Generation and functional characterisation of motor neurons derived through transcription factor mediated programming of human pluripotent stem cells

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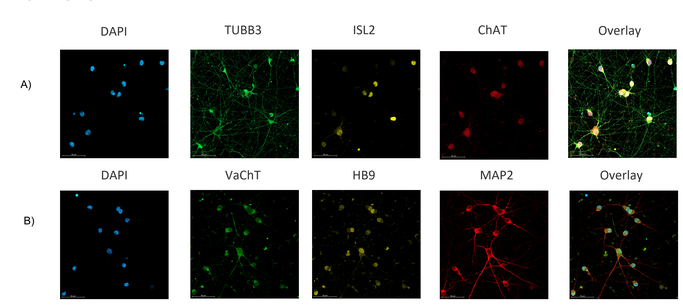
Abstract

Motor neurons consist of distinct neuronal subtypes that control the activity of muscles and glands in direct or indirect manners. Motor neurons form a large neuronal network that receives inputs from interneurons, sensory neurons or other motor neurons to control complex behaviours such as locomotion. Pathological perturbation of these motor circuits can lead to the development of motor neuron diseases (MNDs) such as spinal muscular atrophy and amyotrophic lateral sclerosis. Development of therapies to treat MNDs is hampered by the limited translatability of existing preclinical animal models as well as the lack of reliable and consistent sources of in vitro models. Human induced pluripotent stem cells (hiPSCs) can be used to generate motor neurons for in vitro applications, however current differentiation protocols are often lengthy, inconsistent, and difficult to scale.

We have used opti-ox* technology to rapidly reprogram hiPSCs into motor neurons, termed ioMotor Neurons, which are a homogenous population of cells with classical neuronal morphology and neurite outgrowth. As early as 4 days in culture, cells express the pan-neuronal markers MAP2 and TUBB3, the cholinergic markers ChAT and VAChT and the motor neuron-specific markers MNX1 and ISL1/2, as assessed by both ICC and RT-qPCR. Bulk RNA sequencing of ioMotor Neurons demonstrates a rapid acquisition of a motor neuron signature, with an indicated spinal motor neuron identity (cervical region). A high-density microelectrode array (HD-MEA) system has been used to assess ioMotor Neuron functional activity, and has revealed spontaneous neuronal activity with increasing firing rate over 40 days in culture. Finally, next generation sequencing methods have shown consistency between three different batches produced through opti-ox deterministic cell programming.

opti-ox technology can be utilised for the scalable and consistent production of functional hiPSC-derived motor neurons. ioMotor Neurons have the potential to advance the development of new therapeutics for MNDs and to further our understanding of motor neuron development and maturation in vitro.

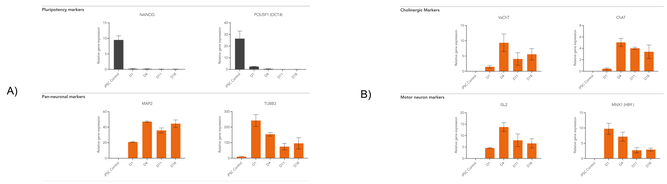
1. ioMotor Neurons homogeneously express key motor neuron markers



A) Immunofluorescent staining on day 11 of reprogramming demonstrates that ioMotor Neurons are positive for TUBB3 (green), ISL2 (yellow) and ChAT (red). DAPI was used as counterstain (blue). MAP2 positive neurons co-localize with the motor neuron markers ISL2 and ChAT indicating that cells have a motor neuron identity. Scale bar is 50um.

B) Immunofluorescent staining on day 11 of reprogramming showing that ioMotor Neurons are all positive for LHX3 (green), HB9 (yellow) and MAP2 (red). DAPI was used as counterstain (blue). Scale bar is 50um.

