Robust and scalable generation of stem cell-derived glutamatergic neurons (ioNEURONS/glut) in support of drug discovery and high throughput screening

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1 INTRODUCTION

The advent of stem cell technologies has opened up new opportunities in drug discovery and development by providing new tools for drug screening, target identification and toxicity testing. The discovery of reprogramming patient derived somatic cells into induced pluripotent stem cells (iPSCs) has offered a promising alternative as they can be used to generate large numbers of cells and varied tissue types including human neurons which are relevant to CNS related diseases.

Although iPSC derived neurons have promising applications in drug discovery platforms, their use for high throughput screening (HTS) has been limited by the traditional differentiation methods, which are difficult to reproduce at scale. Here we have characterised with immunocytochemistry (ICC) and branched DNA (bDNA) assays human glutamatergic neurons (ioNEURONS/glut) differentiated from iPSCs cells using forward reprogramming technology developed by Bit Bio[1]. Only 2 days after plating cells show complex neuronal morphology and high expression of pan neuronal markers MAP2 and βIII-tubulin.

To determine suitability for HTS, ioNEURONS/glut plated in 384-well microplates were homogeneously differentiated up to 9 days with even cell distribution and minimal well to well variability. A cytotoxicity (Cell Titre Glo, CTG) assay in neurons treated with tool compound showed excellent assay statistics and high suitability for HTS. TR-FRET (HTRF) assays for Huntington (HTT) and protein kinase B (AKT) proteins showed lower signal but low variability, and may also be suitable for HTS platforms.

2 METHODS

NGN2 opti-ox™ iPSCs were generated by Bit Bio according to their published protocol[1]. Fig. 1 shows a schematic representation of the Glutamatergic neuronal culture provided by Bit Bio and a comparison with traditional neuronal differentiation protocols. In brief NGN2 opti-ox™ iPSCs were primed to glutamatergic neurons for 3 days by Bit Bio prior freezing. Cells were then revived in glutamatergic neuronal media supplemented with doxycycline and Rock inhibitor and cultured for 48 hours.

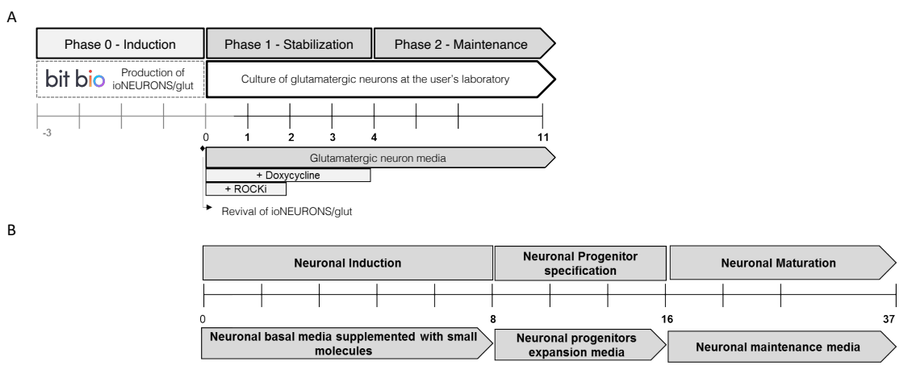


Figure 1. (A) Schematic representation of Bit Bio glutamatergic neuronal culture provided by Bit Bio. (B) Schematic representation of traditional neuronal differentiation protocols and timelines. Neuronal differentiation protocols were adapted from Shi et al[2] and Telezhkin et al[3].

We then aimed to assess suitability of the cells for HTS assays. HTRF and CTG assays were used as example assay formats to assess suitability for screening. Fig. 2 below shows a schematic representation of an example HTS assay workflow.

