

Supplementary Material

Multiplex imaging of human induced pluripotent stem cell-derived neurons with CO-Detection by indEXing (CODEX) technology

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Supplementary Material

Supplemental Table 1: Antibody Panel for iPSC-derived Neurons for Figure 3

Antibody	Cell type-specific	AB clone	Vendor	Cat. No.	Barcode	Dilution	Exposure (ms)	Fluorophore
S100B	Astrocyte	RM304	RevMab		BX032	1:50	350ms	550
GFAP	Astrocyte	134B1	Synaptic Systems	173011	BX007	1:50	350ms	488
TBR2	Cortical layer transcriptional activator	EPR21950-241	Abcam	ab261913	BX002	1:50	350ms	550
CTIP2	Cortical Layer V, Medium Spiny Neurons	EPR23120-25	Abcam	ab269367	BX043	1:50	250ms	488
Tyrosine Hydroxylase	Dopaminergic neuron	E2L6M	Biologend	818001	BX049	1:50	350ms	488
Neurofilament-L (NEFL)	Mature neuronal cells	C28E10	Cell Signaling Tech	2837BF	BX022	1:50	250ms	488
MAP2ab	Mature neuronal cells	MT-08	Antibodies Online.com	ABIN125739	BX023	1:50	350ms	550
Synapsin	Synaptic protein	7H10G6	Antibodies Online.com	ABIN5542390	BX001	1:50	250ms	488
CAMK2	100% CaMK2 beta, 93% CaMK2 gamma and 73% CaMK2 alpha and delta, cytosol, synapse and dendrites	EPR6686(2)	Abcam	ab227108	BX004	1:50	350ms	488

Supplemental Table 2: Antibody Panel for iPSC-derived Neurons for Figure 4

Antibody	Cell type-specific	AB clone	Vendor	Cat. No.	Barcode	Dilution	Exposure (ms)	Fluorophore
S100B	Astrocyte	RM304	RevMab	31-1189-00	BX036	1:50	350ms	550
GFAP	Astrocyte	134B1	Synaptic Systems	173011	BX007	1:50	350ms	488
Tyrosine Hydroxylase	Dopaminergic neurons	E2L6M	Biologend	818001	BX049	1:50	250ms	488
Engrail1 (EN1)	Ectodermal Transcription factor	1F5	Thermo Fisher	H00002019-M06	BX041	1:50	350ms	550
H2A.X	Histone H2A, DNA repair	2F3	Biologend	613402	BX042	1:50	350ms	Cy5
Neurofilament -L (NEL)	Mature neuronal cells	C28E10	Cell Signaling Tech	2837BF	BX022	1:50	250ms	488
MAP2ab	Mature neuronal cells	MT-08	Antibodies Online.com	ABIN125739	BX023	1:50	350ms	550
SOX2	Neuroprogenitor	EPR3131	Abcam	ab215970	BX045	1:50	350ms	Cy5
Synaptophysin (SYP)	Presynaptic vesicles of neurons	Sy38	Invitrogen/Thermo	MA1-213	BX028	1:50	250ms	488
Synapsin 1 (SYN)	Synaptic protein	7H10G6	Antibodies Online.com	ABIN5542390	BX001	1:50	250ms	488
Doublecortin (DCX)	Mature neuronal cells	EPR19997	Abcam	ab222921	BX034	1:50	250ms	488

Supplemental Table 3: Antibody Panel for iPSC-derived Neurons for Figure 5

Antibody	Cell type-specific	AB clone	Vendor	Cat. No.	Barcode	Dilution	Exposure (ms)	Fluoro-phore
MAP2ab	Mature neuronal cells	MT-08	Antibodies Online.com	ABIN125739	BX023	1:50	350ms	550
α Synuclein	Synaptic protein	354A10	Synaptic Systems	128211	BX004	1:50	250ms	488
CAV2.3	Voltage gated calcium channel	62C10	Synaptic Systems	152403	BX042	1:50	350ms	Cy5
Piccolo	Synaptic active zone marker	Neu 287	Synaptic Systems	142003	BX003	1:50	350ms	Cy5
DARPP32	Marker for striatal medium spiny neurons	polyclonal	Abcam	382003	BX024	1:50	350ms	Cy5

Supplemental Table 4: Antibody Panel for iPSC-derived Neurons for Figure 6

Antibody	Cell type-specific	AB clone	Vendor	Cat. No.	Barcode	Dilution	Exposure (ms)	Fluoro-phore
MAP2ab	Mature neuronal cells	MT-08	Antibodies Online.com	ABIN125739	BX023	1:1000	350ms	550
Neurofilament-L (NEL)	Mature neuronal cells	C28E10	Cell Signaling Tech	2837BF	BX022	1:50	250ms	488
α Synuclein	Synaptic protein	354A10	Synaptic Systems	128211	BX004	1:50	250ms	488
CAV2.3	Voltage gated calcium channel	62C10	Synaptic Systems	152403	BX042	1:50	350ms	Cy5
Piccolo	Synaptic active zone marker	Neu 287	Synaptic Systems	142003	BX003	1:50	350ms	Cy5
Synaptophysin (SYP)	Presynaptic vesicles of neurons	Sy38	Invitrogen/Thermo	MA1-213	BX028	1:50	250ms	488
FOXA2	Floorplate marker expressed in Dopaminergic neuron	7E6	Abcam	ab60721	BX036	1:50	350ms	Cy5

Supplemental Table 5: Antibody Panel for iPSC-derived Neurons for Supplemental Figure 2

Antibody	Cell type-specific	AB clone	Vendor	Cat. No.	Barcode	Dilution	Exposure (ms)	Fluoro-phore
Neurofilament-L (NEFL)	Mature neuronal cells	C28E10	Cell Signaling Tech	2837BF	BX022	1:50	250ms	488
Synaptophysin (SYP)	Presynaptic vesicles of neuron	Sy38	Invitrogen / Thermo	MA1-213	BX028	1:50	250ms	488
Tyrosine Hydroxylase	Dopaminergic neuron	polyclonal	Synaptic Systems	213104	BX045	1:50	250ms	Cy5

(TH)								
α Synuclein	Neuronal protein	354A10	Synaptic Systems	128211	BX004	1:50	250ms	488
FOXA2	Floorplate marker expressed in Dopaminergic neuron	7E6	Abcam	ab60721	BX036	1:50	350ms	Cy5
VAMP2	Vesicle protein, Synaptic marker	69.1	Synaptic Systems	104008	BX010	1:50	250ms	488

