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ab259259 ioGlutamateric Neurons – Human iPSC-Derived Glutamatergic Neurons

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For conversion into consistent, mature, functional glutamatergic neurons providing a high quality human model for the study of neurological activity and disease.

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1. Overview

ioGlutamatergic Neurons rapidly mature into functional glutamatergic cortical neurons after revival in the recommended medium. They are delivered in a convenient cryopreserved format and provide a homogeneous and reproducible model for human excitatory neurons.

ioGlutamatergic Neuron cultures consist mainly of glutamatergic neurons (>85%) characterized by the expression of the glutamate transporter genes VGLUT1 and GLUT2. The minor remaining fraction of the neuronal population express marker genes of cholinergic neurons. A bulk RNAseq analysis shows that ioGlutamatergic Neurons have a rostral CNS identity and express the classical cortical maker genes FOXG1 and TBR1.

The protocol for the generation of ioGlutamatergic Neurons is a three-phase process. Phase 0 - Induction is carried out at bit.bio before distribution (Fig 1).

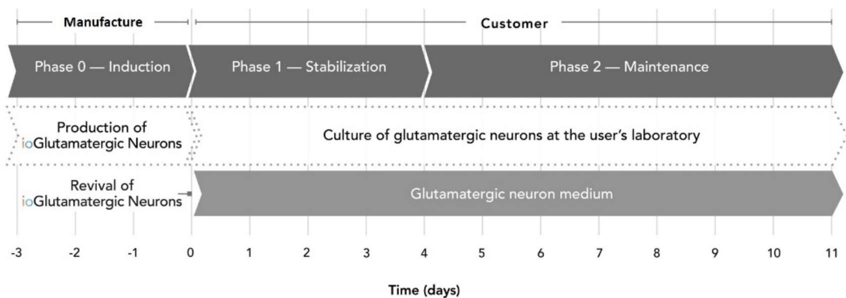


Figure 1 Schematic representation of the three-phase protocol to produce and culture ioGlutamatergic Neurons.

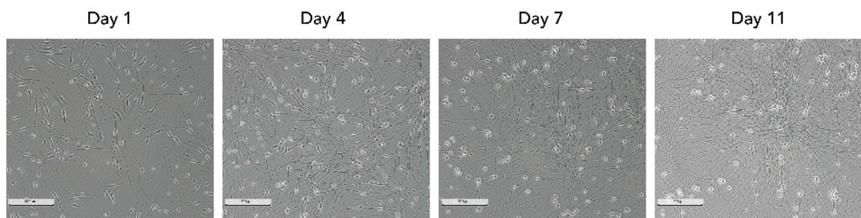
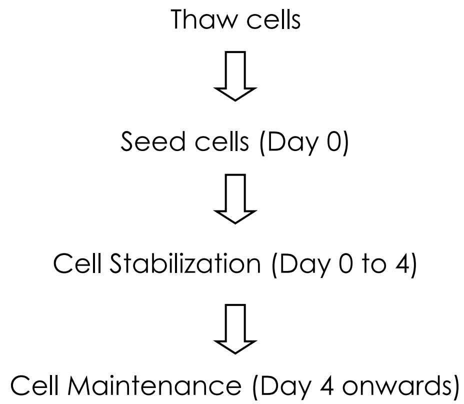


Figure 2 Photos of glutamatergic neurons after revival over the course of the first 11 days of culture (Day 1 to 11 post-thawing; 10X magnification; scale bar: 200µm).

Manufacture r	**Phase 0 — Induction (day -3 to 0):** Human iPSCs are exposed to a 3-day induction protocol. This leads to irreversible loss of pluripotency, and the synchronised homogenous production of glutamatergic neurons. The ioGlutamatergic Neurons are subsequently cryopreserved for distribution.
User	**Phase1 — Stabilization (day 0 to 4):** The ioGlutamatergic Neurons are revived at the user's laboratory using the recommended medium supplemented with doxycycline (96h) and DAPT (day 2-4) for sustained induction.
	Phase 2 — Maintenance (day 4 onwards): Depending on assay requirements, the ioGlutamatergic Neurons can be used over different lengths of time in the maintenance medium. Note: ioGlutamatergic Neurons have been maintained up to 28 days in the above conditions without impairment to neuronal health, function and culture attachment.

