Multielectrode array characterization of human iPSC-derived motor neuron disease model for ALS drug discovery.

A rapid 20-day, thaw-to-data protocol using Axol Bioscience motor neurons, Accelerator supplement and Axion BioSystems' Maestro Pro.

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KEY FINDINGS:

- Four human iPSC lines, 3 from donors with ALS and 1 unaffected donor, were successfully differentiated to functional motor neurons.

- The use of the 'Accelerator' supplement to mimic *in vivo* like environment enabled assay ready, functional firing of motor neurons in ~10 days.

- Used in conjunction with the Maestro Pro, the "thaw-to-data" time was ~20 days.

- Motor neurons derived from ALS donor iPSCs were morphologically different to those from unaffected donors.

- Through MEA analysis, morphological differences corresponded to functional phenotype differences including the key attribute of hyperexcitability.

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INTRODUCTION:

Amyotrophic Lateral Sclerosis (ALS) is a progressively fatal neurodegenerative disease that damages nerve cells in the central nervous system (CNS), but its effects are manifested in the peripheral nervous system (PNS) due to the loss of motor neuron function resulting in muscle weakness and atrophy. Although ~85-90% of ALS cases are sporadic, mutations in over 40 genes have been identified that contribute to the development of ALS (1) .

Despite considerable research efforts and funding, there is no cure for ALS and approved treatments are primarily aimed at addressing symptoms. A significant hurdle in early-stage drug discovery for ALS is the lack of standardized, easy-to-use, and readily accessible human cell models. As a result, scientists have been constrained to animal models and cell lines that differ considerably from human disease biology, posing a considerable challenge for lead optimization.

One specific trait relating to ALS neuronal pathology is hyperexcitability. Hyperexcitability refers to an increased responsiveness of motor neurons to synaptic inputs, leading to an abnormal increase in firing rate. This altered electrical activity is believed to contribute to the degeneration of motor neurons and the progression of ALS and, as such, should be an essential attribute in an *in vitro* model.
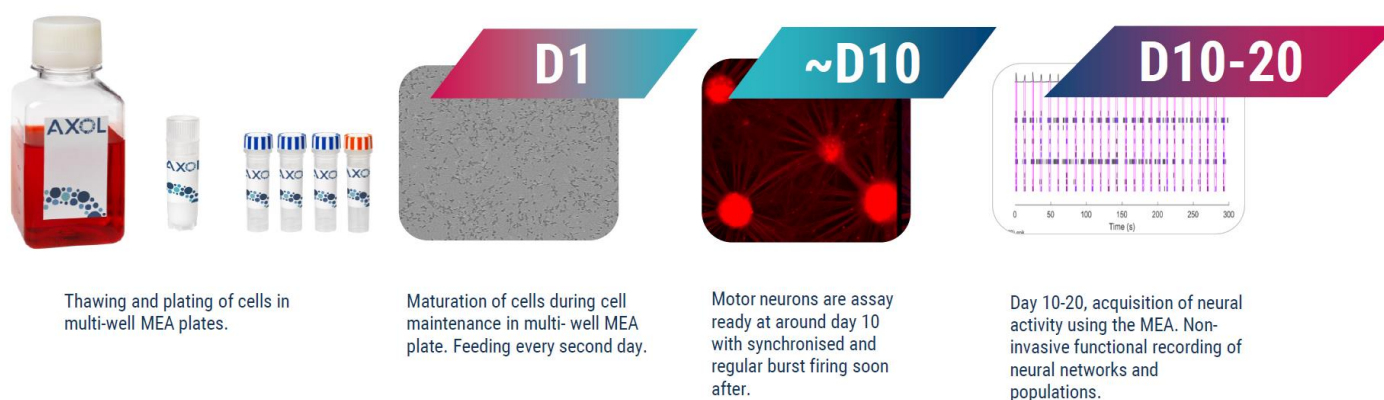
Human induced pluripotent stem cell (iPSC)-derived disease models, with their direct connection back to a human donor, offer the promise of increased translational relevance when compared to traditional *in vitro* models. These also allow for a reproducible and scalable supply of cells providing representation of clinically relevant disease phenotypes.