Supplemental Table 6: Other antibodies tested against iPSCs (failed or no staining observed in iPSC culture samples)

Antibody	Cell type-specific	AB clone	Vendor	Cat. No.	Barcode	Dilution	Exposure	Fluorophore
VGlut1	Glutamate transporter of synaptic vesicles	EPR22269	Abcam	ab242017	BX050	1:50	250ms	488
GAD67	Catalyzes the production of GABA	EPR20578	Abcam	ab246335	BX029	1:50	350ms	550
DAT	Transmembrane protein to Reuptake of dopamine	EP19695	Abcam	ab221845	BX025	1:50	350ms	488
MAG	Axon-myelin stabilization	EPR24276-125	Abcam	ab277535	BX024	1:50	350ms	Cy5
Serotonin transporter	Regulates serotonergic neurotransmission	EPR23530-3	Abcam	ab275094	BX013	1:50	250ms	488
TUBB3	Mature neuronal cells	Tu-20	Antibodies Online.com	ABIN93911	BX010	1:50	350ms	488
ACTIN	Cytoskeletal protein	S12-I	Antibodies Online.com	ABIN870295	BX006	1:50	350ms	Cy5
SLC32	Uptake of GABA into Synaptic vesicles	SLC32A1	Antibodies Online.com	ABIN2855225	BX031	1:50	250ms	488
Cortactin	Regulate Ca leveling in neurons	Polyclonal	Antibodies Online.com	ABIN2854674	BX014	1:50	350ms	550
TMEM119	Microglia	E3E4T	Cell Signalling Tech	1134BF	BX027	1:50	350ms	Cy5
Basson	Synaptic protein	Polyclonal	Antibodies Online.com	ABIN863198	BX019	1:50	250ms	488
Shank3	Synaptic protein	S367-51	Antibodies Online.com	ABIN1741251	BX020	1:50	350ms	550
SaTB	Cortical Layer IV	EPNCIR130A	Abcam	ab212177	BX047	1:50	350ms	550
Calbindin	Calcium-binding protein	EP3478	Abcam	ab233018	BX046	1:50	350ms	488

PSD-95	Synaptic protein	6G6	Antibodies Online.com	ABIN361694	BX026	1:50	350ms	550
TAU	Mature neuronal cells	EPR22524-94	Abcam	ab255271	BX003	1:50	350ms	Cy5
GRIN2B	Myelin sheath of oligodendrocytes and Schwann cells	6E9A8	Antibodies Online.com	ABIN5611338	BX016	1:50	250ms	488
SLC17	Glutamate transporter at SLC family	S28-9	Antibodies Online.com	ABIN1027710	BX017	1:50	350ms	550
MBP	Myelin sheath of oligodendrocytes and Schwann cells	EPR21188	Abcam	ab218011	BX037	1:50	250ms	488
CD34	Endothelium	Qbend10	Invitrogen/Thermo	MA1-10202	BX005	1:50	350ms	550
Nestin	Pre-neuronal	10C2	Biolegend	656802	BX035	1:50	350ms	550

Supplemental Table 7: Materials and Reagents

Item name	Vendor	Catalog #
iPSC maintenance:		
Matrigel	Thermo Fisher Scientific	CB-40230
KnockOut DMEM	Thermo Fisher Scientific	10829018
Bambanker	Thermo Fisher Scientific	NC9582225
Stemflex media kit	Thermo Fisher Scientific	A3349401
Thiazovivin	ReproCell	04-0017
ReLeSR	StemCell Technologies	05872
Neuronal differentiation of iPSC		
KnockOut™ Serum Replacement	Thermo Fisher Scientific	10828028
Penicillin Streptomycin	Thermo Fisher Scientific	15140-122
GlutaMAX	Thermo Fisher Scientific	35050-061
NEAA	Thermo Fisher Scientific	11140050
b-Mercaptoethanol	Thermo Fisher Scientific	21985023
Knockout DMEM/F12	Thermo Fisher Scientific	12660-012
B27 Supplement 50X without Vit.A	Thermo Fisher Scientific	12587010
Bd-cAMP	Peprotech	6099240
L-Ascorbic Acid	Peprotech	5088177
DAPT	Peprotech	2634
Neurobasal A Medium	Thermo Fisher Scientific	12349015
BDNF	Peprotech	450-02
GDNF	Peprotech	450-10
FBS	Thermo Fisher Scientific	SH3091003
N2 Supplement 100X	Thermo Fisher Scientific	17502-048
Neurobasal™ Medium	Thermo Fisher Scientific	21103049
DMEM/F-12, no glutamine	Thermo Fisher Scientific	21331020
neurotrophin-3	Peprotech	450-03
Doxycycline hyclate	Sigma-Aldrich	D9891-5G
Cultureone™ Supplement (100X)	Thermo Fisher Scientific	A3320201
Gene expression using SYBR green		
2-mercaptoethanol	Sigma Aldrich	M2650

DNase I, Amplification Grade	Thermo Fisher Scientific	18068015
Ethanol, Molecular grade	Thermo Fisher Scientific	BP2818500
Homogenizer spin column	Thermo Fisher Scientific	12183-026
PureLink RNA Mini Kit	Thermo Fisher Scientific	12183025
RNase Away	Thermo Fisher Scientific	10328011
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368814
MicroAmp 8 tube strip	Thermo Fisher Scientific	A30589
384-well PCR plate	Thermo Fisher Scientific	AB1384W
Nuclease-free water	US Biological LifeSciences	W0900
PowerUp™ SYBR™ Green master mix	Thermo Fisher Scientific	A25780
CODEX coverslip preparation and NPCs seeding		
Coverslips	Electron Microscopy Science	72204-10
Poly-L-Lysine	Sigma-Aldrich	P8920
Laminin (mouse)	Thermo Fisher Scientific	23017-015
Fixation, hydration, and Pre-staining fixation of neurons of coverslip		
PFA	Thermo Fisher Scientific	50-980-495
Acetone 100%	Millipore-Sigma	AX0120-6
Drierite absorbent beads	W.A. Hammond Drierite Co	21001
Hydration Buffer	Akoya Biosciences	7000008
Staining buffer	Akoya Biosciences	7000008
Storage buffer	Akoya Biosciences	7000008
Blocking Buffer	Akoya Biosciences	7000008
PBS	MP Biomedicals	091860454
Methanol	Sigma-Aldrich	646377
BS3 substrate	Thermo Fisher Scientific	21580
Primary Antibody processing		
CODEX conjugation kit	Akoya Biosciences	7000009
BSA removal kit	Abcam	Ab173231
Primary Astrocyte culture		
Human Astrocytes	ScienceCell	1800
Ara-C	Sigma-Aldrich	C6645

