ioGlutamatergic Neurons

User Manual

ioGlutamatergic Neurons
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User Manual
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Before starting

- bit.bio recommends reading this protocol in its entirety before the revival of the glutamatergic neurons, referred to as ioGlutamatergic Neurons throughout this user manual.
- 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 Geltrex (Appendix 5.5).
- The recommended reagents for the revival and maintenance of ioGlutamatergic Neurons can be found in Appendix 3.3.

Notes:

- ioGlutamatergic Neurons are for Research and Development use only. User agrees to use the Product in compliance with all applicable statutes and regulations, but not to use the Product for any administration or application to humans. Moreover, User agrees not to use the Product in human subjects for human clinical use for therapeutic, diagnostic or prophylactic purposes, or in animals for veterinary use for therapeutic, diagnostic or prophylactic purposes, including but not limited to clinical applications, cell therapy, transplantation, and/or regenerative medicine without an appropriate license.
- ioGlutamatergic Neurons are cryopreserved in DMSO: the safety data sheet (SDS) of dimethyl sulfoxide (DMSO) is available on request.
- ioGlutamatergic Neurons should only be used by personnel qualified in handling human biological materials following local health and safety regulations.

1. Overview of ioGlutamatergic Neurons

bit.bio glutamatergic neurons, referred to as ioGlutamatergic Neurons throughout this user manual, rapidly mature into functional glutamatergic 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 Neurons cultures consist mainly of glutamatergic neurons (>80%) characterised by the expression of the glutamate transporter genes VGLUT1 and VGLUT2. The minor remaining fraction of the neuronal population express marker genes of cholinergic neurons. A bulk RNA-sequencing analysis shows that ioGlutamatergic Neurons have a rostral CNS identity and express the classical cortical marker genes FOXG1 and TBR1.

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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 1A).

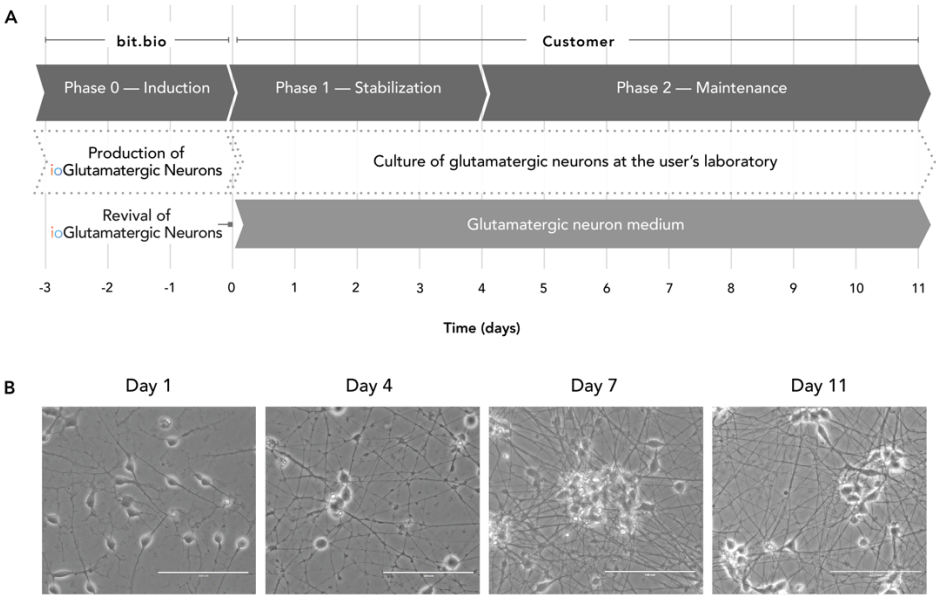

Figure 1
A. Schematic representation of the three-phase protocol to produce and culture ioGlutamatergic Neurons. B. Photos of glutamatergic neurons after revival over the course of the first 11 days of culture (Day 1 to 11 post-thawing; 400X magnification; scale bar: 100µm).

bit.bio	**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	**Phase 1 – Stabilization (day 0 to 4):** The ioGlutamatergic Neurons are revived at the user's laboratory using the recommended medium supplemented with doxycycline (96h) for sustained induction. **Phase 2 – Maintenance (day 4 to 11):** Depending on assay requirements, the ioGlutamatergic Neurons can be used over different lengths of time in the maintenance medium (typically up to day 11 after revival).

