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For any questions, contact our experts at technical@bit.bio

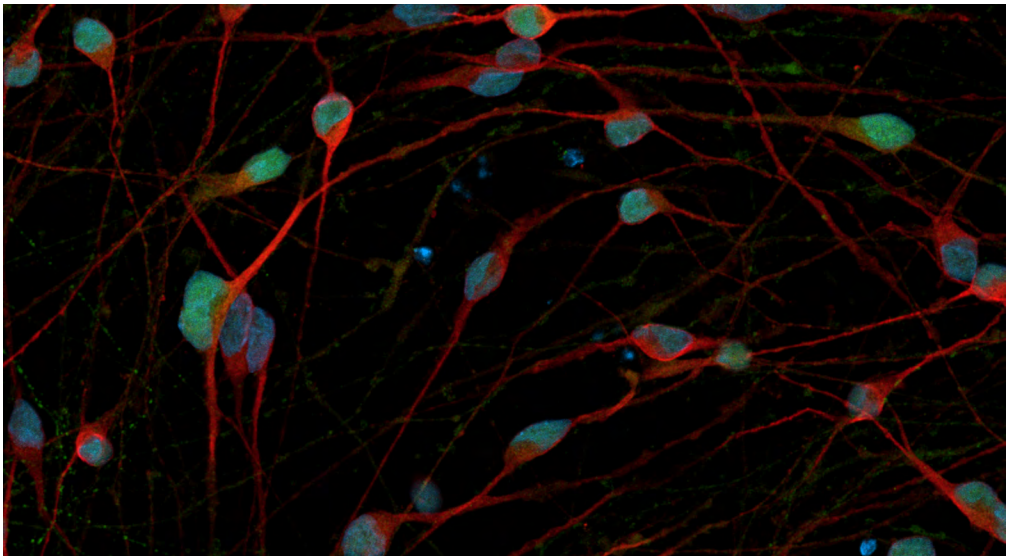
Cell culture hacks

Human iPSC-derived GABAergic Neurons

Human iPSC-derived inhibitory GABAergic neurons offer scientists a valuable in vitro system to better understand neurological circuitry, neurodevelopmental disorders, and neurodegenerative diseases. Understanding the practical nuances of human iPSC-derived GABAergic neuron cell culture, including bit.bio's ioGABAergic Neurons, can help you achieve success with your experiments.

In this blog, Christopher Aruthan, a Lead Production Scientist at bit.bio who works extensively with ioGABAergic Neurons, shares his first-hand cell culture experience of these inhibitory neurons, and offers his top tips for cell culture success.

By adding neuronal cell culture media slowly to thawing cryopreserved cells, correctly coating cell culture surfaces, maintaining a constant temperature after plating out cells, and checking cells under the microscope regularly, you can realise cell culture success – getting more robust cell cultures and reliable results from your downstream experiments!



What are GABAergic neurons?

20-30% of the brain's cerebral cortex is composed of two types of neurons - excitatory, like glutamatergic neurons, and inhibitory, like GABAergic neurons (Figure 1).[1] "The main job of inhibitory neurons is to inhibit the excitation caused by excitatory neurons, so that the neurons in our body aren't constantly firing - providing an excitation-inhibition balance that enables the brain to integrate and process information," explains Christopher.

GABAergic neurons make the main inhibitory neurotransmitter found in the central nervous system, called gamma-aminobutyric acid (GABA). GABA helps regulate brain network connectivity, activity and plasticity. When excitatory neurons constantly fire and aren't kept in check, which can be caused by poor GABAergic neuron performance, serious and life-threatening neurological and neurodegenerative disorders can arise. These include Alzheimer's disease, autism spectrum disorder, Parkinson's disease, epilepsy, and schizophrenia.[2,3]

About Christopher Aruthan
Christopher Arunthan is a Senior Scientist with 9 years total experience in the biotechnology industry, and 4 years of experience working in the manufacturing department at bit.bio. He specialises in the optimisation and execution of protocols for the manufacturing and utilisation of human iPSC derived cell types. He was involved in the launch of ioGABAergic Neurons product and is responsible for overseeing the manufacture and launch of several cell types within bit.bio's ioCell portfolio.