Astrocyte Media	ScienceCell	1801
Barcodes		
BX001	Akoya Biosciences	5450013
BX002	Akoya Biosciences	5450023
BX003	Akoya Biosciences	5450026
BX004	Akoya Biosciences	5450014
BX005	Akoya Biosciences	5450024
BX006	Akoya Biosciences	5450027
BX007	Akoya Biosciences	5450015
BX010	Akoya Biosciences	5450016
BX013	Akoya Biosciences	5450017
BX014	Akoya Biosciences	5450025
BX016	Akoya Biosciences	5150001
BX017	Akoya Biosciences	5250001
BX019	Akoya Biosciences	5150002
BX020	Akoya Biosciences	5250002
BX022	Akoya Biosciences	5150003
BX023	Akoya Biosciences	5250003
BX024	Akoya Biosciences	5350003
BX025	Akoya Biosciences	5150004
BX026	Akoya Biosciences	5250004
BX027	Akoya Biosciences	5350004
BX028	Akoya Biosciences	5150005
BX029	Akoya Biosciences	5250005
BX031	Akoya Biosciences	5150006
BX032	Akoya Biosciences	5250006
BX035	Akoya Biosciences	5250007
BX036	Akoya Biosciences	5350007
BX037	Akoya Biosciences	5150008
BX041	Akoya Biosciences	5250008
BX042	Akoya Biosciences	5350008
BX043	Akoya Biosciences	5150010

BX045	Akoya Biosciences	5350009
BX046	Akoya Biosciences	5150011
BX047	Akoya Biosciences	5250009
BX049	Akoya Biosciences	5150012
BX050	Akoya Biosciences	provided by Akoya

Supplemental Table 8: Primers used for SYBR Green qPCR assay

Gene	Function	Primer direction (5' to 3')	Sequence
FOXA2	Dopaminergic neuron marker	Forward	CTGGGAGCGGTGAAGATGGAA
		Reverse	TTCATGTTGCTCACGGAGGAGTAG
EN1	Dopaminergic neuron marker	Forward	TGCTAGATAAGAACGAGCGATCCA
		Reverse	GAGGAAGGAGGCAGGCGAAG
Doublecortin	Neurodevelopmental marker	Forward	TCTGACAACATCAACCTGCCTCA
		Reverse	TTCCTCCAGTTCATCCATGCTTCC
LMX1A	Dopaminergic neuron marker	Forward	CTTCTGCTGCTGTGTCTGCG
		Reverse	CTCCCGCTCCTTCTCATAGTCC
SOX2	Pluripotency marker	Forward	TACATGAACGGCTCGCCAC
		Reverse	GGACTTGACCACCGAACCCA
MAP2	Neuronal maturation marker	Forward	GGACATGATCTTCTCCTTGCTT
		Reverse	AGGTGTGGTGGCTGGAAGGT
TH	Dopaminergic neuron marker	Forward	ATTGCTGAGATCGCCTTCCAGTA
		Reverse	GTGGTGTAGACCTCCTTCCAGG
GFAP	Astrocyte marker	Forward	GGGACAATCTGGCACAGGAC
		Reverse	GGGTGGCTTCATCTGCTTCC
S100B	Astrocyte marker	Forward	ATATTCTGGAAGGGAGGGAGACAA
		Reverse	AGAAATGGGAAAGCTCATTGTTGATG
H2A.X	Cell stress and damage	Forward	GCAGTCTGGAGTACCTCACC
		Reverse	CTCCTCGTCGTTGCGGATGG

CAMKII	Neurodevelopmental marker	Forward	CAGACTTCGGCCTAGCTATCGAG
		Reverse	GGCTTGCCATACGCCTCTTTG
CTIP2	Striatal & Cortical neuron marker	Forward	ATTCCTGGGCGACAGCAACC
		Reverse	GACTGAAGAGAGGGCGGCGTG
Synapsin	Synaptic marker	Forward	GGTGAAGGTCGTGCGGTCTC
		Reverse	GTAGTCTCCGTTGCGTGCCAT
Synaptophysin	Synaptic marker	Forward	TCCCTTTCCCTGCATCCCTTG
		Reverse	ACACAGCCGAGGTCTGTTC
TBR2	Cortical Development	Forward	AATGTGTTCTAGAGGTGGTGCT
		Reverse	CCCTGCATGTTATTGTCGGCTTT
SNCA	Presynaptic protein, Disease marker for Parkinson's	Forward	GCAGAAGCAGCAGGAAAGACAAA
		Reverse	CACTTGCTCTTTGGTCTTCTCAGC
Cortactin	Neurodevelopmental marker	Forward	TGTCTTTCAAGAGCATCAGACCCT
		Reverse	CCACACCAAATTCCTCCATAGC
MAPT	Neuronal maturation marker	Forward	AGTCGAAGATTGGGTCCCTGG
		Reverse	GGAAGGTCAGCTTGTGGGTTTC
GAD65	Striatal/GABA-ergic marker	Forward	TTGGATATGGTTGGATTAGCAGCAG
		Reverse	GCACAATACTGGAGCAATTCATAGG
VGAT	Striatal/GABA-ergic marker	Forward	GAGCAAGCGGAGATAGCGACTTT
		Reverse	GGACAGCGGAAAGGACAGAAGG
Neurofilament	Neuronal marker	Forward	CAAGACCTCCTCAACGTGAAGATG
		Reverse	TGAAACTGAGTCGGGTCTCCTC
GAPDH	Housekeeping Gene	Forward	AAGGCTGTGGGCAAGGTCATC
		Reverse	GGCAGGTCAGGTCCACCACT

Supplemental Table 9: Thermal cycling settings for qPCR reaction

Step	Temperature	Duration	Cycles
UDG activation	50 °C	2 min	Hold

Dual-Lock™ Taq DNA polymerase	95 °C	2 min	Hold
Denature	95 °C	15 s	40
Anneal/ extend	60 °C	1 min	