Table 1 Description of the three-phase protocol for the production and culture of ioGlutamatergic Neurons.

2. Protocol Summary



3. Materials Supplied and Storage

Transfer the vials of ioGlutamatergic Neurons to liquid nitrogen or to -150°C immediately after receipt.

Before the revival of ioGlutamatergic Neurons, prepare the tissue culture plates or flasks coated with PDL-Geltrex (refer to Section 6.3)

The recommended reagents for the revival and maintenance of ioGlutamatergic Neurons can be found in Section 4.

4. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully culture the cells:

– Biological Safety Cabinet
– Normoxic cell culture incubator (37°C, 5% CO_2)
– 37°C water bath or equivalent
– Haemocytometer or automatic cell counter
– Liquid Nitrogen Storage Unit
– Standard tissue culture wares (pipettes, tips, culture plates)
– Bench Top Centrifuge
– Geltrex (Reduced GF)
– PDL-hydrobromide
– Borate buffer (20x)
– Sterile water
– DMEM/F-12
– Neurobasal
– B27
– Glutamax
– 2-Mercaptoethanol
– NT3
– BDNF
– DAPT
– Doxycycline (ab141091)
– Bovine Serum Albumin
– Cytarabine (ara-C) (ab141924)

5. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For typical data produced using the cells, please see the ioGlutamatergic Neurons cells datasheet on our website.

6. Reagent Preparation

6.1 Preparation of stock solutions

Reagent	Stock Solution	Working concentration
NT3	50 µg/mL (5,000X solution) To prepare, reconstitute 25 ug in 500 µL of PBS containing 0.1% BSA	10 ng/mL 0.2 µL of stock solution per 1 mL of medium
BDNF	10 µg /mL (2,000X solution) To prepare, reconstitute 5 ug in 500 µL of PBS containing 0.1% BSA	5 ng/mL 0.5 µL of stock solution per 1 mL of medium
DAPT	20 mM (2,000X solution) To prepare, reconstitute 10 mg in 1156 µL of DMSO	10 µM 0.5 µL of stock solution per 1 mL of medium
Doxycycline (DOX) (ab141091)	2 mg/mL (2,000X solution) To prepare, reconstitute 20 mg in 10 mL of H_2O	1 µg/mL 0.5 µL of stock solution per 1 mL of medium
Cytarabine (ara-C) (ab141924)	20 mM (10,000X solution) To prepare, reconstitute 10 mg in 2 mL of water	2 µM 0.1 µL of stock solution per 1 mL of medium

6.2 Preparation of Glutamatergic Neuron Medium

b:GN: Basal Glutamatergic Neuron Medium

Reagent/Media	For 200 mL	For 500 mL
Neurobasal	200 mL	500 mL
Glutamax (100x)	2 mL	5 mL
2-Mercaptoethanol (Final conc. 25 µM)	100 µL	250 µL

Δ Note: The basal medium is stable for 3 weeks at 4°C; Pen/Strep antibiotics can be added if required.

comp:GN: Complete Glutamatergic Neuron Medium

Reagent/Media	For 50 mL	For 200 mL
b:GN	50 mL	200 mL
B27	1 mL	4 mL
NT3 (final conc. 10 ng/mL)	10 µL	40 µL
BDNF (final conc. 5 ng/mL)	25 µL	100 µL

Δ Note: The complete medium is better prepared fresh before each feeding. It is not recommended to use the complete medium for more than 4 days after preparation while stored at 4°C.

comp:GN+D: Doxycycline Supplemented Complete Glutamatergic Neuron Medium

Reagent/Media	For 10 mL	For 50 mL
comp:GN	10 mL	50 mL
Doxycycline (final conc. 1 µg/mL)	5 µL	25 µL

Δ Note: The supplemented complete medium is better prepared fresh before each feeding. It is not recommended to use the supplemented complete medium for more than 4 days after preparation while stored at 4°C.

comp:GN+D+DAPT:Doxycycline and DAPT Complete Supplemented Glutamatergic Neuron Medium

Reagent/Media	For 10 mL	For 50 mL
comp:GN	10 mL	50 mL
DAPT (final conc. 10 µM)	5 µL	25 µL
Doxycycline (final conc. 1 µg/mL)	5 µL	25 µL

Δ Note: The supplemented complete medium is better prepared fresh before each feeding. It is not recommended to use the supplemented complete medium for more than 4 days after preparation while stored at 4°C.

