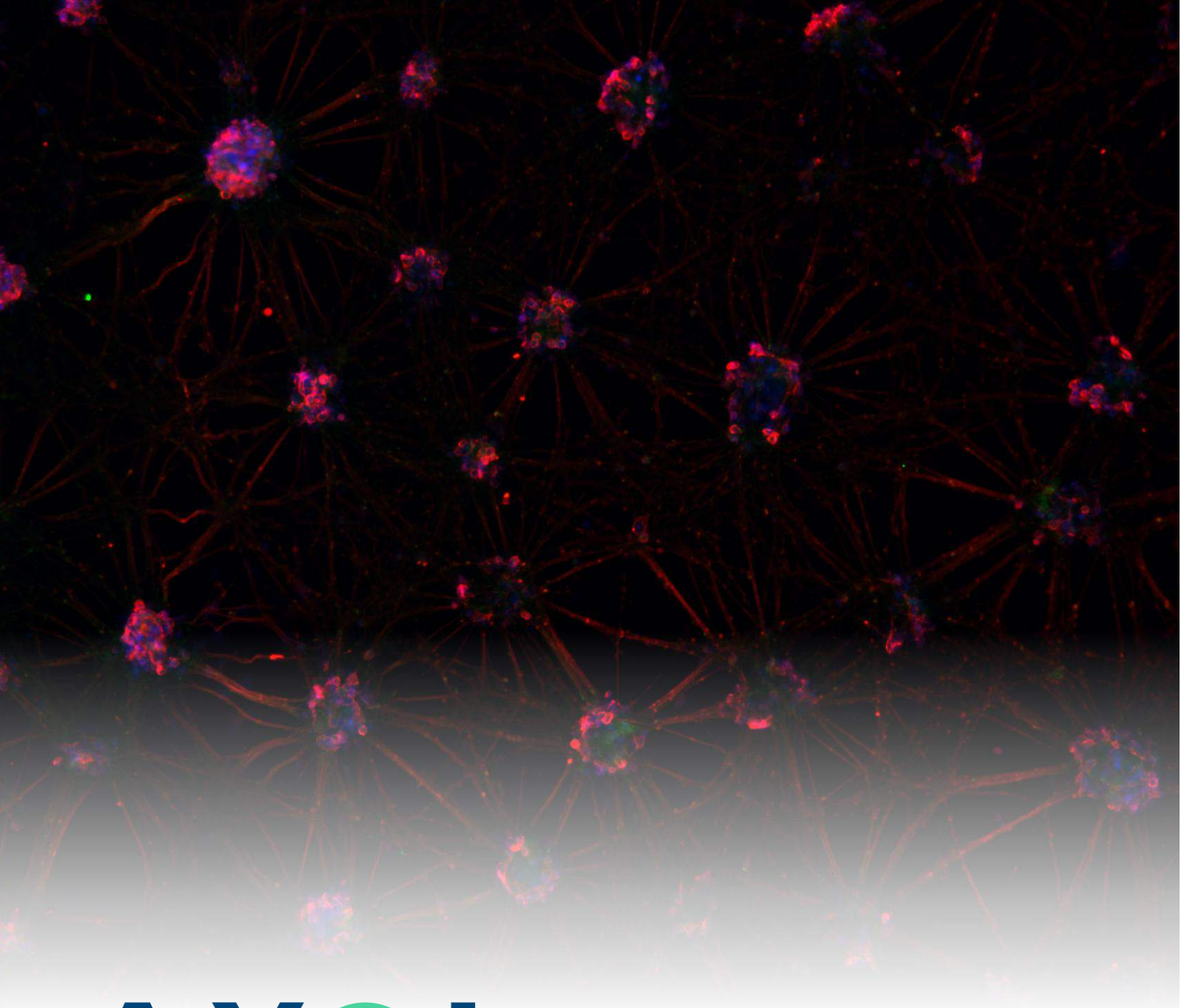


AXOL

Functional motor neurons from patient iPSC lines to support ALS research and drug discovery

axoCells™ ALS toolbox
Cells and specialist service provision

www.axolbio.com

In vitro models for ALS/MND drug discovery

ALS (amyotrophic lateral sclerosis) is the most common form of motor neuron disease, where the progressive destruction of motor neurons leads to loss of muscular functions.