Supplemental Table 10: Settings for qPCR dissociation step

Step	Ramp rate	Temperature	Time
Denature	1.6 °C/s	95 °C	15 s
Anneal	1.6 °C/ s	60 °C	1 min
Dissociation	0.15 °C/ s	95 °C	15 s

Supplementary Methods

1. iPSC maintenance

Human iPSCs were stored in Bambanker media in liquid nitrogen. Before thawing the iPSCs, one well of a 6-well plate was coated with Matrigel diluted 1:80 in Knockout Dulbecco's modified Eagle's (KO-DMEM) for 1 h at 37°C. Frozen vial with cells was quickly thawed for 2 min in a 37°C water bath. The cell suspension was transferred to 5 ml of Stemflex media and pelleted by centrifugation at 1000 rpm for 3 min. The supernatant was removed by aspiration and the cell pellet was resuspended in 1 ml of Stemflex media containing 1 µM of Thiazovivin (THZ). After removing the Matrigel solution from the coated well and adding 1 ml of Stemflex media containing 1 µM THZ, the cell suspension was added and mixed by gently shaking the plate. 48 h after plating, the media was changed to 3 ml Stemflex media without THZ. Subsequently, media was changed every 48 hr. Once cells reached confluency they were passaged non-enzymatically with ReLeSR passaging solution. For passaging, media was aspirated and cells were washed once with 1ml phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ and then incubated with 1 ml ReLeSR for 4-6 min at room temperature (RT). After carefully aspirating off the ReLeSR, cells were detached by gently pipetting with 1 ml Stemflex media containing 1 µM THZ.

Details on vendors and catalog numbers are listed in Supplemental Table 7 (Materials and Reagents).

2. Protocols for neuronal differentiation of iPSCs

2.1.1 Neuronal differentiation by chemical induction

To generate iPSC-derived cortical, striatal, and dopaminergic neurons, we adapted three different protocols (Calatayud et al., 2021). First, forebrain PAX6-positive neural progenitor cells (NPCs) pre-patterned to derive into cortical neurons were generated from iPSCs by chemical induction using an adapted protocol (Shi et al., 2012). To increase the outcome of PAX6 positive cells, we applied dual SMAD-inhibition by LDN/SB431542 instead of noggin/SB431542 (Surmacz et al., 2012). We further applied wingless/integrated (Wnt) pathway inhibition by exposure to IWP-2 (1 µM) during the first 4 days (Moya et al., 2014). Second, lateral ganglionic eminence (LGE) NPCs pre-patterned to derive

into GABAergic medium-sized spiny neurons (MSNs) were generated from iPSCs by chemical induction using a modified protocol (Arber et al., 2015). Exposure to SB431542 was reduced to 6 days (Day 0 to Day 6), while we started to supplement Activin A on day 8. We further adapted the LDN concentration to 500 nM and included IWP-2 (1 μ M) during the first 4 days (Day 0 to Day 4). Third, floorplate NPCs pre-patterned to derive into dopaminergic neurons were generated from iPSCs by chemical induction using a modified dual SMAD-inhibition protocol (Kriks et al., 2011). The concentration of LDN was adapted (500 nM) and the timepoint of FGF8 exposure was optimized starting subsequently to sonic hedgehog/purmorphamine exposure from day 7.

For all three protocols, human KOLF2.1 iPSCs were first pre-patterned to NPCs. For generating NPCs, human iPSCs were passaged with ReLeSR and resuspended in a 1:1 mixture of Stemflex and KnockOut Serum Replacement (KSR) media (15% KSR, 1X Penicillin Streptomycin, 1X GlutaMAX, 1X non-essential amino acids (NEAA), 0.1 mM beta-mercaptoethanol in KO-DMEM/F12) containing 1 μ M THZ. Cells were plated at a concentration of 300,000 cells/cm² on freshly-coated Matrigel plates (1:50 in KO-DMEM).

For differentiation of NPCs into the respective neuron types, NPCs were plated onto primary human astrocytes grown on poly-L-lysine (PLL)/laminin-coated coverslips (Section 2.4.2). One day prior, 300,000 cells of 2 mM Ara-C-inactivated primary human astrocytes were thawed and plated in 400 μ l Astrocyte media onto the CODEX coverslip (22x22 mm) only and allowed to attach for 10 min. Then 1 ml of astrocyte media was carefully added to the 6-well plate.

The next day, NPCs were passaged using ReLeSR and collected in N2B27 (Neurobasal medium: DMEM/F12 (1:1), 0.5X B27 without vitamin A (Vit.A), 0.5X N2, 1X PenStrep, 1X GlutaMAX, 1X NEAA) media containing 1 μ M THZ. By careful pipetting NPCs were plated onto the coverslip with the pre-seeded primary astrocytes and allowed to attach for 10 min. Then 2 ml of N2B27 media containing 1 μ M THZ was gently added to the well. To induce differentiation of the NPC the media was changed to terminal differentiation media (1X B27 without Vit. A, 1X Glutamax, 1X Pen Strep, 1X NEAA, 0.5 mM dbcAMP, 0.2 mM L-Ascorbic Acid, 1 μ M DAPT, 100 nM SR11237, freshly added: BDNF 10 ng/ml, GDNF 10 ng/ml, and 0.5% fetal bovine serum (FBS) in Neurobasal A) the next day. For the first 14 days, half of the media was changed every 3 days and 1 μ g/ml of mouse laminin was added to the media every second time of media change.

After day 14, half of the media was changed every 7 days and 1 μ g/ml of mouse laminin was added to the media every second time of media change. After growing the culture in terminal differentiation media for 7 days, Culture One treatment (1X) was added to the media for three weeks to stop the growth of undifferentiated progenitor cells.