6.3 Preparation of the PDL-Geltrex coating solution and coated vessels

For the preparation of PDL-Geltrex coated plates, treat culture vessels first with PDL solution and subsequently with Geltrex according to the protocol below:

6.3.1 Preparation of PDL coating solution:

6.3.1.1 Make up 50 mL of 1x borate buffer, by diluting 2.5 mL of the 20x stock with 47.5 mL of sterile water.
6.3.1.2 Resuspend a 5 mg vial of PDL in 50 mL of the 1x borate buffer, for a working concentration of 100 µg/mL.
6.3.1.3 PDL coating solution can be kept at 20°C for long-term storage.

6.3.2 PDL coating:

6.3.2.1 Calculate the total surface area to be coated.
6.3.2.2 Coat the surface area of your culture vessel with the PDL coating solution. Recommended coating volumes are shown in the table below (circal 100 µL per cm^2).
6.3.2.3 Incubate the coated plates, overnight at 37°C or for at least 3h at 37°C.

6.3.2.4 Aspirate PDL solution and then wash 3 times with sterile water. For each wash, use the same volume used for coating.

6.3.2.5 Asiprate the water and allow coated surfaces to dry completely in a laminar flow hood (without lids). This typically requires 30-60 minutes.

6.3.2.6 Proceed with Geltrex coating as described below.

For the preparation of Geltrex aliquots and PDL-Geltrex coated plates, please follow the manufacturer's instructions. In brief:

6.3.3 Preparation of Geltrex aliquots:

6.3.3.1 Remove Geltrex stock aliquots from -80°C and thaw on ices in a 4°C fridge overnight. The next day, prepare aliquots according to foreseen use in order to minimize further freeze-thawing; store at -80°C.
Δ Note: Geltrex solidifies quickly at temperatures above 4°C — keep Geltrex on ice at all times.

6.3.3.2 Depending on the volume, the smaller aliquots should take about 30 minutes to thaw on ice.

6.3.4 Geltrex coating

6.3.4.1 Calculate the total surface area to be coated.

6.3.4.2 Dilute the Geltrex 1:100 in chilled DMEM/F12 (e.g. 100 µL in 10 mL)

6.3.4.3 Coat the surface area of your culture vessel with the Geltrex:DMEM/F12 coating solution.

6.3.4.4 We recommend the follow coating volumes (circa 100 µL per cm^2)

Coating solution	384 well	96 well	24 well	12 well	6 well	10 cm dish
Geltrex:DMEM	15 µL	50 µL	400 µL	500 µL	1 mL	6 mL

6.3.4.5 Incubate the coated plates at 37°C for a minimum of 60 minutes. At the time of use, we recommend keeping the plates at room temperature for one hour before aspirating. Carefully aspirate off the excess Geltrex, then immediately plate the cells.

Δ **Note:** For long-term storage of Geltrex-coated plates, maintain the excess Geltrex in each well and seal the plate with parafilm. Geltrex coated plates can be kept at 4°C for 1 month.

7. Assay Procedure

7.1 Culture of ioGlutamatergic Neurons:

7.1.1 Before starting:

- Each ioGlutamatergic Neurons vial contains either >0.75 $x10^6$ (small vial) or >1.5 $x10^6$ cells (large vial). A seeding density of 30,000 cells/cm^2 is recommended. Prepare enough tissue culture vessels with Geltrex coating prior to reviving the cryovial(s) (see Section 6.3).
- Warm-up the water bath to 37°C.
- Warm-up basal glutamatergic neuron medium (b:GN) medium to 37°C (see Section 6.2).
- Prepare the complete glutamatergic neuron (comp:GN) medium supplemented by 1 µg/mL Doxycycline (comp:GN+D) for revival (see Section 6.2).

