
StemPro Accutase	NA	NA	NA	Life Technologies	A1110501
DPBS	NA	NA	NA	Gibco	14190144
UltraPure 0.5M EDTA	NA	0.5 mM	0.5 M	Life Technologies	15575020
Distilled Water	NA	NA	NA	Life Technologies	15230089
Y-27632	NA	10 μ M	1mM	Abcam	ab120129-10mg
ReLeSR	NA	NA	NA	Stemcell Technologies	5872
Consumables (Droplet Printing)					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
Silicone oil AR20	NA	NA	NA	Sigma	10836
Undecane	NA	NA	NA	Sigma	1120-21-4
DPHPC	NA	NA	NA	Avanti	850356
Trimethoxysilane	NA	5% v/v	NA	Sigma	281778
Matrigel	NA	NA	NA	Corning	354230
Consumables (Brain Explant)					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
EBSS	NA	NA	NA	Life Technologies	24010043
Culture Insert	NA	NA	NA	Merck	PICMORG50
X30 Cell Imaging Dish,	NA	NA	NA	Fisher Scientific UK	15670537
BrainPhys Imaging Optimized Medium	NA	NA	NA	Stemcell Technologies	5796
UltraPure Low Melting Point Agarose	NA	NA	NA	Life Technologies	16520050
Microtome blade	NA	NA	NA	Fisher Scientific	11912355
Needle	NA	NA	NA	Fisher Scientific	10749891
Super Glue	NA	NA	NA	Office Depot	4086446
Consumables (qPCR)					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
LunaScript(R) RT SuperMix Kit	NA	NA	NA	New England Biolabs	E3010L
Monarch(R) Total RNA Miniprep Kit	NA	NA	NA	New England Biolabs	T2010S
Luna(R) Universal qPCR Master Mix	NA	NA	NA	New England Biolabs	M3003L
MicroAmp Fast Optical 96-Well Reaction Plate	NA	NA	NA	Life Technologies	4346906
Nuclease-Free water	NA	NA	NA	QIAGEN	129114
Consumables (Immunostaining, live/dead assay and Fluo-4 imaging)					
Item	volume	Final Conc	Stock Conc	Supplier	Cat no
Ibidi μ -Slide 18 Well	NA	NA	NA	ThistleScientific	SKU 81816
Paraformaldehyde 4%	NA	4%	4%	Alfa Aesar	J61899.AK
Glycine 1 M Solution	NA	NA	NA	Merck	67419-1ML-F
Triton X-100	NA	NA	NA	Merck	93443-100ML
Tween-20	NA	NA	NA	Alfa Aesar	P9416-50ML
Normal Goat Serum	NA	NA	NA	Abcam	ab7481
Normal Donkey Serum	NA	NA	NA	Abcam	ab7475
DAPI Solution	NA	1X	10000X	Merck	MBD0015-1ML
Mounting Medium With DAPI	NA	1X	1X	Abcam	ab104139
Fluo-4 Direct Calcium Assay Kit	NA	1X	2X	Life Technologies	F10471
Calcein-AM	NA	2.5 μ M	NA	Cambridge bioscience	1755-50
Propidium iodide	NA	5.0 μ M	NA	Sigma	P4170
Plastics and others					
1.8ml Cryogenic Vial	NA	NA	NA	STARLAB	E3090-6222
10 μ l Pipette Tip	NA	NA	NA	STARLAB	S1121-2710
20 μ l Pipette Tip	NA	NA	NA	STARLAB	S1120-1710
200 μ l Pipette Tip	NA	NA	NA	STARLAB	S1126-7810
1000 μ l Pipette Tip	NA	NA	NA	STARLAB	S1120-8810
6 Well Tissue Culture Plate	NA	NA	NA	Greiner Bio-One	657160
12 Well Tissue Culture Plate	NA	NA	NA	Greiner Bio-One	665180
24Well Tissue Culture Plate	NA	NA	NA	Greiner Bio-One	662160
48 Well Tissue Culture Plate	NA	NA	NA	Greiner Bio-One	677180
96 well Tissue Culture Plate	NA	NA	NA	Greiner Bio-One	655180
96 well Assessment Plate	NA	NA	NA	Corning	CLS3603
5mL Stripette	NA	NA	NA	Scientific Laboratory Supp	4487
10mL Stripette	NA	NA	NA	Scientific Laboratory Supp	4488
25mL Stripette	NA	NA	NA	Scientific Laboratory Supp	4489
Cryo Container	NA	NA	NA	VWR International	479-3200
Microslides	NA	NA	NA	VWR International	631-0448
PAP Pen	NA	NA	NA	Merck	Z672548-1EA