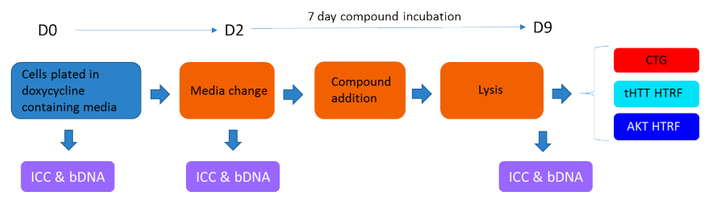


Figure 2. Schematic representation of assay workflow

3 RESULTS

Cell Characterisation

Bit Bio ioNEURONS/glut were characterised by ICC for a small subset of neuronal and precursor markers at day 2 and 9 after plating. Branched DNA (bDNA) was also used to characterise RNA expression at various time points using a large panel of markers (41 genes) including those for pluripotent, progenitor and neuronal cell types. ioNEURONS/glut show a neuronal phenotype immediately after plating with low progenitor and pluripotency markers and increased expression of pan-neuronal markers at both protein and RNA level (Fig. 3 and 4). Removal of doxycycline at day 2 of culture did not significantly affect gene expression and could simplify workflow. A small subset of bDNA genes from neurons differentiated with traditional protocols is represented as well in Figure 4 as comparison to ioNEURONS/glut.

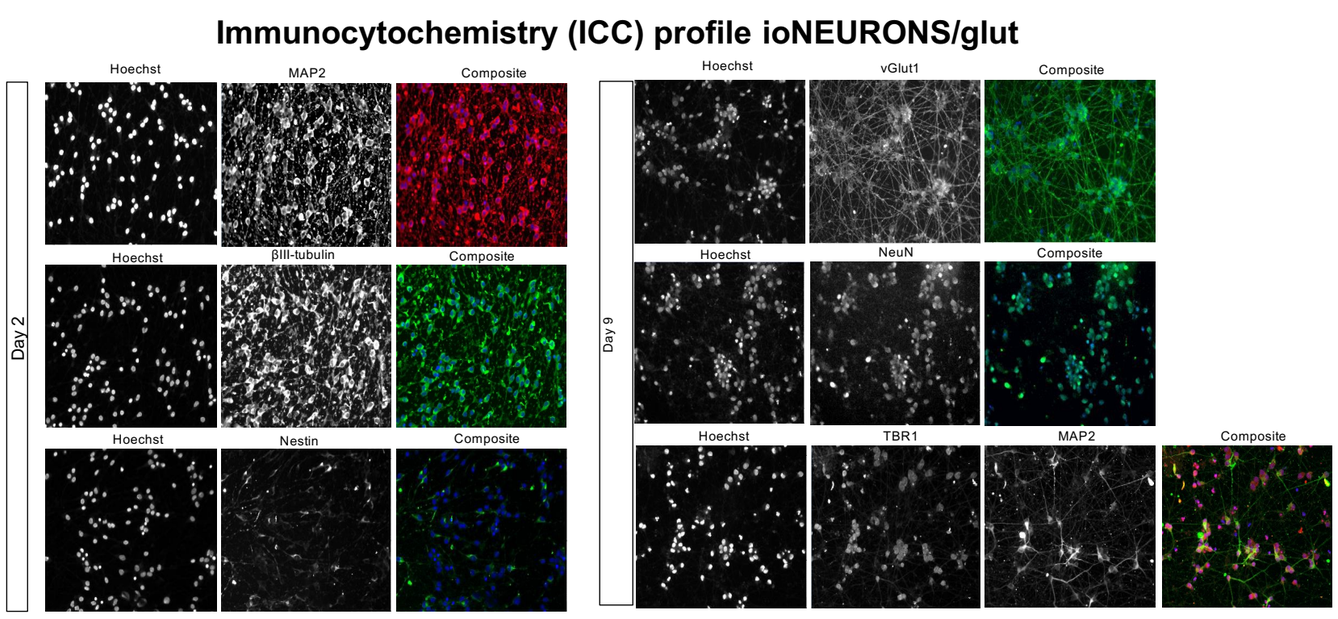


Figure 3. Representative images of Bit Bio induced neurons (ioNEURONS/glut) differentiated in glutamatergic neuronal media. Cells showed neuronal phenotype and expression of pan neuronal markers (MAP2 and βIII-tubulin) at day 2 and high vGlut1 and TBR1 expression at day 9.