2.1.2 Generation of Ngn2-induced neurons

The Neurogenin 2 (Ngn2) iPSC line (name: WTC11_G3) was differentiated into neurons with a two-step differentiation protocol (pre-differentiation and maturation) (Wang et al., 2017). For pre-differentiation (Days -3 to -1), iPSCs were seeded (Day -3) in KO-DMEM/F12 medium containing 1X N2 supplement, 1X NEAA, 1x GlutaMAX, 0.2 mg/mL mouse laminin, 10 ng/mL BDNF, 10 ng/mL neurotrophin-3 (NT3), 2 mg/mL doxycycline and 1 μ M THZ at a density of 200,000 cells/cm² in a 6-well plate coated with Matrigel. The medium (without THZ) was then changed daily for two more days, and THZ was removed from day-2. 300,000 cells of 2 mM Ara-C-inactivated primary human astrocytes were thawed and plated in 400 μ l astrocyte media onto the PLL/laminin-coated coverslip only and allowed to attach for 10 min. Then 1 ml of astrocyte media with 0.5% FBS was carefully added to the 6-well plate. For maturation at day 0, pre-differentiated precursor cells were

dissociated using ReLeSR, and plated at 150,000 cells/cm² density on astrocyte-feeder plates in maturation medium containing 50% DMEM/F12, 50% Neurobasal-A medium, 0.5X B27 supplement, 0.5X N2 supplement, 1X GlutaMax, 1X NEAA, 1 mg/mL mouse laminin, 10 ng/mL BDNF, 10 ng/mL NT3, 0.5% FBS and 1 μM THZ. Half of the medium was replaced on Day 1, Day 3, Day 7, and again on Day 14. Thereafter, half of the medium was replaced weekly until the cells were fixed for staining.

3. Characterization and validation of iPSC-derived neurons using Q-PCR by SYBR Green multi-well array

iPSCs and iPSC-derived neurons were characterized and validated using a previously established 96 - well SYBR Green qPCR expression array (Srinivasaraghavan et al., 2022). A confluent well from a 12-well plate of iPSCs (~2,500,000 cells) or one 6-well of iPSC-derived neurons (~500,000 cells) were dissociated with ReLesR. iPSCs were pelleted for 5 min by centrifugation (5,000 g at 4°C), washed with 1 ml of PBS again, followed by another 5 min centrifugation step (5,000 g at 4°C). RNA was then extracted using the Purelink™ RNA Mini Kit. To increase RNA yield, differentiated neurons were in some cases treated with lysis buffer directly on the plate without dissociating and pelleting cells first. Cells were washed with 1 ml PBS twice, then 300 μL of lysis buffer containing 3 μL of 2-mercaptoethanol was added directly to the well for 1-2 min at RT. The cell lysis was directly transferred to the homogenization tube. All subsequent steps of the Purelink™ RNA extraction protocol were identical for iPSCs and neurons. The volume of lysis buffer was adjusted based on cell numbers, according to the Purelink™ kit protocol. To eliminate contamination with genomic DNA, 1 μg of RNA were treated with DNase I for 15 min at RT followed by 65°C inactivation for 3 min and then directly used for cDNA reverse transcription. using the High-Capacity cDNA Reverse Transcription kit. Negative control was generated with nuclease-free water instead of RNA, and a reverse transcriptase control was generated with RNA but no reverse transcriptase. Afterward, 80 μL of nuclease-free water was added to each cDNA tube for a final concentration of 10 ng/μL and a final volume of 100 μL before storing at -20°C.

For the SYBR Green array, both forward and reverse primers (10 μM in nuclease-free water) of the target gene were mixed in a stock solution and coated at 0.3 μL (final concentration of 300 nM each primer) into a 384-well of an optical PCR plate in triplicates. On every plate, a negative control containing GAPDH primers and the negative control sample (mentioned above) was included. After finishing the pre-coating of all primers for all genes of interest, the 384 well-plate was centrifuged at 2,000 rpm for 2 min. Plates were then placed in a box or drawer and pre-coated primers were left to dry overnight at RT. The SYBR Green array reaction was run in a QuantStudio 6 Flex with cycling conditions as indicated in Supplemental Tables 9 and 10. A reaction mix containing 2.5 μL of PowerUp SYBR Green Master Mix, 1.5 μL of nuclease-free water, and 1 μL of sample cDNA at 10 ng/μL (final cDNA concentration of 2 ng/μL) was prepared per well. After pipetting the reaction mix to the wells with the pre-coated primers, the plate was sealed with optical adhesive film and centrifuged at 2,000 rpm for 2 min. The PCR conditions in Supplemental Table 9 are run first to amplify cDNA, while the conditions in Table 9 were run at the end to generate a melt curve from the resulting PCR product.

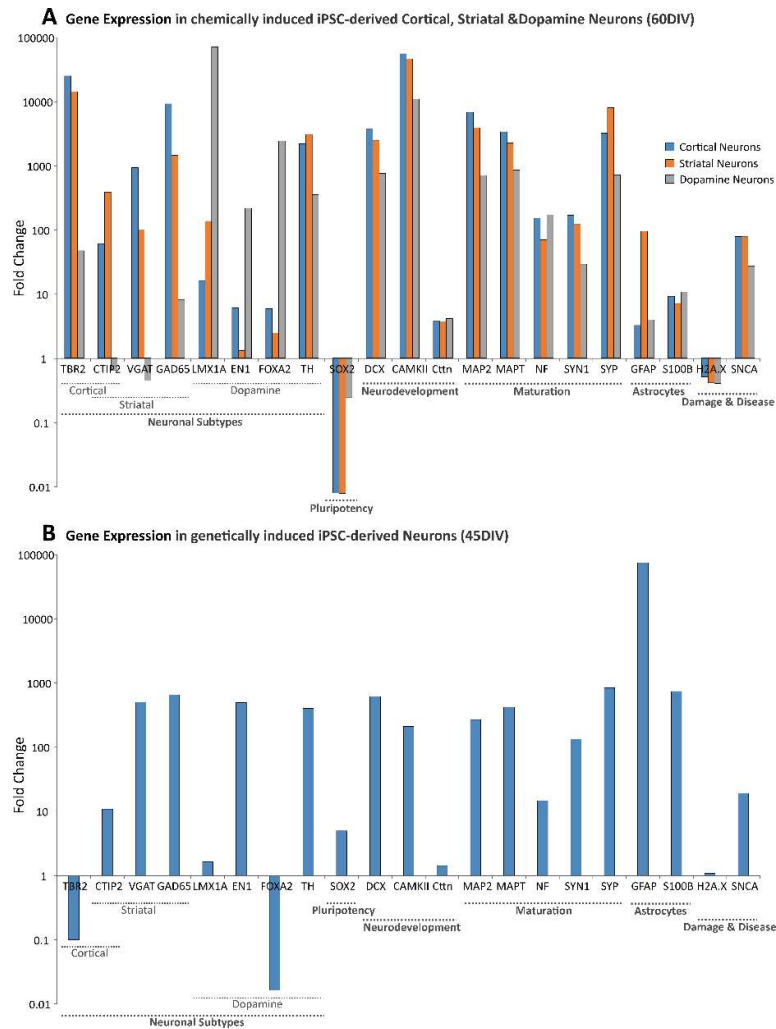
To quantify relative gene expression among different cell types, the fold change was calculated from cycle threshold (Ct) values. Mean Ct values for each gene and sample were calculated among each triplicate. ΔCt was then calculated using $\Delta Ct = \text{mean } Ct_{\text{target gene}} - \text{mean } Ct_{\text{housekeeping gene (GAPDH)}}$. $\Delta\Delta Ct$ was then calculated using $\Delta\Delta Ct = \Delta Ct_{\text{treated cell (neuron)}} - \Delta Ct_{\text{untreated cell (iPSC)}}$. Finally, fold change was calculated using $\text{Fold change} = 2^{-\Delta\Delta Ct}$. ΔCt represents the difference in gene expression between the gene of interest and the housekeeping gene. $\Delta\Delta Ct$ normalizes the relative gene expression to an