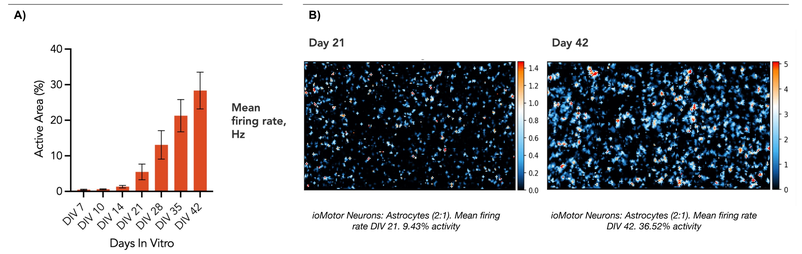
2. RT-qPCR – Gene expression of key neuronal & lower motor neuron markers



A) RT-qPCR data showing expression of key motor neuronal markers at 5 timepoints day 0 (iPSCs), 1, 4, 11, and 18. A swift downregulation of pluripotency markers NANOG and POU5F1 (OCT4) can be seen by Day 1. From day 1, ioMotor neurons show consistent expression of the pan-neuronal markers MAP2 and TUBB3.

B) From day 1, ioMotor neurons show upregulation of cholinergic markers VaChT and ChAT. For motor neuron specific markers, ioMotor Neurons show upregulation of markers ISL2 and MNX1 (HB9) as early as Day 1.

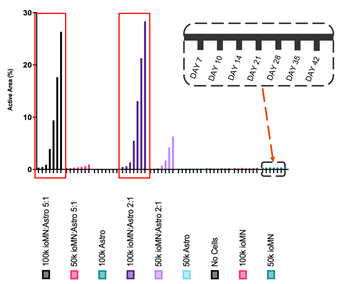
3. ioMotor Neurons rapidly gain functional activity over time.



A) ioMotor Neurons were plated with astrocytes at a 2:1 ratio to perform Multi-Electrode Array (MEA) analysis over a period of 42 days. ioMotor Neurons show electrical activity as early as 14 days post-thaw, with the percentage of active area increasing throughout the length of the experiment.

B) Heatmap showing increase in % active area from day 21 (9.43%) to day 42 (36.52%) in culture.

4. Active area comparison between different co-culture and mono-culture conditions.



Bar graph showing active area percentage for multiple experiment conditions. Conditions include variations of densities and mono- and or co-cultures.

Areas highlighted in red show significant increase in active area over time demonstrating astrocytes accelerate maturation and support synaptic formation; thus; robust and abundant signal bursts resulting from microcircuit assembly in cocultures.

Mono-culture active areas are negligible, some detection – but significantly lower than co-culture scenarios. This indicates that ioMotor Neurons are of high purity and unable to form synapses without the presence of other cell types (see 100/50k Astro* & 100/50k ioMN**).

*100,000 / 50,000 astrocytes (cells)

**100,000 /50,000 ioMotor Neurons (cells)

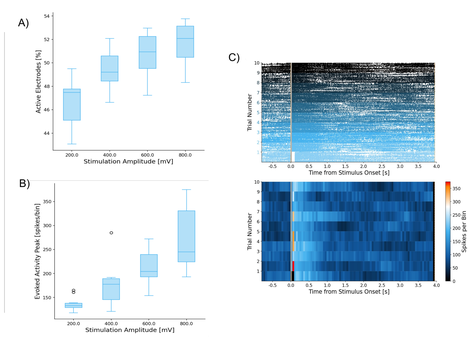
5. ioMotor neurons respond to electrical stimulation

ioMotor Neurons were stimulated by electrical activity at day 42 for four different voltages (200mV, 400mV, 600mV, and 800mV) over 10 different trials. ioMotor Neurons were plated with astrocytes at a 2:1 ratio.