7.1.2 Cell Thawing:

7.1.2.1 Remove the cryovial(s) from dry ice and immediately immerse into a 37°C waterbath (or similar) while maintaining a constant gentle agitation.

7.1.2.2 Remove the cryovial(s) from the water bath when only a very small ice cube is left visible (this should take approximately 1 minute).

7.1.2.3 Spray the cryovial(s) with 70% ethanol and take it to a biological safety cabinet.

7.1.2.4 Transfer the cells from each vial into a 15 mL tube containing 1 mL of b:GN medium.

Δ Note: Freezing medium contains DMSO: minimize the time between thawing and centrifugation of cells.

7.1.2.5 Add a further 3 mL of b:GN medium per tube in a dropwise manner.

7.1.2.6 Carefully wash the cryovial(s) with 1 mL of b:GN medium and add it to the tube(s).

7.1.2.7 Centrifuge the cells at 200 x *g* for 3 minutes at room temperature.

7.1.2.8 Carefully remove the supernatant by aspiration.

7.1.2.9 Add 3 mL of comp:GN+D medium to the cell pellet and gently resuspend the cells by pipetting up-and-down with a 1 mL micropipette.

7.1.2.10 Count the cells including a cell viability marker. The typical recovery from one cryovial is >0.75 x10^6 (small vial) or >1.5 x10^6 cells (large vial).

7.1.3 **Cell Seeding (Day 0)**:

7.1.3.1 Dilute the cell suspension to the required cell concentration using comp:GN+D medium to achieve the required seeding density for your desired experimental conditions (see Section 7.3 for 96 and 384 well plate culture). A seeding density of 30,000 cells/cm^2 is routinely used.

Plate format	Surface (cm^2)	mL/well	Cells/well	Cells/mL
6 well	9.5	2.5	285,000	114,000
12 well	3.8	1	114,000	114,000
24 well	1.9	0.5	57,000	114,000
48 well	0.95	0.25	28,500	114,000

7.1.3.2 Aspirate the Geltrex coating solution from the culture vessel(s).

7.1.3.3 Directly add the required volume of cell suspension to the culture vessel(s).

7.1.3.4 Immediately transfer the culture vessel(s) to a standard normoxic tissue culture humidified incubator at 37°C, 5% CO_2.

7.1.3.5 To ensure an even cell distribution, gently cross-shake the plate once on the incubator shelf (back and forth, side to side, 2-3 times).

7.1.4 **Cell Stabilization (Day 0 to 4) and Maintenance (Day 4 onwards)**:

7.1.4.1 Day 2: 48 hours post thawing, completely replace the culture medium with fresh pre-warmed comp:GN+D medium supplemented with 10 µM of DAPT (comp:GN+D+DAPT)

Δ Note: Culture of ioGlutamatergic Neurons should be carried out with special care as neuronal cells are prone to

mechanical stress which may cause detachment. It is recommended that for all medium replacements, medium aspiration and addition should be performed slowly and on the side of the well, using micropipettes instead of serological pipettes.

7.1.4.2 Day 4: 96 hours post thawing, completely replace the culture medium with fresh pre-warmed comp:GN medium (no Doxycycline).

7.1.4.3 Day 6 onwards: for optimal glutamatergic neuron maintenance, it is recommended a half-medium change regime every 48 hours, i.e., replacing 50% of the medium with fresh comp:GN medium (no Doxycycline).

7.2 Culture of ioGlutamatergic Neurons in 96 or 384 well plate:

The following protocol has been optimized for the revival and culture of ioGlutamatergic Neurons directly into 96 or 384 well plates. Note that the optimal cell seeding density will depend on the specific experimental aims defined by the user.