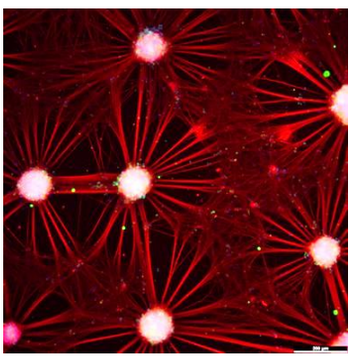
With limited treatment options and a predicted 69% increase in cases by 2040[2], attention has turned to implementing *in vitro* ALS models that use human induced pluripotent stem cell (iPSC)-derived cells from healthy or patient donors to accelerate preclinical drug discovery.

The cells retain the disease characteristics and genetic background of the donor, enabling researchers to generate more predictive *in vitro* ALS models for basic research and drug discovery.

Major cell types involved in ALS

Motor neurons
Central to ALS pathophysiology is the progressive destruction of motor neurons, preventing effective communication between the brain/spinal cord and muscles.

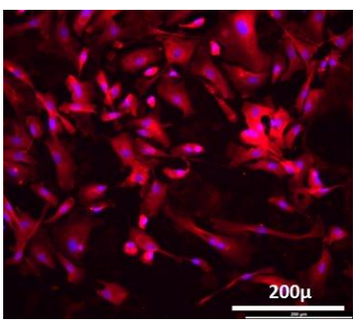
axoCells motor neurons are functionally active in 10 days and demonstrate key marker expression (including HB9 and ChAT) and functional relevance in assays including electrophysiology and calcium imaging. ALS patient donor-derived motor neurons that carry known mutations, such as in C9orf72 (ax0074), demonstrate phenotypic and functional differences compared to healthy donor -derived motor neurons, including more fibrous neurites and higher firing frequency.



axoCells Motor Neurons **S**tained for TUJ-1 marker (red) at day 21.

Microglia
An overlap has been observed between neurodegenerative disorders, ALS and frontotemporal dementia (FTD) with several genes implicated in both diseases, including C9orf72, the most common cause of familial ALS. Neuroinflammation is thought to be an important contributor to the pathology of both of these diseases.

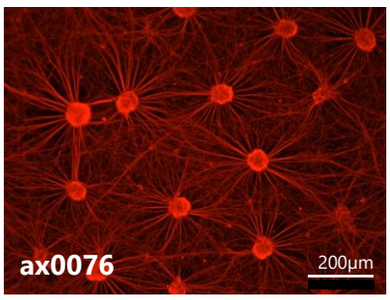
axoCells microglia are assay-ready in 7 days and express key markers (including Iba1, TMEM119, CX3CR1 and P2RY12), with functional relevance in assays including phagocytosis, chemotaxis and cytokine release. ALS-derived microglia (C9orf72) exhibit reduced phagocytosis of myelin basic protein compared to healthy control.



axoCells Microglia stained for CX3CR1 marker (red) and DAPI (blue) at day 7.

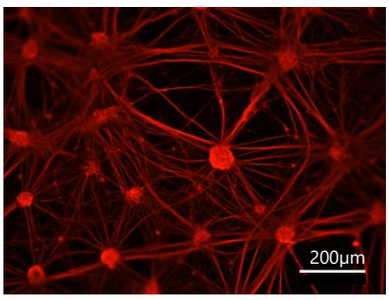
Characterization of iPSC-derived motor neurons

Unaffected donors (including an asymptomatic donor with C9orf72 mutation)



ax0076, male donor, 40-50 years

- Large uniform cell-body clusters
- Thick cabling between cell bundles
- Few fibrous thin neurites

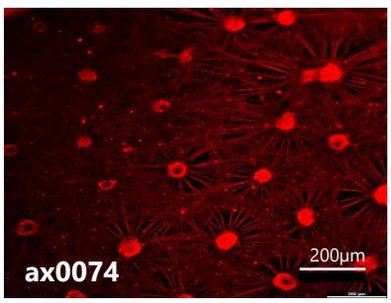


ax0073, male donor, 62 years, C9orf72 carrier, asymptomatic at donation‡

- Smaller irregular cell-body clusters that are dispersed
- Thinner cabling between cell bundles
- More fibrous thin neurites