untreated control sample. For this study, the respective untreated iPSC line was used as a reference sample. Markers specific for various neuron types, pluripotency, neuronal maturation, and stress were used. Supplemental Table 8 enlists all primers and Supplemental Table 7 shows details on reagents and materials.

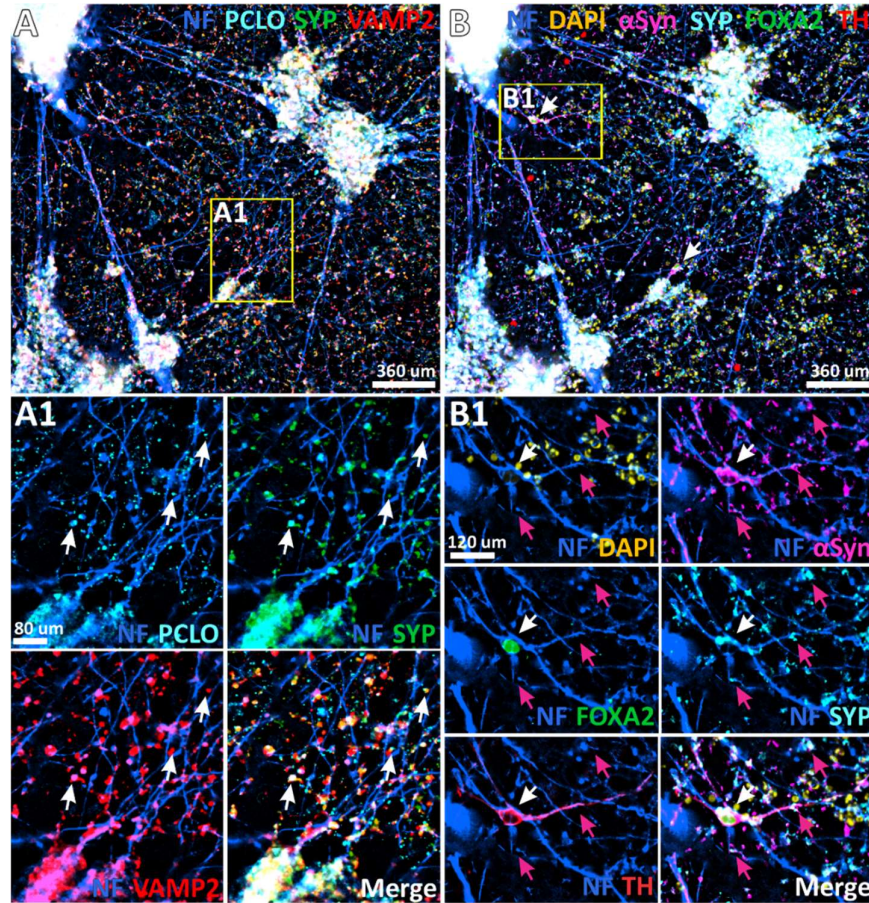
Supplementary Data

Supplemental Table 11: Comparative table of CODEX method for neurons and fresh frozen brain tissue

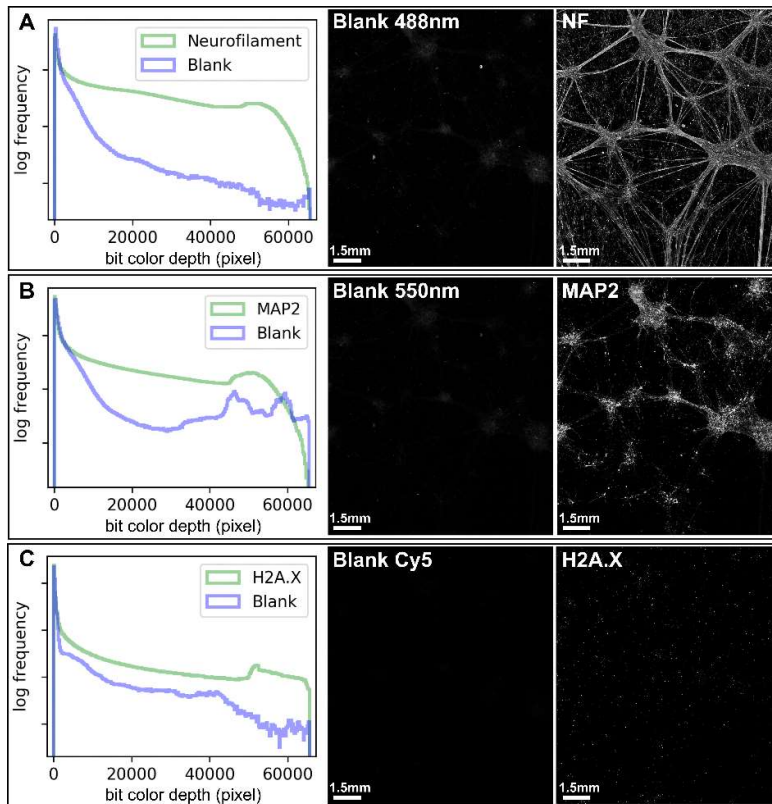
Step	CODEX/ neurons	CODEX/ tissue
Pre-fixation step	2% PFA/PBS for 10 min RT 4% PFA/PBS for 5 min RT	Not applicable
	PBS wash	Not applicable
Drying	DrieRite Beads 2 min RT	DrieRite Beads 2 min RT
Dehydration	Acetone 100% 10 min	Acetone 100% 10 min
Acetone removal	DrieRite Beads 2 min RT	DrieRite Beads 2 min RT
Hydration	Not applicable	Hydration buffer
Pre-staining fix	Pre-staining fix (hydration buffer/1.6% PFA) 10 min	Pre-staining fix (Hydration buffer/ 1.6% PFA)
Hydration	PBS wash	Hydration buffer 2 min RT
Quenching	PBS, 2 cycles 45 minutes LED RT	(PBS/4.5% H ₂ O ₂ / 2.6% 1M NaOH), 1 cycle of 45 min LED
	Not applicable	Distilled water
	Not applicable	Hydration buffer 2 min RT
	Staining buffer	Staining buffer 2 min RT