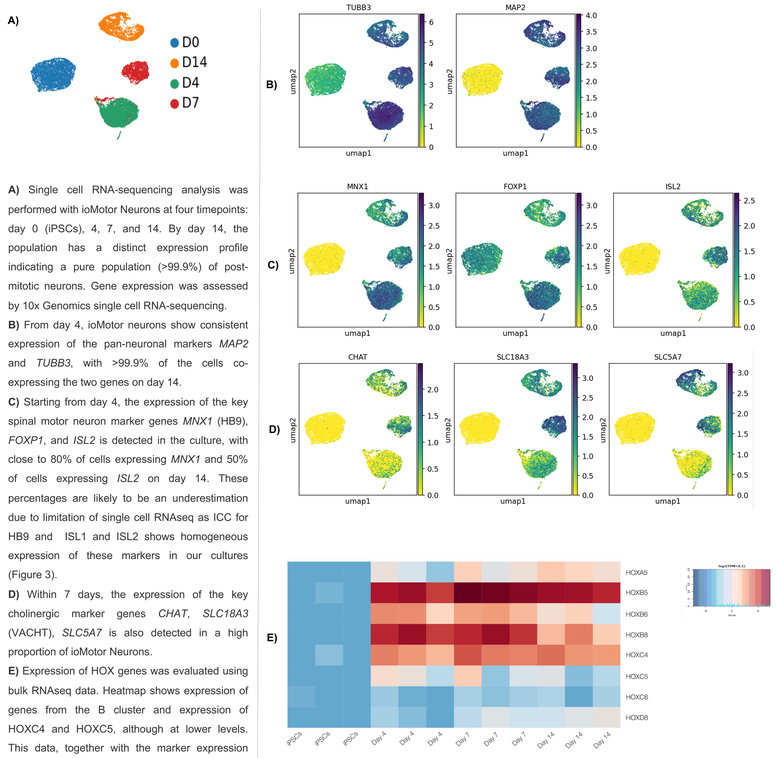
A) ioMotor Neurons become more active as the intensity of the stimulus increases.

B) ioMotor Neurons evoke a higher peak activity as the stimulus increases.

C) Plots showing immediate response to the stimulus before returning to baseline.



6. Single cell RNA-sequencing show ioMotor Neurons are a pure population of neurons with lower motor neuron identity



A) Single cell RNA-sequencing analysis was performed with ioMotor Neurons at four timepoints: day 0 (iPSCs), 4, 7, and 14. By day 14, the population has a distinct expression profile indicating a pure population (>99.9%) of post-mitotic neurons. Gene expression was assessed by 10x Genomics single cell RNA-sequencing.

B) From day 4, ioMotor neurons show consistent expression of the pan-neuronal markers *MAP2* and *TUBB3*, with >99.9% of the cells co-expressing the two genes on day 14.

C) Starting from day 4, the expression of the key spinal motor neuron marker genes *MNX1* (HB9), *FOXP1*, and *ISL2* is detected in the culture, with close to 80% of cells expressing *MNX1* and 50% of cells expressing *ISL2* on day 14. These percentages are likely to be an underestimation due to limitation of single cell RNAseq as ICC for HB9 and ISL1 and ISL2 shows homogeneous expression of these markers in our cultures (Figure 3).

D) Within 7 days, the expression of the key cholinergic marker genes *CHAT*, *SLC18A3* (VACHT), *SLC5A7* is also detected in a high proportion of ioMotor Neurons.

E) Expression of HOX genes was evaluated using bulk RNAseq data. Heatmap shows expression of genes from the B cluster and expression of HOXC4 and HOXC5, although at lower levels. This data, together with the marker expression from single cell RNAseq, suggests that ioMotor Neurons have a posterior hindbrain or spinal cord (cervical region) identity.

Note, this data is from cells in continuous culture, so minor variations may exist between this data and data from cryopreserved cells.

ioMotor Neurons are a pure population of neuronal cells with homogeneous expression of key lower motor neuron markers including MNX1 (HB9), FOXP1, ISL2 as characterized by single cell RNA sequencing and ICC.

ioMotor Neurons have a defined cholinergic identity as shown by the expression of key cholinergic genes CHAT, SLC18A3 and SLC5A7.

ioMotor Neurons offer rapid functionality – spontaneous activity is demonstrated by HD-MEA.

Mono-cultures are devoid of activity due to high purity resulting in a lack of synapse formation.

Electrical stimulation of ioMotor Neurons shows response to stimulus. Further, as voltage increases, as does the activity and intensity of the culture.

ioMotor Neurons can be generated with high consistency, as shown by bulk RNA-sequencing.

Rapidly maturing motor neurons that express key motor neuron already from day 4 post-revival.

Cells are easy to culture using a simple, 3-step protocol – ideal for researchers without iPSC expertise.

References:
Pawlowski, et al Stem Cell Reports. 2017 Apr 11;8(4):803-812.