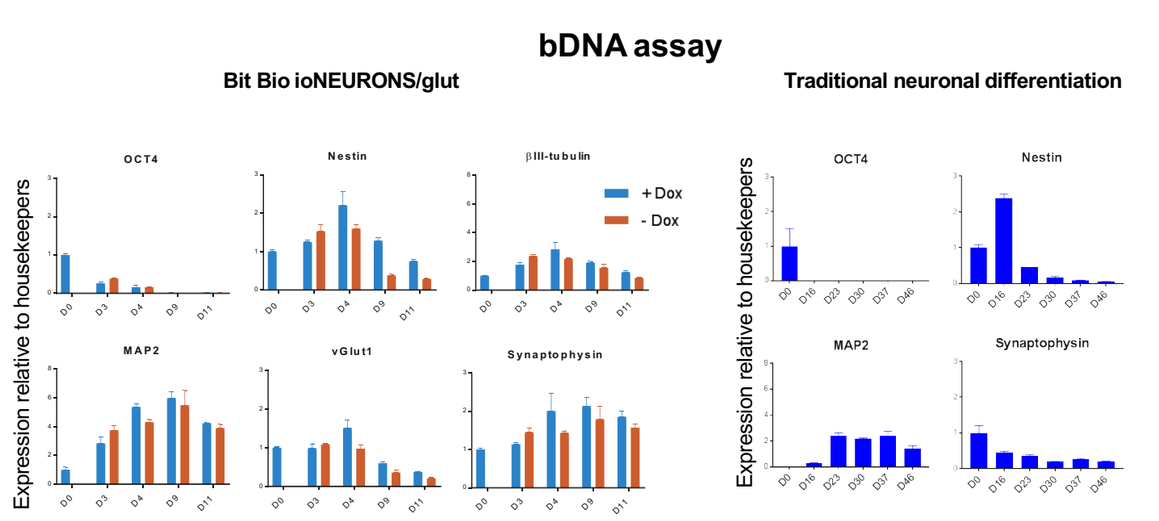
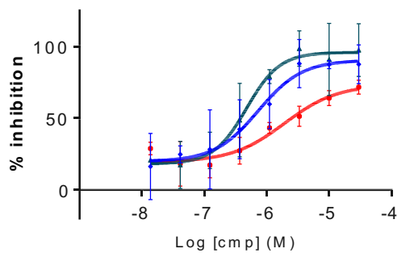


Figure 4. bDNA analysis of Bit Bio ioNEURONS/glut and neurons differentiated with traditional protocols at different time points. Example of data from bDNA analysis. Expression of 8 genes in ioNEURONS/glut showed higher level of expression of neuronal markers already after 3 days in culture. Cells were plated in doxycycline containing media for 48 hours and then differentiated up to day 11 in differentiated media either with or without doxycycline (+/- Dox conditions). Neurons differentiated with traditional protocols showed increased level of pan neuronal gene MAP2 only from day23 onwards.

HTS Compatibility - HTRF and Cell Titer Glo assays

HTRF and CTG assays were used to determine assay signal and potential for compound profiling in neurons after only 2 days of culture (Fig. 5). The CTG assay signal was high and variability low, suggesting high potential for compound profiling. Despite the low signal achieved with HTRF assays for both HTT and AKT, treatment for 7 days with a tool compound suggests pharmacology can be reproducibly determined. Further optimisation of cell plating density and compound incubation time would be required for HTRF and other assay formats.



	Signal / Background
CTG	327
HTT HTRF	1.65
AKT HTRF	1.95

Figure 5. HTRF and CTG assays in Bit Bio neurons treated with tool compound. Compound titration shows concentration response curve in all three assays (mean±sd of 2 replicates). Average signal/background of 2 experiments is excellent for CTG, but lower for both HTRF assays due to lower assay sensitivity.

4 CONCLUSIONS

Preliminary assessment of Bit Bio induced neurons technology showed robust and scalable generation of human glutamatergic neurons suitable for HTS. A short exposure (2 days) of doxycycline and not a sustained treatment was already sufficient to get a complex neuronal network and high expression of pan neuronal markers. Further validation to assess neuronal subtypes is ongoing as well as suitability for other phenotypic assays such as high content quantification and viewRNA.

5 REFERENCES

1. Matthias Pawlowski, Daniel Ortmann, Alessandro Bertero, Joana M. Tavares, Roger A. Pedersen, Ludovic Vallier, and Mark R.N. Kotter. Inducible and Deterministic Forward Programming of Human Pluripotent Stem Cells into Neurons, Skeletal Myocytes, and Oligodendrocytes. Stem Cell Reports j Vol. 8 j 803–812 j April 11,2017.
2. Yichen Shi, Peter Kirwan & Frederick J Livesey. Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. Nature Protocols, 13 September 2012; doi:10.1038/nprot.2012.116.
3. Telezhkin et al., Am J Physiol Cell Physiol., 2016, 310(7); https://www.ncbi.nlm.nih.gov/pubmed/26718628