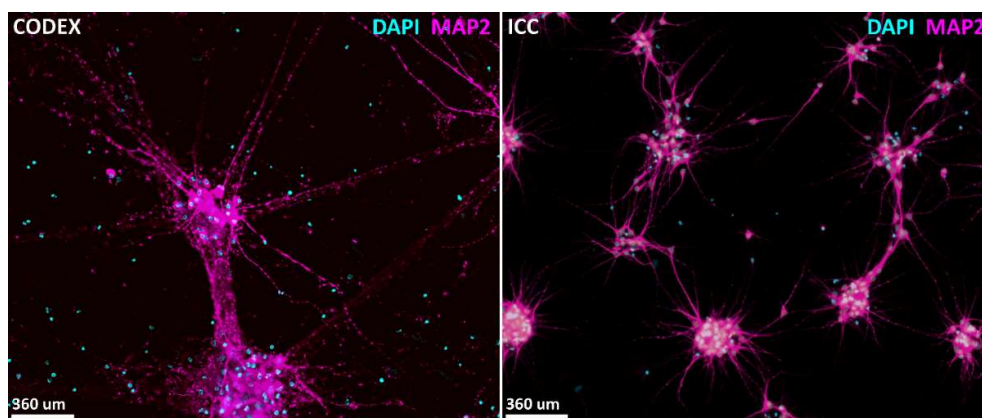
Supplemental Figure 1: Analyzing the gene expression of human iPSC-derived neurons using the SybrGreen assay. Gene expression was analyzed in human induced pluripotent stem cells (iPSCs)-derived neurons co-cultured with primary astrocytes and the undifferentiated iPSC line (reference sample; (A) KOLF2.1 or (B) Neurogenin 2 (WTC11_G3) iPSC line) by qPCR using the SYBR Green assay for the 21 genes of the CODEX panel. Expression of glyceraldehyde-3-phosphate dehydrogenase (GADPH) was measured as a loading control. Per gene, the cycle threshold (Ct) value was measured and ΔCt was calculated by normalizing the Ct value of the target gene to the Ct value of the loading control ($\Delta Ct = Ct$ of the target – Ct of the reference). Then $\Delta\Delta Ct$ was calculated by normalizing ΔCt of the target gene in our neuronal samples to ΔCt of the same gene in the undifferentiated iPSC line ($\Delta\Delta Ct = \Delta Ct(a\ target\ sample) - \Delta Ct(a\ reference\ sample)$). Finally, the fold change was calculated (fold change = $2^{-\Delta\Delta Ct}$). [A] Human KOLF2.1 iPSCs were differentiated into cortical (blue), GABAergic striatal (orange), or dopaminergic (grey) neurons using chemical induction. After 60 days in vitro (DIV), qPCR by SYBR Green assay was conducted to analyze gene expression. Gene expression was also measured in the undifferentiated KOLF2.1 iPSC line to calculate the fold change. [B] Inducible human Ngn2 iPSCs were differentiated into neurons. After 45 DIV qPCR by SYBR Green assay was conducted to analyze gene expression. Gene expression was also measured in the undifferentiated Ngn2 iPSC line to calculate the fold change.



Supplemental Figure 2: CODEX imaging of iPSC-derived neurons (70 DIV). Human KOLF2.1 iPSCs co-cultured with primary astrocytes were differentiated into a mixed culture of cortical, striatal, and dopaminergic neurons. Codex imaging is shown for 7 different markers. Scale bars represent either 360 μm, 120 μm, or 80 μm. **[A]** Representative image of Neurofilament (NEFL, blue), piccolo (PCLO, cyan), Synaptophysin (SYP, green), and the vesicle-associated membrane protein 2 (VAMP2, red) staining. **[A1]** Magnification of the selected area (yellow square) highlighting PCLO (cyan), SYP (green), and VAMP2 (red) colocalization (white arrows) in NEFL-positive cells. **[B]** Representative image of Neurofilament (NF, blue), DAPI (yellow), alpha-synuclein (αSyn, magenta), forkhead-box-protein A2 (FOXA2, green), SYP (cyan), and tyrosine hydroxylase (TH, red) staining. **[B1]** Magnification of selected area (yellow square) highlighting FOXA2 (green) and TH (red) colocalization (white arrows) in NEFL labeled neurons. DAPI staining was higher in potentially damaged cells (bright yellow dots) and was therefore detectable but difficult to see in nuclei of healthy neurons (e.g. FOXA2-positive nuclei).

