Characterisation of iPSC-derived motor neurons using an accelerated maturation protocol

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Abstract

Understanding neurodegenerative conditions such as amyotrophic lateral sclerosis (ALS) requires a supply of cells for *in vitro* modelling and drug screening. **Motor neurons derived from induced pluripotent stem cells (iPSC)** provide an unlimited source of such cells and can potentially facilitate precision medicine approaches to compound testing when derived from somatic cells from patients. Furthermore, in co-culture with iPSC-derived muscle cells, **a fully human preclinical screening model of the Neuromuscular Junction is possible**. However, iPSC-derived motor neurons traditionally required up to six weeks maturation to exhibit relevant functional phenotypes.

We demonstrate use of an *in vivo* environment-mimicking supplement, to produce a more physiological, modified growth medium for maturation, which **reduces the time to reach functional maturity from six weeks to 10 days**. Cells showed typical morphology with abundant neurites and were positive for expression of the mature motor neuron marker **SMI-32 along with HB9, ChAT and Isl-1**, as assessed by immunocytochemistry.

Recordings using a 48-well multi-electrode array (MEA) demonstrate synchronised spontaneous firing at a similar timepoint. Motor neurons matured using the accelerated protocol formed junctions with muscle cells in a microfluidic co-culture system, as demonstrated by staining with fluorescently labelled alpha-bungarotoxin. The phenotype of motor neurons from an ALS patient was also investigated.

These rapidly matured cells provide an invaluable tool for research into neuromuscular disease and a model allowing a **higher throughput for compound screening**.

METHODS

Cell culture of iPSC progenitor cells to mature cell types was performed using Axol Bioscience user protocols and media (ax0072) for motor neurons (ax0078 control and ax0074 ALS C9ORF72, greater than 145 GGGGCC repeats). Initial studies used Motor Neuron Accelerator (ax0179) with a poly-D-lysine and SureBond XF matrix (day 18 plus) . Subsequently the process was accelerated further using a vitronectin matrix (day 10 plus).

Immunostaining:

Immunostaining was conducted using standard PFA fixing, 0.3% Triton, using commercially available antibodies.

For visualisation of the neuromuscular junction, cultured motor neuron progenitor or skeletal muscle progenitor cells were seeded into a microfluidic chamber with 450um channels

MEA Recording:

Extracellular field potentials were acquired at 37ºC, 5% CO_2 using the Axion Maestro Pro high-throughput MEA platform. 48-wells plates with a 350µm separation between each well-floor mounted electrode were used. This enabled simultaneous recording of extracellular potentials from 16 electrodes per well at a sampling rate of 20kHz/channel. Cultured motor neuron progenitor cells were spotted onto the array in a 10µl volume of media. These were then differentiated in-situ to form their mature cell types.

Figure 1. MEA Configuration.
Figures A, B and C show the axion MEA system, well plate and MEA array that resides in wells of the plate.
Figure D. (phase contrast) of MEA coated motor neuron culture.

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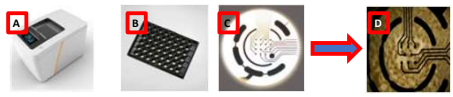


Spontaneous Neuronal Activity (SNA) Recording:

Motor neurons were transfected at day 2 and day 7 post-thaw with Incucyte® Neuroburst Orange Lentivirus, encoding a calcium reporter driven from the synapsin promoter. Spontaneous activity was recorded using Incucyte® Neuronal Activity Analysis Software on an Incucyte® S3.

RESULTS

Motor Neuron Activity Measurement Via MEA Detection

Synchronized clustered firing

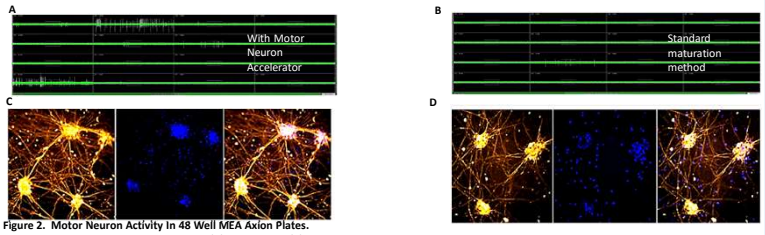
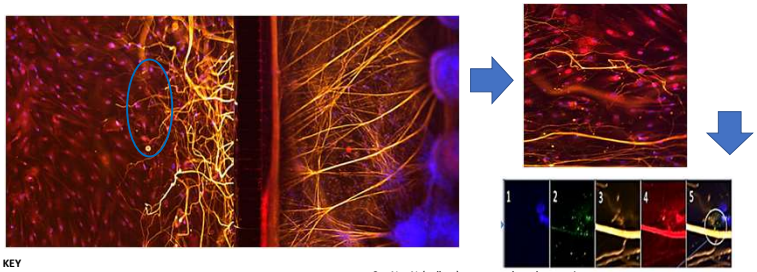