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

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2. Culture of ioGlutamatergic Neurons

Before starting:

- Each ioGlutamatergic Neurons vial contains either ≥0.75.0x10^6 (Small vial) or ≥1.5x10^6 (Large vial) viable cells. bit.bio recommends a seeding density of 30,000 cells/cm^2. Prepare enough tissue culture vessels with Geltrex coating prior to reviving the cryovial(s) (Appendix 5.5).
- Warm-up the water bath to 37°C.
- Warm-up **basal glutamatergic neuron (b:GN) medium** to 37°C (Appendix 5.4).
- Prepare the **complete glutamatergic neuron (comp:GN) medium** supplemented by **1µg/mL doxycycline** (D) **(comp:GN+D)** for revival (Appendix 5.4).

Revival and culture procedures:

2.1 – Cell thawing

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.
2. Remove the cryovial(s) from the water bath when only a very small ice cube is left visible (this should take approximately 1min).
3. Spray the cryovial(s) with 70% ethanol and take it to a biological safety cabinet.
4. Transfer the cells from each vial into a 15mL tube containing 1mL of **b:GN medium** (⚠Freezing medium contains DMSO: minimise the time between thawing and centrifugation of cells).
5. Add a further 3mL of **b:GN medium** per tube in a dropwise manner.
6. Carefully wash the cryovial(s) with 1mL of **b:GN medium** and add it to the tube(s).
7. Centrifuge the cells at 200g for 3 min at room temperature.
8. Carefully remove the supernatant by aspiration.
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.
10. Count the cells including a cell viability marker. The typical recovery from one cryovial is ≥0.75.0x10^6 viable cells (Small vial) or ≥1.5x10^6 viable cells (Large vial).

2.2 – Cell seeding (Day 0)

11. Dilute the cell suspension to the required cell concentration (table below for reference) using **comp:GN+D medium** to achieve the optimal seeding density for your desired experimental conditions (Section 3 for 96 and 384 well plate cultures). A seeding density of 30,000 cells/cm^2 is routinely used at bit.bio.

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

12. Aspirate the Geltrex coating solution from the culture vessel(s).
13. Directly add the required volume of cell suspension to the culture vessel(s).
14. Immediately transfer the culture vessel(s) to a standard normoxic tissue culture humidified incubator at 37°C, 5% CO_2.
15. 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).

2.3 – Cell stabilization (Day 0 to 4) and maintenance (Day 4 to 11)

16. Day 2: 48h post thawing, completely replace the culture medium with fresh pre-warmed **comp:GN medium** supplemented with **1µg/mL doxycycline**. (⚠Culture of ioGlutamatergic Neurons should be carried out with special care as neuronal cells are prone to mechanical stress which may cause detachment. bit.bio recommends 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).
17. Day 4: 96h post thawing, completely replace the culture medium with fresh pre-warmed **comp:GN medium** (no doxycycline).
18. Day 6-11: for optimal glutamatergic neuron maintenance, bit.bio recommends half-medium change every 48h, i.e., replacing 50% of the medium with fresh **comp:GN medium** (no doxycycline).

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3. Culture of ioGlutamatergic Neurons in 96 or 384 well plates

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.

1. Pre-coat the required wells with Geltrex (Appendix 5.5).
2. Thaw the cells as per the standard protocol described in section 2.1.
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)	384well (0.056cm², 30 µL)		96well (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

Recommendation: bit.bio recommends a seeding density between 30,000 to 50,000 cells/cm^2 in a final volume of 30µL per 384 well or 100µl per 96 well.
Note: when calculating the total volume of cell suspension required, consider preparing 10% more to accommodate for volume losses during cell handling.