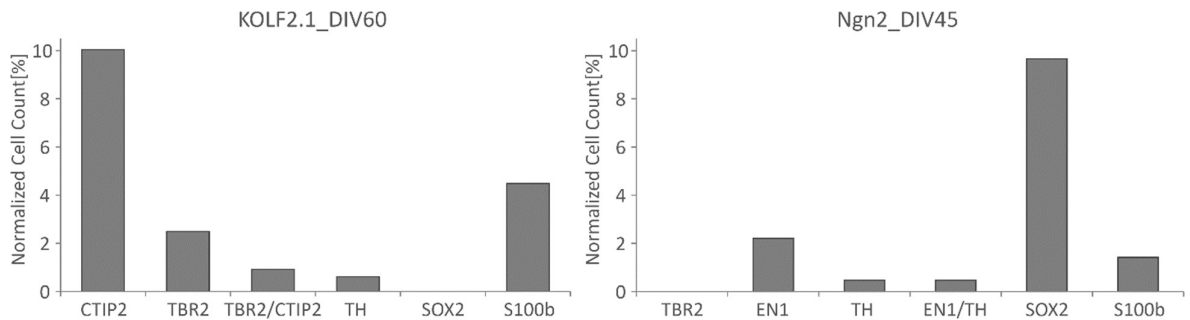


Supplemental Figure 3: Qualitative control data for representative CODEX staining on iPSC-derived neurons. Representative image analysis report(s) of CODEX stained human Ngn2 iPSCs co-cultured with primary human astrocytes and differentiated into neurons for 45 days. Histograms display pixel frequencies of signal intensities detected within the imaged region for the autofluorescence or 'Blank' (purple line; sample imaged without reporter incubation) and a marker (green line) for the three applied excitation wavelengths (**Panel A:** 488 nm, **Panel B:** 550 nm, and **Panel C:** Cy5). The x-axis covers the color pixels of the 16-bit color depth (0 ~ 65535). The y-axis gives the amount (frequency) of a given intensity pixel within the imaged region on a logarithmic scale (Akoya Biosciences, 2020). Representative images are shown of every respective Blank and marker with the scale bar representing 1.5 mm.

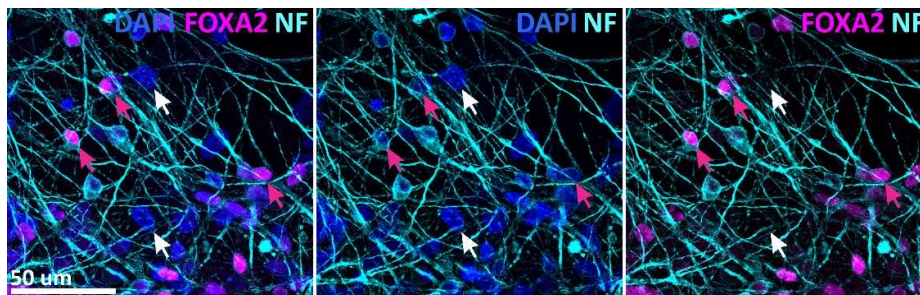


Supplemental Figure 4: Comparative images of MAP2 stained with CODEX versus standard immunocytochemistry on iPSC-derived cortical neurons. Human Ngn2 iPSCs were co-cultured with primary human astrocytes and differentiated into neurons. Scale bars represent 360 μ m. [CODEX] Cortical neurons were

differentiated for 45 days and stained for MAP2 (magenta) using the CODEX technique. DAPI (cyan) was used as a nuclear stain. [ICC] Cortical neurons differentiated for 21 days were stained for MAP2 (magenta) using standard immunocytochemistry. Hoechst (cyan) was used as a nuclear stain.



Supplemental Figure 5: Relative cell composition of iPSC-derived neurons co-cultures with primary human astrocytes. Different iPSC-derived neuronal cultures were stained using CODEX for various cell and neuronal subtype-specific markers. Relative proportion (y-axis) of positively stained cells for a given marker (x-axis) was quantified and normalized to the total cell count quantified by counting DAPI puncta. For quantification, the Image J software was used. [KOLF2.1_DIV60] KOLF2.1 iPSC-derived mixed culture of cortical, striatal, and dopaminergic neurons co-cultured with primary human astrocytes were differentiated for 60 days. [Ngn2_DIV45] Ngn2 iPSC-derived neurons co-cultured with primary human astrocytes were differentiated for 45 days and imaged with CODEX.



Supplemental Figure 6: Validation of FOXA2 antibody for CODEX multiplex imaging of iPSC-derived neurons (70DIV). Human iPSC-derived neural progenitor cells co-cultured with astrocytes were differentiated into neurons with dopaminergic identity. Codex imaging was conducted for 6 different markers using a confocal microscope equipped with a 63x oil-immersion lens. Scale bars represent either 50 μm. DAPI (blue), FOXA2 (magenta), and neurofilament (NF, cyan) staining. Arrows highlighting nuclei with (magenta arrows) or without (white arrows) FOXA2 staining.

References used in Supplements

- Arber, C., Precious, S. v., Cambray, S., Risner-Janiczek, J.R., Kelly, C., Noakes, Z., Marija, F., Heuer, A., Ungless, M.A., Rodriguez, T.A., Rosser, A.E., Dunnett, S.B., Li, M., 2015. Activin A directs striatal projection neuron differentiation of human pluripotent stem cells. *Development* 142, 1375–1386.
- Calatayud, C., Muñoz-Pedraza, E., Fernández-Gallego, S., Verstreken, P., 2021. Modular generation of cortical, striatal and ventral midbrain progenitor cells [WWW Document]. URL [dx.doi.org/10.17504/protocols.io.btmsnk6e](https://doi.org/10.17504/protocols.io.btmsnk6e) (Accessed 1.9.22).
- Kriks, S., Shim, J.W., Piao, J., Ganat, Y.M., Wakeman, D.R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., Yang, L., Beal, M.F., Surmeier, D.J., Kordower, J.H., Tabar, V., Studer, L., 2011. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480, 547–551. <https://doi.org/10.1038/nature10648>
- Moya, N., Cutts, J., Gaasterland, T., Willert, K., Brafman, D.A., 2014. Endogenous WNT signaling regulates hPSC-derived neural progenitor cell heterogeneity and specifies their regional identity. *Stem Cell Reports* 3, 1015–1028. <https://doi.org/10.1016/j.stemcr.2014.10.004>
- Shi, Y., Kirwan, P., Livesey, F.J., 2012. Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nature Protocols* 7, 1836–1846. <https://doi.org/10.1038/nprot.2012.116>
- Surmacz, B., Fox, H., Gutteridge, A., Lubitz, S., Whiting, P., 2012. Directing differentiation of human embryonic stem cells toward anterior neural ectoderm using small molecules. *Stem Cells* 30, 1875–1884. <https://doi.org/10.1002/stem.1166>
- Srinivasaraghavan, V.N., Zafar, F., Schüle, B., 2022. Gene Expression Analysis in Stem Cell-derived Cortical Neuronal Cultures Using Multi-well SYBR Green Quantitative PCR Arrays. *Bio-101* e4283. <https://doi.org/10.21769/BioProtoc.4283>