Supplementary Table 3. Antibodies and Primers

Primary Antibodies					
Target	Original Species	Manufacturer		Dilution Factor	Cat. No
CUX1	Ms	Santa Cruz Biotechnology		100	sc-13024
CUX2	Rb	AbCam		200	ab216588
BRN2	Ms	Santa Cruz Biotechnology		100	sc-393324
SATB2	Rb	AbCam		200	ab92446
CTIP2	Rat	AbCam		200	ab18465
TBR1	Rb	Merck		500	AB10554
SOX2	Rb	Millipore		100-200	ab5603
TUJ1	Ms	AbCam		500-1000	ab78078
GFAP	Rat	Invitrogen		200	13-0300
HNCAM	Rb	AbCam		200	ab75813
Secondary Antibodies					
Target Species	Original Species	Fluorophore	Manufacturer	Dilution Factor	Cat. No
Rb	Goat	Alex488	Invitrogen	1000	a11006
Ms	Goat	Alex488	Invitrogen	1000	a32723
Ms	Goat	Alex633	Invitrogen	1000	a21052
Rat	Goat	Alex647	Invitrogen	1000	a21247
Rb	Goat	Alex647	Invitrogen	1000	a21245
qPCR Primer					
Target	Forward or Backward	5' -3' Sequence			Manufacturer
PAX6	F	GCCAGCAACACACCTAGTCA			Life Technologies
	R	TGTGAGGGCTGTGTCTGTTC			Life Technologies
Nestin	F	GGAAGAGAACCTGGGAAAGG			Life Technologies
	R	CTTGGTCCTTCTCCACCGTA			Life Technologies
CTIP2	F	GAGTACTGCGGCAAGGTGTT			Life Technologies
	R	TAGTTGCACAGCTCGCACTT			Life Technologies
BRN2	F	GACCTTTGCAGGCGAGTAAC			Life Technologies
	R	TCAGGAAGCTGCATTTTGTG			Life Technologies
CUX1	F	GCTCTCATCGGCCAATCACT			Life Technologies
	R	TCTATGGCCTGCTCCACGT			Life Technologies
CUX2	F	AAGGAGATCGAGTCGCAGAA			Life Technologies
	R	CTCCAGGATGCTCTTGATGG			Life Technologies
18S	F	GAGGATGAGGTGGAACGTGT			Life Technologies
	R	TCTTCAGTCGCTCCAGGTCT			Life Technologies