7.2.1 Pre-coat the required wells with PDL-Geltrex (see Section 6.3).

7.2.2 Thaw the cells as per the standard protocol described in section 7.1.2.

7.2.3 After the cell counting, adjust the cell suspension concentration using comp:GN+D medium to achieve the targeted seeding density (see table below for reference).

Seeding density (cells/cm²)	**384 well (0.056cm², 30 µL)**		**96 well (0.32cm², 100 µL)**	
	Cells/Well	**Cells/mL**	**Cells/Well**	**Cells/mL**
30,000	1.680	56,000	9,600	96,000
40,000	2,240	74,666	12,800	128,000
50,000	2,800	93,333	16,000	160,000

Δ Note: A seeding density between 30,000 to 50,000 cells/cm² in a final volume of 30 µL per 384 well or 100 µL per 96 well is recommended.

Δ Note: When calculating the total volume of cell suspension required, consider preparing 10% more to accommodate for volume losses during cell handling.

7.2.4 Aspirate the Geltrex coating medium from the plate.

7.2.5 Pour the adjusted seeding cell suspension into a reservoir suitable for multichannel pipettes.

7.2.6 Using a multichannel pipette, add 30 µL or 100 µL of the cell suspension into the wells of the 384 or 96 well plate.

7.2.7 Transfer the culture plate into the incubator at 37°C, 5% CO^2.

7.2.8 Day 2: 48 hours post-thawing completely replace the culture medium with fresh pre-warmed comp:GN+D+DAPT. Use the same volymes as used for seeding. Be gentle during the media replacement to avoid cell detachment.

7.2.9 Day 4: refresh the medium by adding the same amount of fresh pre-warmed comp:GN medium (no doxycycline). For example, if 30µl was used, add an extra 30µl for a final culture amount of 60µl.

7.2.10 Day 6 onwards: For optimal glutamatergic neuron maintenance, it is recommended to use a half-medium change regime every 48 hours ,i.e., replacing 50% of the medium with fresh comp:GN medium (no Doxycycline).

7.3 Co-culture of ioGlutamatergic Neurons with astrocytes:

Possible applications: e.g. to determine the electrophysiological properties of neurons.

7.3.1 Before starting:

- Prepare enough issue culture vessels with PDL-Geltrex coating prior to reviving the cryovial(s) (see Section 6.3). A 1:1 co-culture ratio of ioGlutamatergic Neurons and astrocytes is recommended, and a seeding density of 30,000 cells/cm^2 of each cell type (total of 60,000 cells/cm^2).
- Warm-up the water bath to 37°C.
- Warm-up basal glutamatergic neuron medium (b:GN) medium to 37°C (see Section 6.2)
- Prepare the complete glutamatergic neuron (comp:GN) medium supplemented by 1 µg/mL Doxycycline (comp:GN+D) for revival (see Section 6.2).
- Prepare astrocytes for seeding according to manufacturer/author's protocol, in parallel to section 7.3.2 of

this protocol. If not possible, prepare astrocytes first and keep the cell suspension in a standard normoxic tissue culture humidified incubator at 37°C, 5% CO_2, occasionally shaking the cell suspension. Make sure the cap of the vial or tube is not fully closed. Proceed immediately to section 7.3.2.

7.3.2 **ioGlutamatergic Neurons Thawing**:

7.3.2.1 Remove the cryovial(s) from dry ice and immediately immerse into a 37°C water bath (or similar) while maintaining a constant gentle agitation.
7.3.2.2 Remove the cryovial(s) from the water bath when only a very small ice cube is left visible (this should take approximately 1 minute).
7.3.2.3 Spray the cryovial(s) with 70% ethanol and take it to a biological safety cabinet.
7.3.2.4 Transfer the cells from each vial into a 15 mL tube containing 1mL of b:GN medium

Δ Note: Freezing medium contains DMSO: minimize the time between thawing and centrifugation of cells.