Axol Bioscience is a manufacturing and services company who has worked with neuronal iPSC technology for over a decade. The company aims to develop more effective *in vitro* models for drug discovery through the production of consistent and functionally relevant cells under ISO 9001 conditions. Cell types include neurons, neuroinflammatory cells, and astrocytes.

Media and cell-specific supplements have been developed to simulate a more "*in vivo*-like" environment and have been previously demonstrated to accelerate maturation and produce more mature end cell types.

We characterized motor neurons morphologically and functionally, via multielectrode array (MEA), from 4 different lines. Frozen motor neuron progenitors from healthy patients were assay ready from as early as 10 days post-thaw, while SOD1 and TDP-43 phenotypes required 20 days post-thaw to mature (Fig. 1).

Figure 1. Schematic of protocol from thawing of motor neuron precursors to MEA measurements.

D1

~D10

D10-20

Thawing and plating of cells in multi-well MEA plates.

Maturation of cells during cell maintenance in multi- well MEA plate. Feeding every second day.

Motor neurons are assay ready at around day 10 with synchronised and regular burst firing soon after.

Day 10-20, acquisition of neural activity using the MEA. Non-invasive functional recording of neural networks and populations.

Multielectrode array (MEA) is a key tool for functional electrophysiological measurements in *in vitro* disease modelling. Axion BioSystems' Maestro Pro MEA platform provided label-free assessment of neural activity and connectivity over time, allowing for simple analysis of complex biology. The Maestro Pro detects key aspects of neural networks, including activity, synchrony, and oscillation, to provide a comprehensive functional profile for disease modelling. Standard format multi-well plates allow for evaluation of the differing disease lines.

Materials and Methods:

axoCells cells, media and supplements:

Motor Neuron Progenitors User Guide

≥2 million cells, supplied frozen.

Kit contains cells, media and Accelerator, BDNF, GDNF, CNTF. Other third-party reagents may be required.

| Status	Age at sampling	Sex	mutation	Reprogramming method	Cells / Axol SKU	Bundle Kit
Unaffected	40-50	Male	None	Sendai	ax0076	ax0186
ALS	61	Female	SOD1 Het D109Y (G>T)	Sendai	ax0735	ax01835
	62	Female	TARDBP A382T	Sendai	ax0079	ax0189
	64. Onset at 60. Death 65	Female (Caucasian/ Ashkenazi, familial history of ALS and dementia)	C9orf72	Sendai	ax0074	ax0184

Also used

ax0072 – Motor neuron maintenance medium

ax0178 – Motor neuron Accelerator supplement

Cell plating and measurements

Motor neuron progenitors (Axol Biosciences) were plated at 60K cells per well in a CytoView MEA 48-well plate and cultured for up to 20 days according to manufacturer-recommended protocols (Fig. 2A).