4. Aspirate the Geltrex coating medium from the plate.
5. Pour the adjusted seeding cell suspension into a reservoir suitable for multichannel pipettes.
6. Using a multichannel pipette, add 30µL or 100µL of the cell suspension into the wells of the 384 or 96 well plate.
7. Transfer the culture plate into the incubator at 37°C, 5% CO_2.
8. 48h post-seeding, completely replace the culture medium with fresh pre-warmed **comp:GN medium** supplemented with **1µg/mL doxycycline**. Be gentle during the media replacement to avoid cell detachment. (bit.bio recommends using 30µl per 384 wells and 100µl per 96 well).
9. 96h post-seeding, refresh the medium by adding the same amount of fresh pre-warmed **comp:GN medium** (no doxycycline). For example, if 30µl was used in step 8, add an extra 30µl for a final culture amount of 60µl.
10. For optimal glutamatergic neuron maintenance, bit.bio recommends a half-medium change regime every 48h, i.e., replacing 50% of the medium with fresh **comp:GN medium** (no doxycycline).

Note: the maintenance protocol can be adapted according to the user's experimental requirements (e.g. no further medium change after step.8 and exclusion of doxycycline from step.8 medium for early compound screening experiments).

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4. Co-culture of ioGlutamatergic Neurons with Astrocytes

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

Before starting:

- Prepare enough tissue culture vessels with Geltrex coating prior to reviving the cryovial(s) (Appendix 5.5). bit.bio recommends a 1:1 co-culture ratio of ioGlutamatergic Neurons and astrocytes, 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 (b:GN) medium** to 37°C (Appendix 5.4).
- Prepare the **complete glutamatergic neuron (comp:GN) medium** supplemented by **1µg/mL doxycycline (comp:GN+D)** for revival (Appendix 5.4).
- Prepare astrocytes for seeding according to manufacturer/author's protocol, in parallel to section 4.1 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 4.1.

Revival and culture procedures:

4.1 – ioGlutamatergic Neurons thawing

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.
2. Remove the cryovial(s) from the water bath when only a very small ice cube is left visible (this should take approximately 1min).
3. Spray the cryovial(s) with 70% ethanol and take it to a biological safety cabinet.
4. Transfer the cells from each vial into a 15mL tube containing 1mL of **b:GN medium** (⚠Freezing medium contains DMSO: minimise the time between thawing and centrifugation of cells).
5. Add a further 3mL of **b:GN medium** per tube in a dropwise manner.
6. Carefully wash the cryovial(s) with 1mL of b:GN medium and add it to the tube(s).
7. Centrifuge the cells at 200g for 3 min at room temperature.
8. Carefully remove the supernatant by aspiration.
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.
10. Count the cells including a cell viability marker. The typical recovery from one cryovial is ≥0.75.0x10^6 viable cells (Small vial) or ≥1.5x10^6 viable cells (Large vial).

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4.2 – Cell seeding (Day 0)

11. 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 at bit.bio.
12. Mix both cell suspensions to achieve a homogenous 1:1 ratio mixed-cell suspension.
13. Aspirate the Geltrex coating solution from the culture vessel(s).
14. Directly add the required volume of cell suspension to the culture vessel(s).
15. Immediately transfer the culture vessel(s) to a standard normoxic tissue culture humidified incubator at 37°C, 5% CO_2.
16. 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).

4.3 – Cell stabilization (Day 0 to 4) and maintenance (Day 4 to 11)

17. Day 1: 24h post thawing, completely replace the culture medium with fresh prewarmed **comp:GN medium** supplemented with **1µg/mL doxycycline** (comp:GN+D medium). (⚠Culture of ioGlutamatergic Neurons should be carried out with special care as neuronal cells are prone to mechanical stress which may cause detachment. bit.bio recommends 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).
18. Day 2: 48h post thawing, completely replace the culture medium with fresh pre-warmed **comp:GN+D medium**.
19. Day 4: 96h post thawing, completely replace the culture medium with fresh pre-warmed **comp:GN medium** (no doxycycline).