7.3.2.5 Add a further 3mL of b:GN medium per tube in a dropwise manner.
7.3.2.6 Carefully wash the cryovial(s) with 1mL of b:GN medium and add it to the tube(s).
7.3.2.7 Centrifuge the cells at 200 x *g* for 3 minutes at room temperature.
7.3.2.8 Carefully remove the supernatant by aspiration.
7.3.2.9 Add 3mL of comp:GN+ D medium to the cell pellet and gently resuspend the cells by pipetting up-and-down with a 1mL micropipette.
7.3.2.10 Count the cells including a cell viability marker. The typical recovery from one cryovial is >0.75 $x10^6$ (small vial) or >1.5 $x10^6$ cells (large vial).

7.3.3 **Cell Seeding (Day 0)**:

7.3.3.1 Resuspend astrocytes and ioGlutamatergic Neurons at the appropriate cell concentration in comp:GN+D medium to achieve the required seeding density for your desired experimental conditions. A seeding density of 30,000 cells/cm^2 of each cell type is routinely used.

7.3.3.2 Mix both cell suspensions to achieve a homogenous1:1 ratio mixed-cell suspension.

7.3.3.3 Aspirate the Geltrex coating solution from the culture vessel(s).

7.3.3.4 Directly add the required volume of cell suspension to the culture vessel(s).

7.3.3.5 Immediately transfer the culture vessel(s) to a standard normoxic tissue culture humidified incubator at 37°C, 5% CO_2.

7.3.3.6 To ensure an even cell distribution, gently cross-shake the plate once on the incubator shelf (back and forth, side to side, 2-3 times).

7.3.4 **Cell Stabilization (Day 0 to 4) and Maintenance (Day 4 onwards)**:

7.3.4.1 Day 2: 48 hours post thawing, completely replace the culture medium with fresh prewarmed comp:GN+D+DAPT medium.

Δ Note: Culture of ioGlutamatergic Neurons should be carried out with special care as neuronal cells are prone to mechanical stress which may cause detachment. It is recommended that for all medium replacements, medium aspiration and addition should be performed slowly and on the side of the well, using micropipettes instead or serological pipettes).

7.3.4.2 Day 4: 96 hours post thawing, completely replace the culture medium with fresh prewarmed comp:GN medium (no Doxycycline).

Δ Note: A single addition of 2 µM Cytarabine (ara-C) to arrest further astrocyte growth is recommended at this stage.

7.3.4.3 Day 6 onwards: for optimal astrocyte-glutamatergic neuron co-culture maintenance, it is recommended to use a half-medium change regime every 48 hours ,i.e., replacing 50% of the medium with fresh comp:GN medium (no Doxycycline).

Δ Note: Co-cultures have been carried out with primary astrocytes derived from P0-P2 neonatal Spraque Dawley rats. These astrocytes have demonstrated good long-term survival and functionality in comp:GN medium, without the need for

fetal bovine serum (FBS) in the medium. Co-culture of glutamatergic neurons with rat astrocytes have demonstrated good survival up to 100 days post-thawing, with first instance of electrophysiological activity at 8 days (+/- 2 days) post-thawing. If using astrocytes from other sources, such as human PSC-derived astrocytes, comp:GN may need to be supplemented with growth factors recommended in manufacturer/author's protocol.

8. Typical Data

Data provided **for demonstration purposes only**.

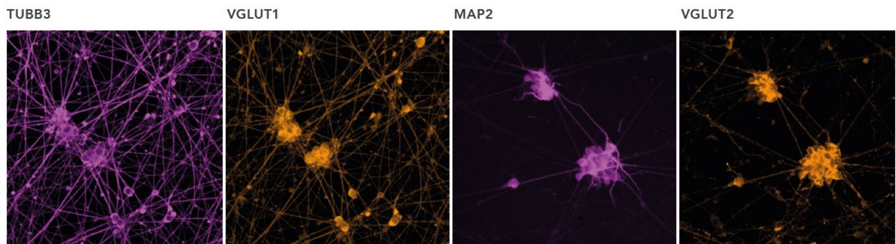


Figure 3. Immunofluorescent staining on post-revival day 11 demonstrates homogenous expression of pan-neuronal proteins (MAP2 and TUBB3) and glutamatergic neuron-specific transporters (VGLUT1 and VGLUT2). Cells exhibit neurite outgrowth.

9. Notes

Technical Support

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