Scientists studying neurodevelopmental disorders often share with the bit.bio team that one of their biggest challenges is the generation of pure, consistent populations of GABAergic neurons, as commonly used directed differentiation protocols generate mixed neuronal populations that could contain excitatory neurons.[6,7] ioGABAergic Neurons form a >99% pure population of inhibitory GABAergic neurons as shown by single cell RNA-sequencing (Figure 2) so researchers can be confident in attributing their results to the inhibitory neurons in their culture. However, if you are used to working with immortalised cell lines, you need to keep in mind that GABAergic neurons are considerably more sensitive and receptive to even small environmental changes. These cells are highly fragile with their long axon projections, and they also have different metabolic needs.

Christopher adds, "In the body, neurons have a supportive environment around them, including other cell types that exchange cellular signals and provide structural support. When grown in mono-culture, in vitro, they lack the support from other types of cells such as astrocytes or oligodendrocytes, making them more fragile."

Follow these tips from Christopher to prepare yourself for some of the common pitfalls of GABAergic neuron cell culture and to maximise your chance of experimental success when using these cells!

TOP TIP 1

Add neuronal cell culture media gently and slowly when thawing cells to improve cell survival

Cell thawing is the most crucial part of many neuronal cell culture methods and protocols. I try not to rush when thawing iPSC-derived GABAergic neurons, as this can cause osmotic shock if media is added too quickly. The trick is to be as gentle as possible. I add the thawed cell suspension to a first millilitre of room temperature DMEM/F12 to the cells, before adding the remaining media slowly to ensure there is no osmotic shock.

Gentle handling throughout your cell culture can also improve cell survival and prevent detachment. I change media using the slowest speed on electric pipette controllers to help prevent cell detachment.

Also, never, ever use an aspirator during the maintenance phase! Instead, I slowly remove spent media manually with a P1000 pipette or, ideally, with a mechanical pipette at the slowest speed possible. Importantly, I introduce new pre-warmed media to cells from the side of the culture plate - not into the centre, again at the slowest speed possible. The culture plate should also be kept horizontal and never tilted during maintenance, to prevent the neurons from detaching.

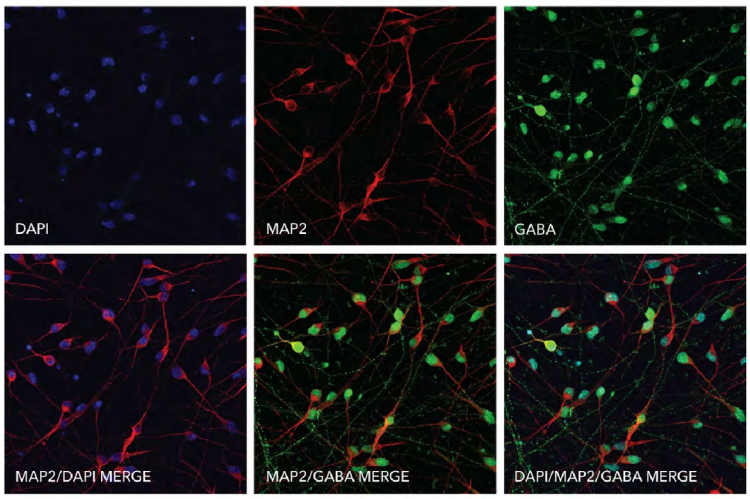


Figure 1. Immunofluorescent staining of ioGABAergic Neurons at day 12 post-revival. The upper panel shows that ioGABAergic Neurons are positive for the pan-neuronal marker MAP2 (red), the key GABAergic neuron marker GABA (green), and the DAPI counterstain (blue). The lower panel shows that all MAP2 positive neurons have a GABAergic neuronal identity.