Figure 2A – Axion Cytoview MEA plate layout.
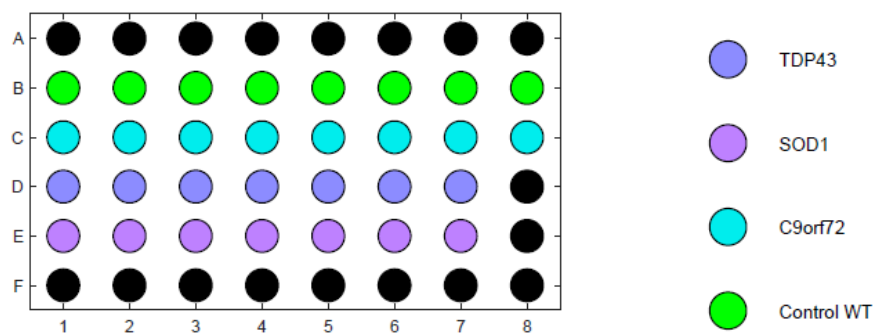
Motor neurons were imaged with brightfield phase microscopy at DIV20; the motor neurons formed a comprehensive monolayer, providing extensive electrode coverage (Fig. 2B).
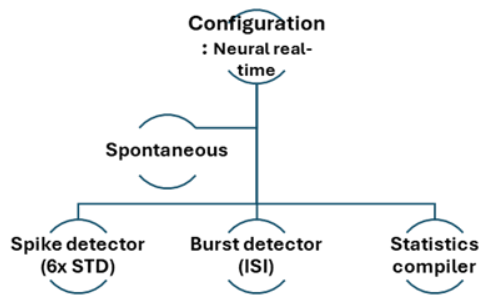
Figure 2B – Motor neuron coverage of the CytoView MEA plates. iPSC-derived motor neurons from four lines:
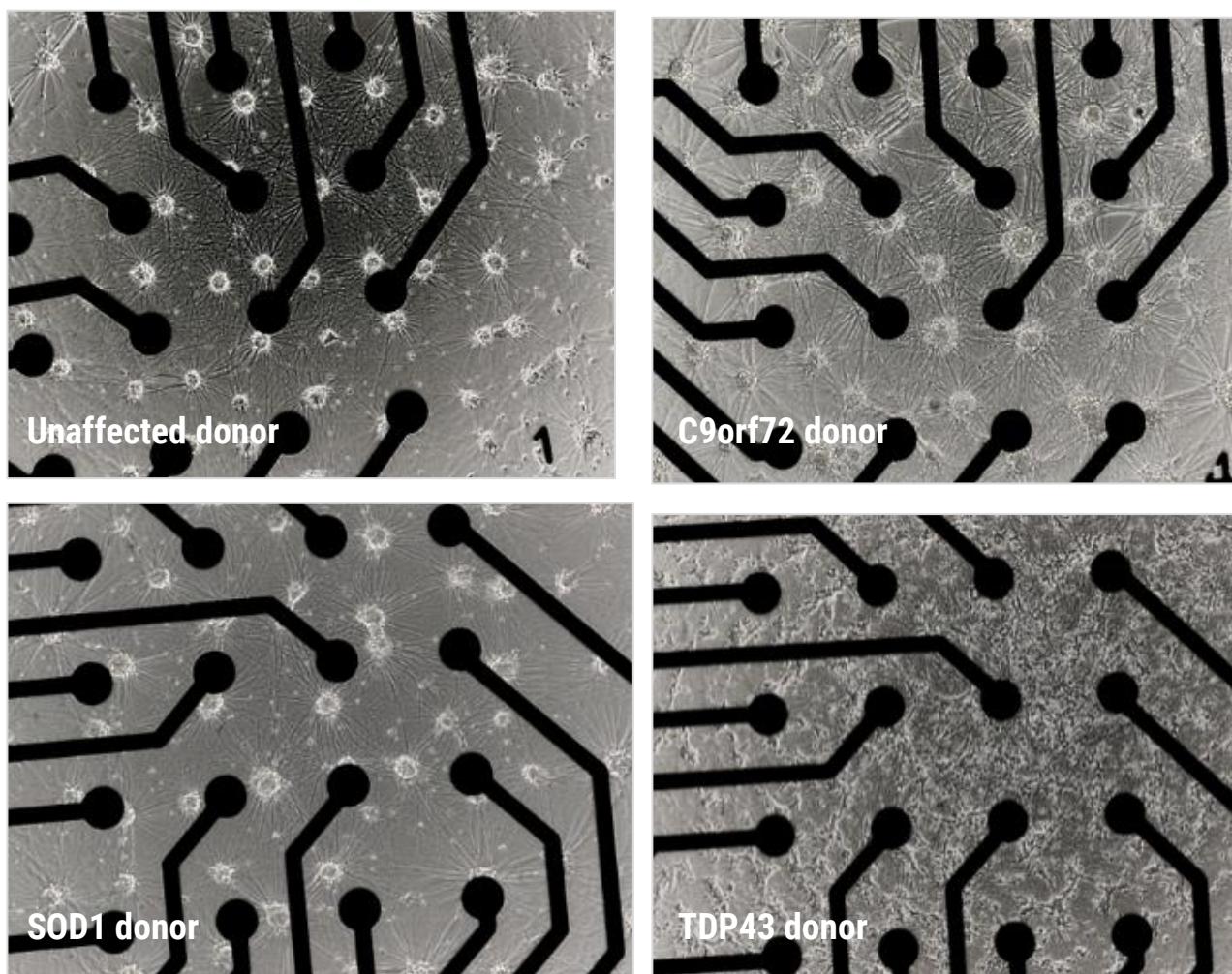
Neuronal activity recording with the Maestro Pro MEA platform:

Data was recorded using the "Neural Spontaneous" configuration in AxIS Navigator software (Axion BioSystems) (Fig. 2C).

Figure 2C

Acquisition Settings:

Neural: Spikes (1000x Gain, 200Hz-3kHz)

Sampling: 12.5kHz

Spike Detection: Adaptive Threshold Crossing (6 x STD)

Temp: 37^0C

CO_2: 5%

- Data analysis performed using Neural Metric Tool and AxIS Metric Plotting Tool (Axion BioSystems)
- All plotted values exclude wells below 0.1 Hz Mean Firing Rate

RESULTS:

**Characterization by cell morphology**

To verify the cells as suitable disease models for functional screening, it is critical to identify whether the disease models demonstrate a disease relevant phenotype when compared to the control.

The motor neurons derived from unaffected and ALS patients were cultured as per the Axol Bioscience user guides and both their morphology and functional neuronal electrical activity were assessed.

The functional activity of the neurons was measured at several time points on the environmentally controlled Maestro Pro MEA platform. Both unaffected and ALS cells developed a stable monolayer in the plates, with suitable coverage of the electrodes.

Brightfield imaging at 10 days *in vitro* (DIV10) showed the expected neuronal morphology of neurite outgrowth and the formation of structural neuronal networks (Table 1).
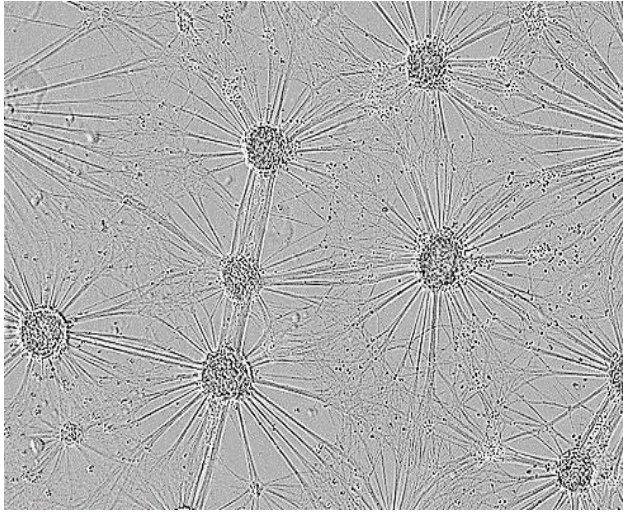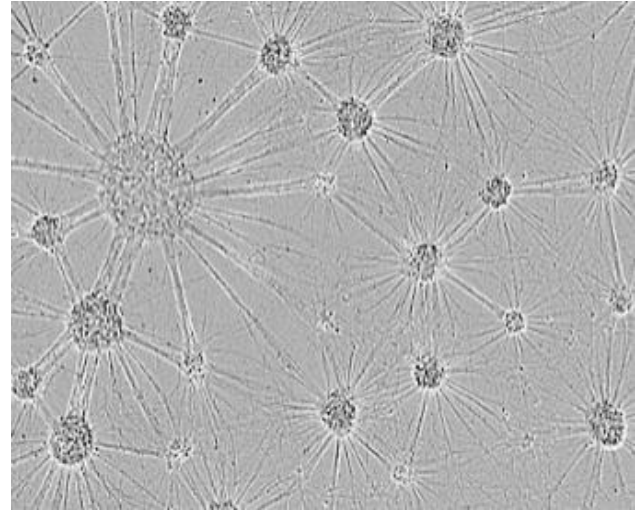