Figure 2. Motor Neuron Activity In 48 Well MEA Axion Plates.
Day 20 post plating – Data generated with (A) and without (B) using Axol Motor neuron accelerator supplement.
Immunocytochemistry staining for SMI32 and DAPI were used to assess the motor neuron cluster formation and development of mature neurites within the culture with (C) and without (D) Axol Motor neuron accelerator supplement, without vitronectin.

2-Dimensional Neuromuscular Junction Model

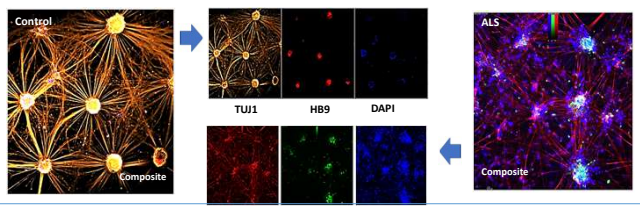


KEY
1. DAPI (blue) - nuclear stain)
2. Alpha-Bungarotoxin (green) – synaptic acetylcholine binding marker
3. NeuN (yellow) – neuronal nuclear antigen
4. Titin (red) – skeletal muscle
5. Composite image

Figure 3. Motor Neuron/ Skeletal Muscle – co-Culture.
Microfluidic co-culture of human iPSC motor neurons and skeletal muscle. The images show development of the neuromuscular junction. The motor neuron nerve (yellow, NeuN) completely overlaps the postsynaptic acetylcholine receptors (green, fluorescent α-bungarotoxin conjugates) and muscle (red, titin)

Expression of markers by immunocytochemistry: WT and C9Orf72 mutant

iPSC used were from a healthy donor (ax0078) and from a donor with C9ORF72 hexanucleotide expansion (greater than 145 GGGGCC repeats) and diagnosed with amyotrophic lateral sclerosis (ALS) (ax0074). Both lines were matured to motor neurons for 10 days with characterization by immunocytochemistry and electrophysiological monitoring.



Results ctd.

Expression of markers by immunocytochemistry: WT and C9Orf72 mutant

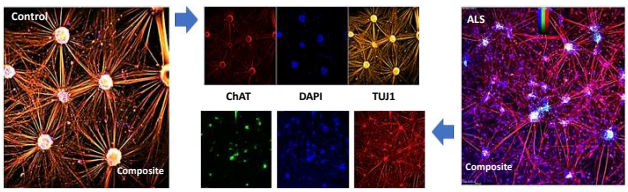


Figure 4. Motor neuron characterization by immunohistochemistry.
Imaged at 20X magnification in 96 well imaging plates. Cells were fixed at day 10 post plating and stained with mature motor neuron markers HB9, ChAT and nuclear marker DAPI. Neurons were matured with Motor Neuron Accelerator supplement and vitronectin matrix.

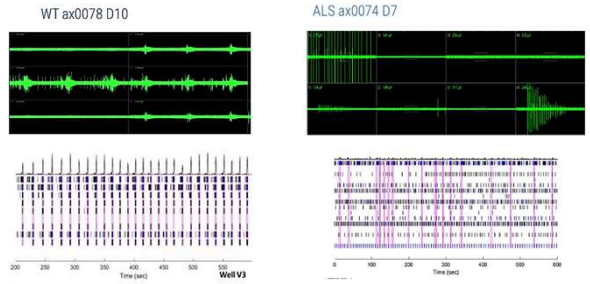
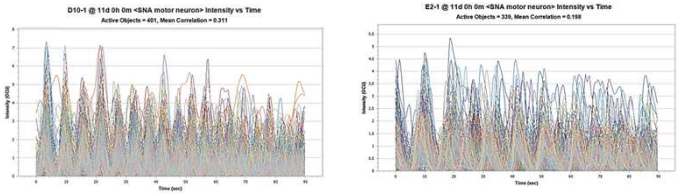