TOP TIP 2

Prevent sudden temperature changes to promote even cell distribution and attachment

Giving human iPSC-derived GABAergic neurons more time to settle at a constant temperature can promote a more even and successful cell attachment and distribution across cell culture surfaces. If the cells experience sudden temperature changes while settling, thermal gradients can occur across the plate, potentially causing 'edge effects,' when cells settle along the edges of wells or cell culture plates. Edge effects can increase noise and variability in cell-based assays.[4]

To avoid edge effects during cell seeding, I pre-warm media and the coated culture vessel(s) to 37°C and work efficiently, ensuring a uniform distribution of the cells. When plating out human iPSC-derived GABAergic neurons, I carefully move the culture vessel forward and backwards and side to side, about three times by hand to ensure an even distribution of cells.

As the neurons can easily detach, beginning with their neural processes, be sure to use extra care when handling them. After placing the culture plate in the incubator, I allow at least two hours for attachment before checking on the neurons again. This provides the neurons time in which to attach undisturbed. Things to look out for include cell clumping and clusters of floating cells, which can be linked to issues with handling, reagents or incubation conditions.

When preparing co-cultures of inhibitory neurons and astrocytes, seeding co-cultures as a mixture of cell types is advised rather than seeding one cell type at a time. With the support of astrocytes, GABAergic neurons reach synchronous electrical activity more quickly.

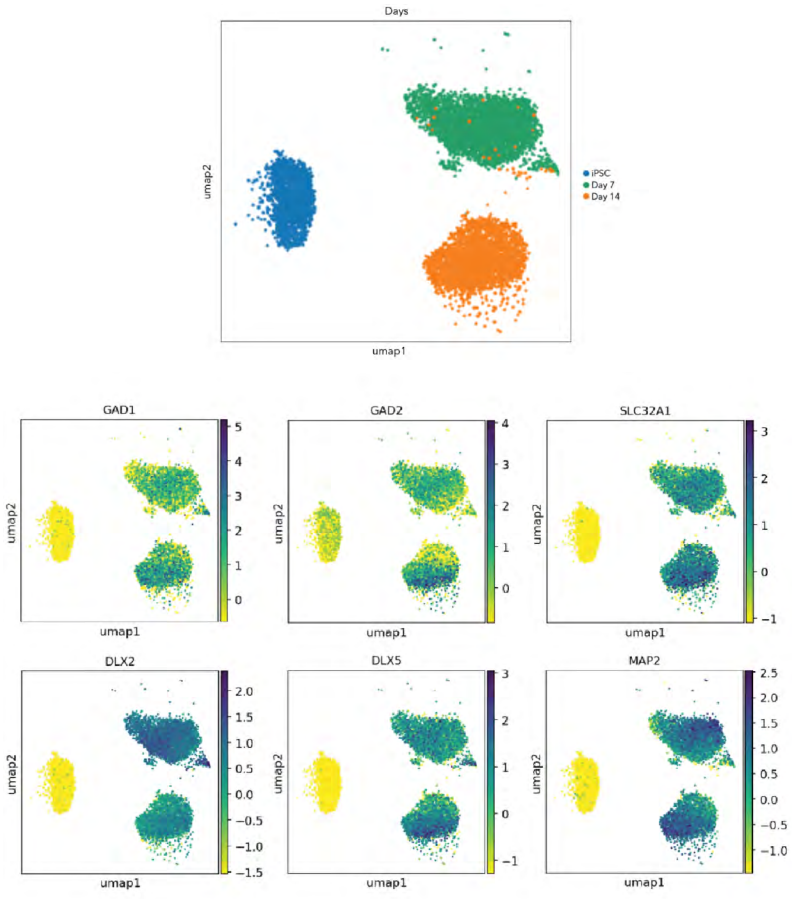


Figure 2. Single-cell RNA-sequencing analysis was performed with ioGABAergic Neurons at three specific timepoints (iPSC stage, days 7 and 14). By day 7, the expression of key GABAergic neuron marker genes [GAD1, GAD2, SLC32A1 (VGAT), DLX2, DLX5], together with the pan-neuronal marker MAP2, could be detected in post-mitotic GABAergic neurons. Gene expression was assessed by 10x Genomics scRNA-sequencing. (Note, this data is from cells in continuous culture, so minor variations may exist between this data and data from cryopreserved cells).