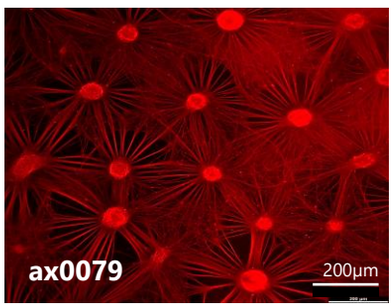
‡sibling to donor for ax0074

ALS patient donors carrying SOD1, TDP-43 or C9orf72 mutations



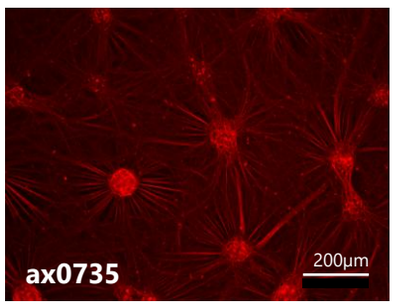
ax0074, female, 64 years, ALS patient donor, C9orf72 mutation

- Smaller irregular cell body clusters that are dispersed
- Thinner cabling between cell bundles
- More fibrous thin neurites



ax0079, female, 62 years, ALS patient donor, TDP-43 mutation

- Smaller irregular cell body clusters
- Thinner cabling between cell bundles
- More fibrous thin neurites

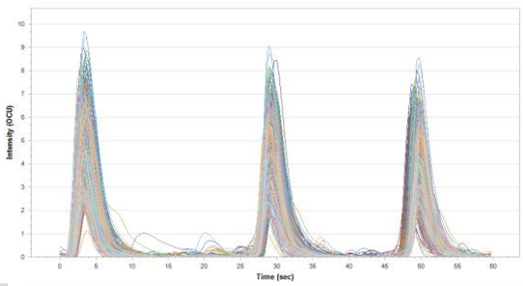
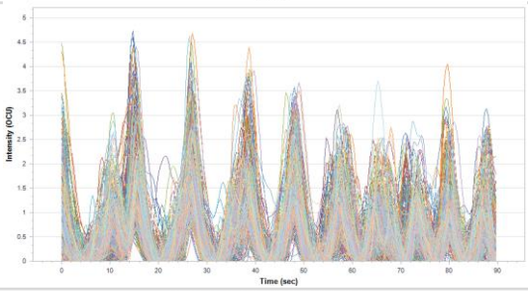
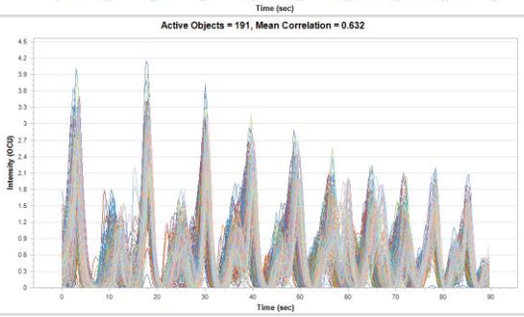
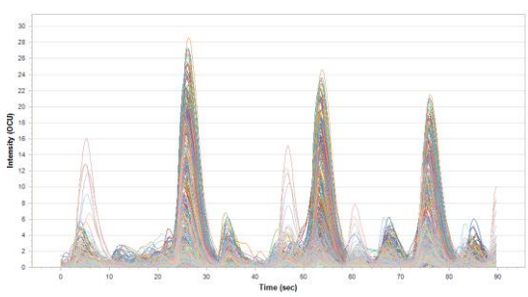
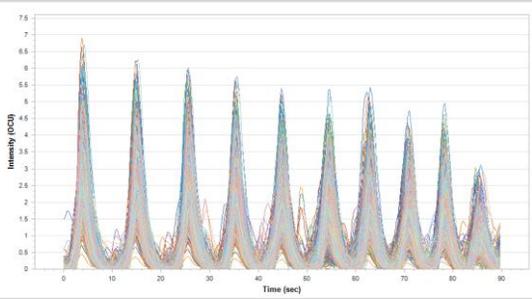


ax0735, female, 61 years, ALS patient donor, SOD1 mutation

- Large, irregular cell body clusters
- Thick cabling between cell bundles
- A mix of thin and thick neurite cabling network

ICC images. Red: TUJ1 (Neuron-specific class III beta-tubulin)