ax0076 Motor Neurons: Unaffected Donor

From a male donor, 40-50 years old at time of donation.

Uniform cultures with large cell bundles and cabling.
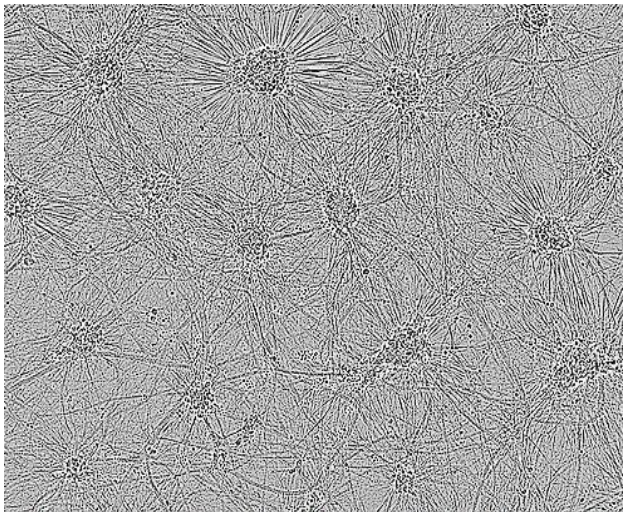
Phase image, d10.

ax00735 Motor Neurons: SOD1 Donor

From a female ALS donor with SOD1 mutation, 61 years old at time of donation.

Heterogenous morphology with large and small clusters, almost as if a mix of control and C9orf72.
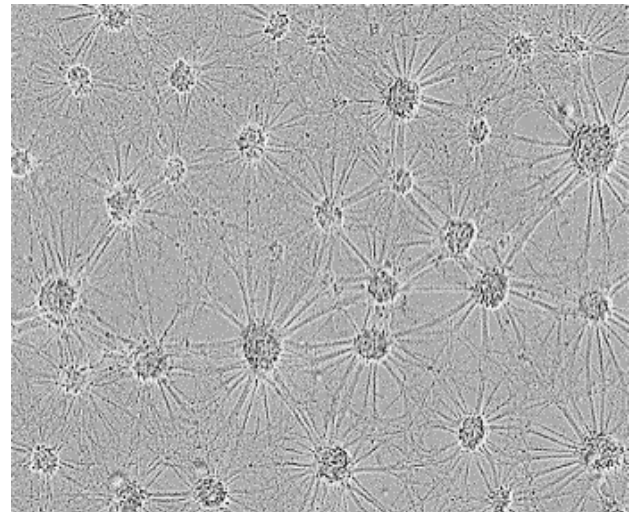
Phase image, d10.

ax0079 Motor Neurons: TDP43 Donor

From a female ALS donor with TDP-43 mutation, 62 years old at time of donation.

Heterogenous with different size clusters, and longer neurites with multiple points of innervation.

Phase image, d10.

ax0074 Motor Neurons: C9orf72 Donor

From a female ALS donor with C9orf72 mutation, 64 years old at time of donation.

Fewer uniform cultures, with smaller bundles and fibrous neurites

Phase image, d10.

Characterization by MEA

MEA enables non-invasive measurement of electrically active cells within a familiar multi-well cell culture format. MEA measurements are based on the detection of extracellular field potentials, which are the electrical signals generated by the activity of neurons or other excitable cells. These potentials arise due to the flow of ions (such as sodium, potassium, and calcium) across the cell membrane during action potentials or other electrical events.