TOP TIP 3

Regularly check cells under the microscope to spot problems early

When I am culturing GABAergic neurons, I check my cells under the microscope at every point to make sure they are attaching to the culture plate nicely. For critical experiments, I come back about a couple of hours after initial seeding to check on the attachment of the cells. Cells can clump together if you've not initially disassociated your cells sufficiently while resuspending them, during the thawing stage, or after spinning them down in a centrifuge. Constantly checking on them saves time in the long run by not putting your experiments at risk.

Given the high (>99%) purity of ioGABAergic Neurons, if you do see clumping under the microscope, it's likely not a cluster of different cell types but a suboptimal resuspension of cells after the initial centrifugation step or suboptimal plate movement for cell distribution after the plating step. It is unlikely, but if you do ever see clumping then you stand a much better chance of success in your experiments by disposing of those cell culture plates and starting fresh with a new vial of cells.

Multi-electrode assays (MEA) are helpful for assessing the functionality of human iPSC-derived GABAergic neurons as they are non-invasive and measure electrophysiological activity[5]. In terms of checking opaque MEA cell culture surfaces to see if cells are healthy and evenly distributed, which is difficult to do using normal light microscopes. I advise to set up a transparent culture plate in parallel. If the cells are dead or clumped on your transparent plate, it's likely that they're exactly the same on the MEA plate.

Additionally, if possible, I use a fluorescent probe for the detection of live neurons (NeuroFluor™ NeuO) at 4 days post-plating. This allows the cells to be viewed on an MEA plate via fluorescence microscopy in an inverted microscope, viewing from the top of the culture (see Figure 3).

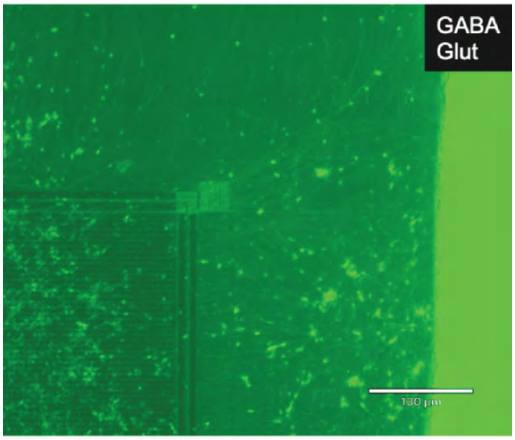


Figure 3. Light microscopy image of a co-culture of ioGABAergic Neurons and ioGlutamatergic Neurons at day 10 post-thawing on an MEA plate (Maxwell Biosystems) labelled with NeuroFluor NeuO. The picture shows strong signals from neuronal cell bodies and reduced signals from neuronal projections.

TOP TIP 4

Use fresh reagents to prevent confounding precipitates

Optimal cell cultures also require optimal cell culture surface coatings. If the coating isn't equally distributed across the plate or if the coating is clumpy, the cells aren't going to attach properly or grow evenly across the cell culture plate, leading to confounding effects in downstream experiments.

To culture ioGABAergic Neurons as mono-cultures, I recommend using poly-L-Ornithine-rhLaminin-521 (PLO-Laminin) coated culture plates. The PLO-Laminin coating protocol is simple, requiring you to first treat your cell culture surfaces with a Poly-L-Ornithine solution followed by rhLaminin- 521. Any excess Poly-L-Ornithine coating solution can be toxic to cells, so it is critical to ensure that any excess coating solution has been sufficiently

rinsed and dried prior to cell seeding. However, for co-cultures with ioGlutamatergic Neurons and hiPSC-derived astrocytes for functional MEA analysis, a Poly-D-Lysine (PDL), followed by a Geltrex coating is optimal to promote cell adherence on specialised MEA culture plates. Read our published protocol for more information. When applying any such coating, scientists should watch out for precipitates.

If there are precipitates in the borate buffer, it will not properly dissolve the PDL, and once coated, the neurons will accumulate around the precipitates. You can check your borate buffer for precipitation by gently swirling the bottle and looking through the buffer to check for crystals in the solution. Another giveaway is crystals forming around the bottle lid. In these instances, it's always best to open a new bottle of borate buffer!