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

20. Day 6-11: for optimal astrocyte-glutamatergic neuron co-culture maintenance, bit.bio recommends a half-medium change regime every 48h, i.e., replacing 50% of the medium with fresh **comp:GN medium** (no doxycycline).

    Note: bit.bio 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 foetal 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.

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5. Appendices

5.1 – Equipment required

- 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

5.2 – Recommended reagents

Reagent	Supplier	Cat. number	Storage
Geltrex (Reduced GF)	ThermoFisher	A1413202	-20°C to -80°C
DMEM/F-12	ThermoFisher	11330032	2°C to 8°C
Neurobasal	ThermoFisher	21103049	2°C to 8°C
B27	ThermoFisher	17504044	-20°C to -80°C
Glutamax	ThermoFisher	35050061	2°C to 8°C
2-Mercaptoethanol	ThermoFisher	31350010	2°C to 8°C
NT3	R&D	267-N3-025	-20°C to -80°C
BDNF	R&D	248-BDB-005	-20°C to -80°C
Doxycycline	Sigma	D9891	2°C to 8°C
Bovine Serum Albumin	Sigma	A7906	-20°C to -80°C
Cytarabine (ara-C)	Sigma	C1768	-20°C to -80°C

5.3 – Preparation of stock solutions

Note: to avoid freeze-thaw cycles, aliquot the stock solutions as appropriate for future use.

Reagent	Stock solution	Working concentration
NT3	50µg/mL (5000X solution) *To prepare, reconstitute 25µg in 500µL of PBS containing 0.1% BSA*	10ng/mL *0.2µl of stock solution per 1mL of medium*
BDNF	10µg/mL (2000X solution) *To prepare, reconstitute 5µg in 500µL of PBS containing 0.1% BSA*	5ng/mL *0.5µl of stock solution per 1mL of medium*
Doxycycline (DOX)	2mg/mL (2000X solution): *To prepare, reconstitute 20mg in 10mL of H_2O*	1µg/mL *0.5µL of stock solution per 1mL of medium*
Cytarabine (ara-C)	20mM (10,000X solution) *To prepare, reconstitute 10mg in 2mL of water*	2µM *0.1µL of stock solution per 1mL of medium*

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5.4 – Preparation of glutamatergic neuron medium

- **b:GN: basal** glutamatergic neuron medium

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

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

Note: The complete medium is better prepared fresh before each feeding. bit.bio does not recommend using the complete medium for more than 4 days after preparation while stored at 4°C.

- **comp:GN+D: supplemented** complete glutamatergic neuron medium

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

Note: The supplemented complete medium is better prepared fresh before each feeding. bit.bio does not recommend using the supplemented complete medium for more than 4 days after preparation while stored at 4°C.

5.5 – Preparation of the Geltrex coating solution and coated vessels

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

Preparation of Geltrex aliquots:

1. Remove Geltrex stock aliquots from -80°C and thaw **on ice in a 4°C fridge overnight**. The next day, prepare aliquots according to foreseen use in order to minimise further freezethawing; store at -80°C. (⚠ Note: Geltrex solidifies quickly at temperatures above 4°C – keep Geltrex on ice at all times)
2. Depending on the volume, the smaller aliquots should take about **30 minutes** to thaw while kept on ice.

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Geltrex coating:

1. Calculate the total surface area to be coated.
2. Dilute the **Geltrex** 1:100 in **chilled** DMEM/F12 (e.g. **100µL** in **10mL**).
3. Coat the surface area of your culture vessel with the Geltrex:DMEM coating solution. We recommend the follow coating volumes (circa **100µL per cm²**):

Coating solution	384well	96well	12well	6well	T25 flask	10cm dish
Geltrex:DMEM	15µL	50µL	500µL	1mL	3mL	6mL

4. 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.

Note: Geltrex coated plates can be kept at 4°C for 1 month if sealed with parafilm.

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Origin

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