'Raw' data is seen in real-time as a continuous voltage trace. A simple way to visualize this neural network behaviour is with a raster plot (Table 2). Each tick mark represents the time, on the x axis, that a spike occurred. Each row of tick marks represents the spikes from a single electrode. Several consecutive spikes make a burst (shown in blue), and when multiple electrodes burst at the same time, this is represented as network burst (pink box).

Table 2.

Donors	MEA Raster Plot	Voltage trace and key characteristics
Unaffected donor		
ax0076: Unaffected From a male donor, 40-50 years old at time of donation		 1. Synchronized burst firing 2. Regular burst events with low IBI SD 3. Short burst durations 4. Constant spike amplitude
ALS donors		
ax0074: C9orf72 From a female ALS donor with C9orf72 mutation, 64 years old at time of donation		 1. Hyperexcitable phenotype 2. Less synchronized burst firing 3. Irregular burst events with higher IBI SD 4. Short burst durations 5. Variable spike amplitude
ax0079: TDP43 From a female ALS donor with TDP43 mutation, 62 years old at time of donation		 1. Less synchronized burst firing 2. Irregular burst events with higher IBI SD 3. Extremely long burst durations 4. Variable spike amplitude
ax0735: SOD1 From a female ALS donor with SOD1 mutation, 61 years old at time of donation		 1. Highly synchronized burst firing 2. Short & medium duration burst trains 3. Constant spike amplitude

The firing activity of the cells was assessed by 5-minute MEA recordings from DIV 10. Synchronous firing was detected from DIV 8 post-thaw. Data was recorded using the "Neural Spontaneous" configuration in AxIS Navigator software (Axion BioSystems). Action potentials were detected via adaptive thresholding to assess a selection of both non-networked and networked parameters which can be looked at in the module and are outlined in Table 3.

The differing ALS disease lines display clear network firing differences in keeping with their phenotype and disease complexity (Table 2). Samples are from a minimum of 3 electrodes in the same well, demonstrating the differences or uniformity of firing patterns within each cell type.

Quantification of the activity profiles and raster plots for the cultures shows that the ALS motor neurons have distinct firing profiles than the unaffected donor motor neurons. Differences are observed in firing rate, spike amplitude, bursting profiles and networking profiles. All the neurons produce clear burst and network burst activity as seen in the raster plots at DIV 20 (Table 2).

Neural activity recording and analysis with the Maestro MEA platform:

Action potentials were detected via adaptive thresholding to be processed and assessed quantitatively as both individual and network parameters, including measurements of firing, bursting, and synchrony. Data analysis was performed using the AxIS Metric Plotting Tool and wells were excluded if below 0.1 Hz Mean Firing Rate to generate the following raster plots (Fig. 3A) and a selection of quantitative endpoints for both non-networked and networked parameters (Fig. 3B). Error bars indicate SEM, n=3 -10 independent technical repeats against 10 wells per condition. Neurons produce clear burst and network burst activity as seen in the raster plots at DIV 20. Table 3 summarises the graphical data into a comparison table with changes in each listed parameter indicated by colour and arrow direction.