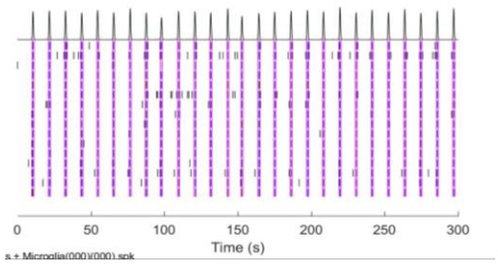
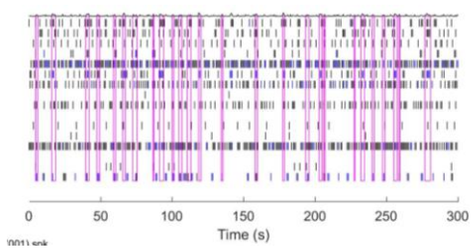
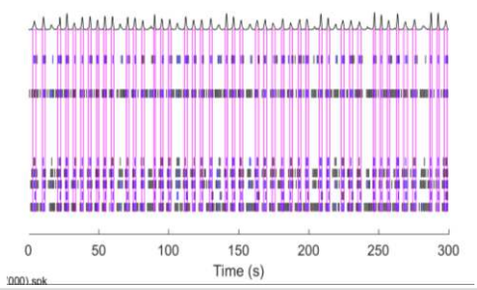
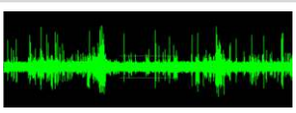
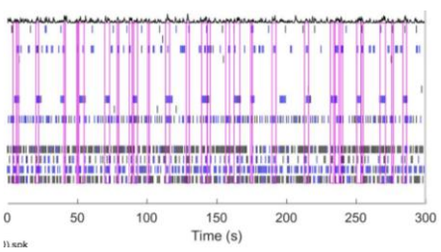
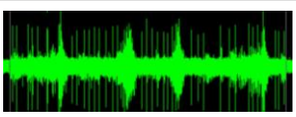
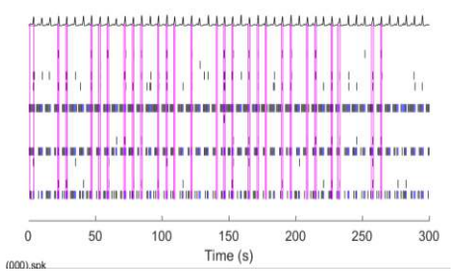
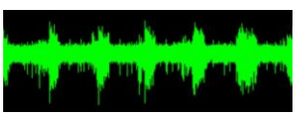
Aberrant excitability of iPSC-derived motor neurons from ALS patient donors

Donors		Key characteristics
Unaffected **ax0076,** male donor, 40-50 years		1. Highly synchronized (mean correlation >0.9) 2. Consistent sustained mean burst duration 3. Regular burst rate
C9orf72 carrier **ax0073,** male donor, 62 years, C9orf72 carrier, asymptomatic at donation, sibling to donor for ax0074		1. Hyperexcitable phenotype 2. Less synchronized 3. Lower mean burst duration 4. Higher burst rate
C9orf72 **ax0074,** female, 64 years, ALS patient donor, C9orf72 mutation		1. Hyperexcitable phenotype 2. Less synchronized (mean correlation ~0.7) 3. Lower mean burst duration 4. Higher burst rate
TDP-43 **ax0079,** female, 62 years, ALS patient donor, TDP-43 mutation		1. Less synchronized 2. Higher burst rate
SOD1 **ax0735,** female, 61 years, ALS patient donor, SOD1 mutation		1. Highly synchronized 2. Higher burst rate