Other PDL-Geltrex™ tips:

- When it comes to the Geltrex coating for MEA plates, it's crucial to ensure that it's thawed on ice, as it polymerises upon warming. To prevent this happening it's best to ensure that the DMEM/F-12 dilution medium is chilled. One recommendation is to limit the number of freeze-thaw cycles that Geltrex undergoes. I prepare aliquots beforehand to limit the number of times the Geltrex is thawed.
- After coating MEA plates, it's good practice to check under the microscope to confirm that the coating on your transparent control plate looks clean and does not contain precipitates of PDL or Geltrex.
- Ideally, you want your network to be uniform, as in Figure 4, especially when you do MEA experiments. Make sure that your reagents for coating are as new and fresh as possible, double-checking that purchased reagents are still in-date and ensuring they haven't undergone too many freeze-thaw cycles. I aliquot reagents into smaller volumes in advance to help prevent this. Otherwise, you will likely end up with cell clusters in some areas of the plate.

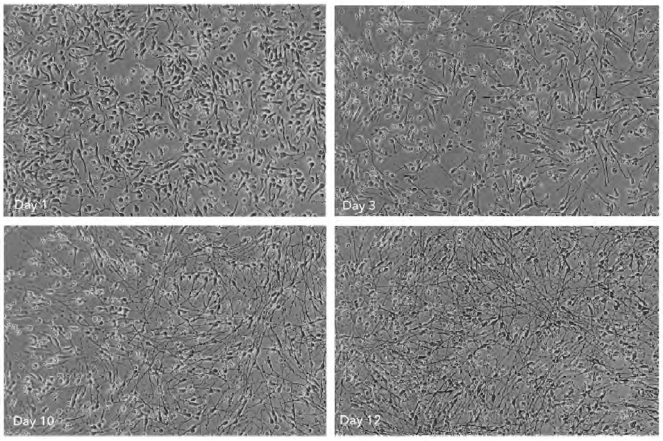


Figure 4. Upon reprogramming, rapid morphological changes are observed in the cells, with neurons identified by day 3 post-revival. Visible neuronal networks are observed by day 10 post-thaw. Images show day 1 to 12 post-thawing; 10X magnification.

Summary

Following Christopher's top cell culture tips will help you culture uniform networks of human iPSC-derived GABAergic neurons for your experiments, and spot the early signs that the neurons are experiencing stress. By adding media slowly when thawing your cells, ensuring that reagents are freshly prepared, avoiding sudden temperature changes when plating out cells, and checking your cells under the microscope regularly, you can ensure that you get the most from your ioGABAergic Neurons!

Follow the link to learn more about bit.bio's human iPSC-derived ioGABAergic Neurons.

REFERENCES

1. Tang X, Jaenisch R, Sur M. The role of GABAergic signalling in neurodevelopmental disorders. Nature Reviews Neuroscience. 2021;22(5):290-307. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9001156/
2. Masuda T, Sankowski R, Staszewski O, et al. Microglia heterogeneity in the single-cell era. Cell reports. 2020;30(5):1271-1281. https://doi.org/10.1016/j.celrep.2020.01.01
3. Raman M, et al. Rapid and consistent generation of functional microglia from reprogrammed hiPSCs to study mechanisms in neurodegeneration and neuroinflammation. Poster at SfN 2022. 11-15 November, Washington D.C.
4. Paolicelli RC, Sierra A, Stevens B, et al. Microglia states and nomenclature: A field at its crossroads. Neuron. 2022;110(21):3458-3483. https://doi.org/10.1016/j.neuron.2022.10.020
5. Guo S, Wang H and Yin Y. Microglia Polarization From M1 to M2 in Neurodegenerative Diseases. Front. Aging Neurosci. 2022;14:815347. https://doi.org/10.3389/fnagi.2022.815347
6. Martinez A, Hériché JK, Calvo M. Characterization of microglia behaviour in healthy and pathological conditions with image analysis tools. Open Biol. 2023 13(1):220200. https://doi.org/10.1098/rsob.220200
7. Haenseler W, Sansom SN, Buchrieser J, et al. A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. Stem Cell Reports. 2017 Jun 6;8(6):1727-1742. https://doi.org/10.1016/j.stemcr.2017.05.017