**Figure 3A: Raster plots of ALS motor neuron activity.**

Raster plots of each ALS phenotype generated by Axion's AxIS Metric Plotting Tool indicating qualitative differences in the firing patterns and network organization for each ALS disease line.
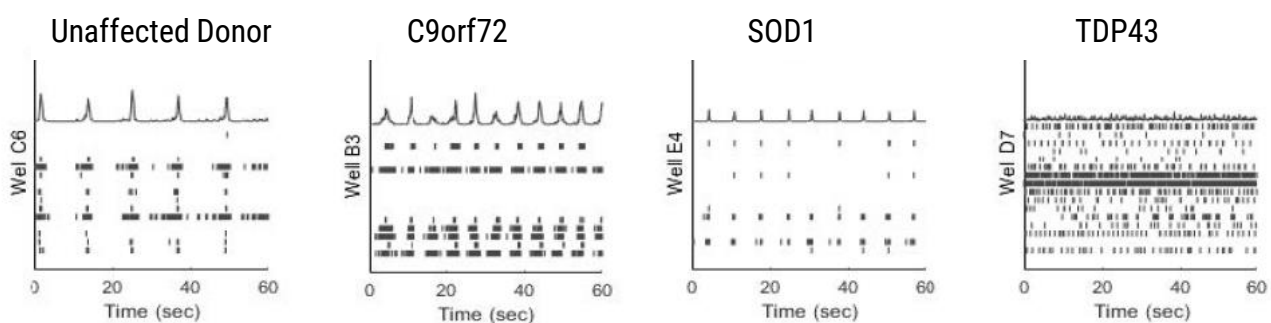
Figure 3B:

Non-network activity analysis.

Non-network parameters were analysed and indicated that spiking and bursting conditions change per ALS phenotype. Quantification of the data showed that, when compared to the unaffected, C9orf72 and SOD1 neurons share some similarities in spiking and network bursting, while TDP-43 neurons display significantly decreased or increased values, depending on the parameter. Distinct trends in firing mechanism were shown in spikes per burst, inter-spike intervals coefficient of variation (ISI-COV), and burst duration across all phenotypes with a downward trend across the ALS phenotype.
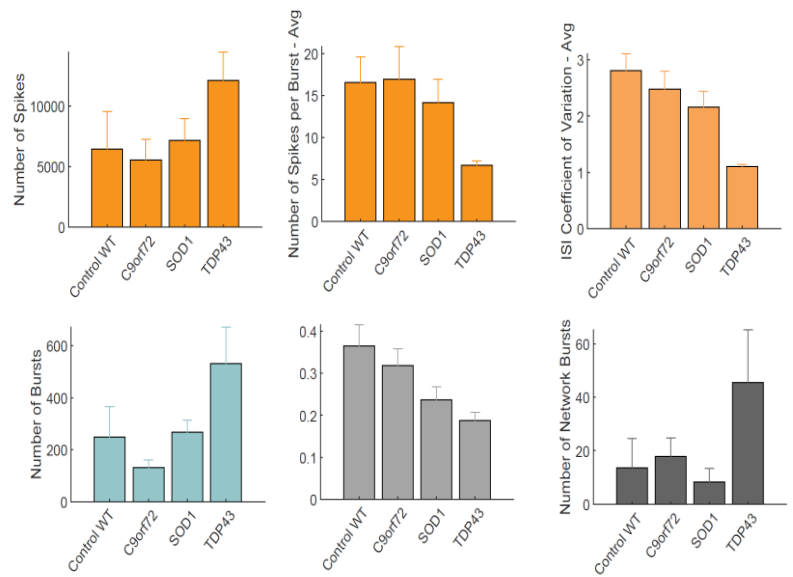
Network activity analysis:

All the cultures produced synchronous network activity, which was quantifiable and assay-ready from DIV 10. Quantification of the data showed that when compared to the unaffected donor cells, none of the phenotypes shared the same network characteristics. C9orf72 displayed significantly increased network burst percentage, and SOD1 displayed decreased network burst duration, percentage, and spike number. TDP-43, in contrast, displayed both increased network burst duration and spikes per burst but significantly decreased synchrony when compared to the other ALS phenotypes.
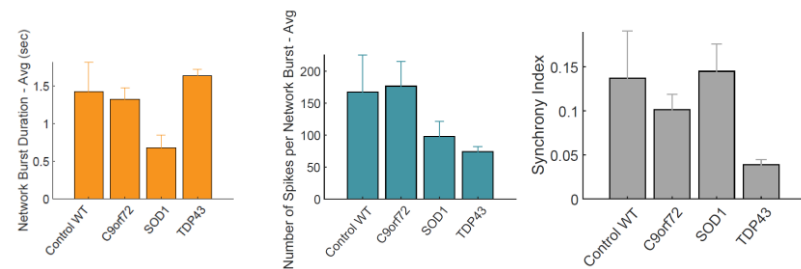
Table 3:

Summary of graphical data.