Supplementary Table 4. Detailed Statistical Test

Figure	Comparison	P value	Catalogue	Label	Test
Fig. 2d	CUX1 vs CTIP2	0.0009	P<0.001	***	One-way ANOVA with Dunnett's test
	BRN2 vs CTIP2	0.0004	P<0.001	***	One-way ANOVA with Dunnett's test
	SATB2 vs CTIP2	0.0008	P<0.001	***	One-way ANOVA with Dunnett's test
Fig. 2e	DNPs CUX1 vs UNPs CUX1	0.022	P<0.05	*	Unpaired Student t-test
	DNPs CUX1 vs UNs CUX1	0.0023	P<0.01	**	Unpaired Student t-test
	UNPs CUX1 vs UNs UCX1	0.7258	P>0.05	ns	Unpaired Student t-test
Fig. 3g	RFP Coverage Upper 4WPP vs 2WPP	0.4265	P>0.05	ns	One-way ANOVA with Dunnett's test
	RFP Coverage Upper 8WPP vs 2WPP	0.1339	P>0.05	ns	One-way ANOVA with Dunnett's test
	RFP Coverage Deep 4WPP vs 2WPP	0.0599	P>0.05	ns	One-way ANOVA with Dunnett's test
	RFP Coverage Deep 8WPP vs 2WPP	0.0115	P<0.05	*	One-way ANOVA with Dunnett's test
	RFP Coverage D/U 4WPP vs 2WPP	0.0074	P<0.01	**	One-way ANOVA with Dunnett's test
	RFP Coverage D/U 8WPP vs 2WPP	0.0003	P<0.001	***	One-way ANOVA with Dunnett's test
	Migration 4WPP vs 2WPP	0.463	P>0.05	ns	One-way ANOVA with Dunnett's test
	Migration 8wpp VS 2WPP	0.0093	P<0.01	**	One-way ANOVA with Dunnett's test
Fig. 3i	2WPP-CUX1 Upper vs Deep	0.0042	P<0.01	**	Unpaired Student t-test
	4WPP-CUX1 Upper vs Deep	0.0032	P<0.01	**	Unpaired Student t-test
	8WPP-CUX1 Upper vs Deep	0.002	P<0.01	**	Unpaired Student t-test
	2WPP-SATB2 Upper vs Deep	0.0403	P<0.05	*	Unpaired Student t-test
	4WPP-SATB2 Upper vs Deep	0.0477	P<0.05	*	Unpaired Student t-test
	8WPP-SATB2 Upper vs Deep	0.027	P<0.05	*	Unpaired Student t-test
	2WPP-CTIP2 Upper vs Deep	0.0045	P<0.01	**	Unpaired Student t-test
	4WPP-CTIP2 Upper vs Deep	0.0553	P>0.05	ns	Unpaired Student t-test
	8WPP-CTIP2 Upper vs Deep	0.3324	P>0.05	ns	Unpaired Student t-test
	2WPP-TUJ1 Upper vs Deep	0.2852	P>0.05	ns	Unpaired Student t-test
	4WPP-TUJ1 Upper vs Deep	0.6063	P>0.05	ns	Unpaired Student t-test
	8WPP-TUJ1 Upper vs Deep	0.6958	P>0.05	ns	Unpaired Student t-test
Fig. 4g	ConditionB -DAPT vs Condition A -DAPT	0.015	P<0.05	*	Unpaired Student t-test
	ConditionB +DAPT vs Condition B -DAPT	0.0077	P<0.01	**	Unpaired Student t-test
	Effect of ConditionA vs ConditionB	0.0008	P<0.001	***	Two-way ANOVA
	Effect of +DAPT vs -DAPT	0.0014	P<0.01	**	Two-way ANOVA
Fig. 4i	14 Days vs 0 Days	0.0211	P<0.05	*	Unpaired Student t-test
Fig. 4k	UNs 1DPIs vs UNs 3DPIs	0.0487	P<0.05	*	One-way ANOVA with Dunnett's test
	UNs 1DPIs vs UNs 5DPIs	0.0015	P<0.01	**	One-way ANOVA with Dunnett's test
	UNs 5DPIs vs 1d DN 5DPIs	0.4676	P>0.05	ns	Unpaired Student t-test
Fig. 5b	1DPI Upper vs Deep	0.2045	P>0.05	ns	Unpaired Student t-test
	3DPI Upper vs Deep	0.204	P>0.05	ns	Unpaired Student t-test
	5DPI Upper vs Deep	0.1179	P>0.05	ns	Unpaired Student t-test
	3 DPI Deep vs 1 DPI Deep	0.0005	P<0.001	***	Unpaired Student t-test
	3 DPI Upper vs 1 DPI Upper	0.0018	P<0.01	**	Unpaired Student t-test
	5 DPI Deep vs 1 DPI Deep	<0.0001	P<0.0001	****	Unpaired Student t-test
	5 DPI Upper vs 1 DPI Upper	<0.0001	P<0.0001	****	Unpaired Student t-test
Fig. 5e	RFP Upper vs Host	<0.0001	P<0.0001	****	One-way ANOVA with Dunnett's test
	RFP Deep vs Host	0.9481	P>0.05	ns	One-way ANOVA with Dunnett's test
	HNCAM Upper vs Host	0.0301	P<0.05	*	One-way ANOVA with Dunnett's test
	HNCAM Deep vs Host	0.117	P>0.05	ns	One-way ANOVA with Dunnett's test

Supplementary Video 1, 3D reconstructed confocal z-projection image showing cross-layer process outgrowth and neuron migration in a printed two-layer tissue at 8 WPP, visualized by RFP (false coloured as fire) expression in UNs and DAPI nucleus staining in both UN and DNs. Scale bars: 500 μm .

Supplementary Video 2, Fluo-4 calcium ion activity recording of the explant implanted with DNPs only at 5 DPI, as indicated in '**Fig. 5h**'. Scale bars: 200 μm .