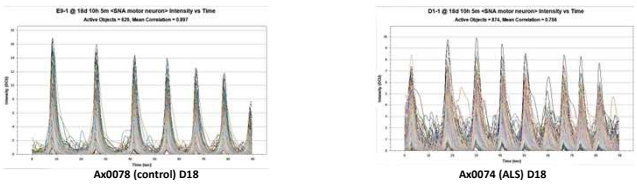
Figure 5. Motor neuron characterization by electrophysiology
MEA recordings were made from day 10-matured iPSC-derived motor neurons from a healthy donor (left) and day 7-matured from a donor with ALS (right)

Motor Neuron Activity Measurement Via Neuroburst® Orange and Incucyte® S3



Mean correlation 0.311
ax0078 (control) day 11 matured

Mean correlation 0.198
ax0074 (ALS) day 11 matured



Ax0078 (control) D18

Ax0074 (ALS) D18

Figure 6a. Analysis from recording of spontaneous neural activity.
Incucyte® Neuroburst Orange lentiviral reagent was used to image spontaneous neuronal firing in iPSC-derived motor neurons from a healthy donor (left) and one with ALS (right), after 11 days maturation

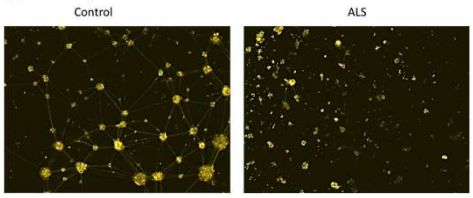
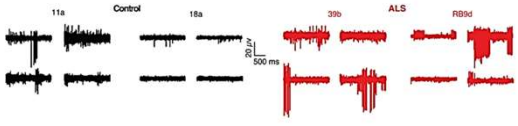


Figure 6b. Images from recording of spontaneous neural activity[1].
Firing in iPSC-derived motor neurons from a healthy donor (left) are more evenly distributed than in similarly differentiated neurons from a donor with ALS (right). Neurites are clearly visible in the control motor neurons and far less so in motor neurons from an ALS donor.

The ALS *in vitro* model recapitulates results from SOD1A4V/+ mutation

To prove the model correlated with clinical findings, the authors[2] used MEA to evaluate iPSC-derived ALS neurons from two patients with the $SOD1^{A4V/+}$ mutation.

- ALS neurons showed increased neural firing compared to control cells. Maestro recordings confirmed clinical findings of neural hyperexcitability compared to controls.
- Below are representative recordings from four out of 64 MEA electrodes in control (11a, 18a) and $SOD1^{A4V/+}$ mutant (39b, RB9d)-derived motor neurons cultured for 28 days on the arrays. MEA recordings revealed increased spontaneous firing in the mutant-derived neurons compared to control-derived neurons, confirming previous clinical findings and further validating the in vitro model.



1. Video available to view at Axol's booth no. 617

2. Wainger BJ. *et al*. Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. Cell Rep. 2014 Apr 10;7(1):1-11.

CONCLUSION

Motor neurons matured for 10 days with a more physiological medium with Motor Neuron Accelerator (ax0179) show typical expression of HB9 and ChAT and a high level of neuraxis. Synchronized spontaneous firing is also evident after only 10 days of maturation, recorded using a multiple electrode array, or using the Incucyte® Neuroburst Orange Lentivirus.

Motor neurons derived from iPSC carrying a C9ORF72 hexanucleotide expansion (greater than 145 GGGGCC repeats) exhibit earlier (day 7) activity, but with longer burst trains and decreased synchronization. The distribution of firing across the well, as visualized on the Incucyte®, is uniform in the wildtype motor neurons, but heterogenous in the ALS motor neurons.

iPSC-derived motor neurons have been shown to exhibit a clinically relevant phenotype by electrophysiology or using the Incucyte® spontaneous neuronal activity module. Using a medium supplement whose components mimic the physiological cellular environment, motor neurons can be matured from progenitors in only 10 days, providing a disease-relevant model for medium throughput compound screening or mechanistic studies.