Changes in the parameter are indicated by color and arrow direction, as described in the key below. Key parameters can then be visualized and analyzed to distinguish among each of the Axol motor neuron ALS phenotypes.

Firing parameter	Wildtype	C9orf72	SOD1	TDP43
No. of spikes	→	→	→	↑
Mean firing rate	→	→	→	→
No. of spikes per burst	→	→	↘	↓
Inter-burst interval	→	↑	↑	↘
ISI-COV	→	↘	↘	↓
No. of bursts	→	↘	→	↑
Burst duration	→	↘	↘	↓
No. of network bursts	→	→	→	↑
Network burst duration	→	↘	→	↑
No. of spikes per NB	→	→	↓	↓
Network burst %	→	→	↓	↘
Network normalised duration IQR	→	→	→	↑
Synchrony index	→	→	→	↓
Spike amplitude	→	↑	→	↑

| Table Key	
Increased parameter value (major / small)	↑ ↗
Decreased parameter value (major / small)	↓ ↘
Comparable to wildtype	→

Discussion:

In the characterization of this set of axoCells™ iPSC-derived motor neurons, we used MEA and imaging to observe key traits. By incorporating this characterization into manufacturing QC and functional QC processes, we recorded multiple lines and multiple production runs (differentiations) to ensure these traits were robust and reproducible and were not just a consequence of a single differentiation procedure.

The use of Accelerator supplement and the Maestro Pro MEA enabled a short 20 day "from thaw to data" experimental regime to detect neuronal firing data and enabled simultaneous brightfield imaging for confirmation of cell spotting accuracy and correlation of cell culture health.

Motor neurons from 4 different lines were assessed for ALS-related morphology and neural activity phenotypes. The unaffected control line exhibited consistent spike amplitude, short burst events, and synchrony. In comparison, motor neurons from the ALS lines demonstrated a reproducible loss- or gain-of-function for certain parameters in each phenotype. C9orf72 motor neurons displayed increase spike amplitude and shortened ISI with increased network burst frequency. SOD1 motor neurons displayed shortened ISI with decreased network burst % and fewer spikes per burst. TDP-43 motor neurons displayed increased spike amplitude and mean firing rate with highly unsynchronised network activity and bursting patterns.

All ALS phenotype lines displayed a reproducible loss of synchronous firing and different degrees of hyperexcitability. This is in accordance with expected ALS clinical pathology and supports the case for the use of these cells in experimental *in vitro* models to study ALS pathology and potential therapeutics.
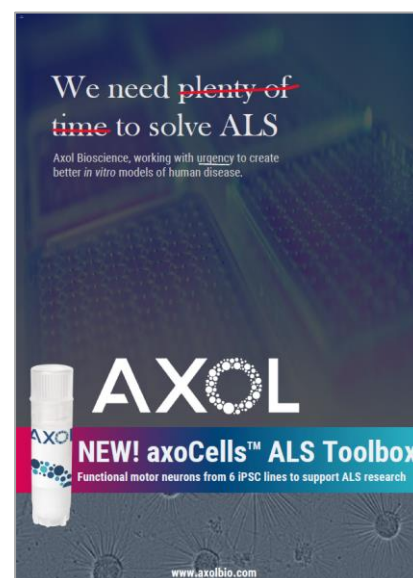
Learn more about iPSC-derived motor neurons in our axoCells™ ALS Toolbox brochure

iPSCs? How can we help.

We work in the spirit of partnership and transparency to unlock the benefits of iPSC technology for ALS drug discovery.

If you have any feedback, would like to discuss a project or receive a quotation, contact us at operations@axolbio.com