sibling

Data generated using the Sartorius IncuCyte® S3

Multi-electrode array (MEA) characterization of iPSC-derived motor neurons

Donors		Key characteristics
Unaffected **ax0076,** male donor, 40-50 years		 1. Synchronized burst firing 2. Regular burst events with low IBI SD 3. Short burst durations 4. Constant spike amplitude
C9orf72 carrier **ax0073,** male donor, 62 years, C9orf72 carrier, asymptomatic at donation, sibling to donor for ax0074		 1. Hyperexcitable phenotype 2. Less synchronized burst firing 3. Irregular burst events with higher IBI SD 4. Short burst durations 5. Variable spike amplitude
C9orf72 **ax0074,** female, 64 years, ALS patient donor, C9orf72 mutation		 1. Hyperexcitable phenotype 2. Less synchronized burst firing 3. Irregular burst events with higher IBI SD 4. Short burst durations 5. Variable spike amplitude
TDP-43 **ax0079,** female, 62 years, ALS patient donor, TDP-43 mutation		 1. Less synchronized burst firing 2. Irregular burst events with higher IBI SD 3. Extremely long burst durations 4. Variable spike amplitude
SOD1 **ax0735,** female, 61 years, ALS patient donor, SOD1 mutation		 1. Highly synchronized burst firing 2. Short & medium duration burst trains 3. Constant spike amplitude

sibling

Data generated using the Axion Maestro PRO

Observing hyperexcitability, a classic trait of ALS, in iPSC-derived motor neurons

A critical aspect of ALS pathophysiology is the hyperexcitability of motor neurons, a phenomenon that has garnered significant attention in the study of this disease. Hyperexcitability refers to an increased responsiveness of motor neurons to synaptic inputs, leading to an abnormal increase in firing rate. In the long term this increased electrical activity can lead to excitotoxicity which is believed to contribute to the degeneration of motor neurons and the progression of ALS.

Mechanisms of hyperexcitability in ALS motor neurons

The hyperexcitability of motor neurons in ALS can be attributed to several underlying mechanisms. One key factor is the altered function of ion channels that regulate neuronal excitability. Motor neurons express a variety of ion channels, including voltage-gated sodium (Na+), potassium (K+), and calcium (Ca^{2+}) channel, all of which play critical roles in the generation and propagation of action potentials. Another factor is that ALS enhances motor neuron vulnerability to glutamate-mediated excitotoxicity via AMPA receptors leading to calcium overload. In ALS, there is evidence that the function of these ion channels is dysregulated.

How hyperexcitability is observed in *in vitro studies using multielectrode array (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.

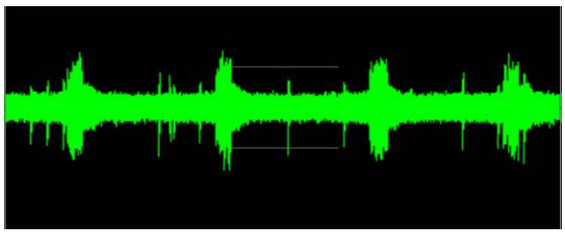
Indications of hyperexcitability in MEA electrode traces

1. Higher frequency - more bursts (synchronised events) happening in the same time scale
2. More activity ('noise') in between the bursts
3. Lack of synchronization of bursts

Raw data is seen in real-time as an electrode trace. A simple way to visualize this neural network behavior is with a raster plot. 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 event (pink box).

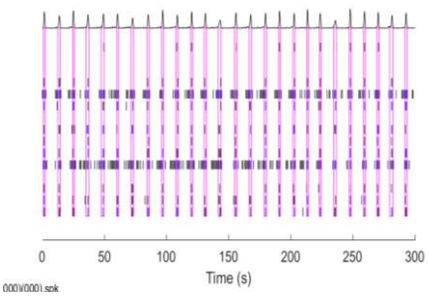
Differences between cells can be observed in connection to the overall firing rate, the synchronization of firing, the length of bursts and the amplitude of bursts.

1a. Electrode trace from ax0078

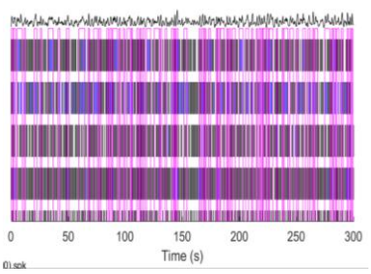


Overview data presented in the form of raster plot captures the difference between the synchronised burst firing of ax0078 unaffected donor motor neurons (1a, 1b) and the increase in firing rate and loss of synchronisation in ax0079 TDP-43 mutant motor neurons from an ALS patient donor (1c)

1b. ax0078 unaffected



1c. ax0079 TDP43



Intrinsic properties of ALS patient-derived motor neurons

ALS motor neurons exhibit intrinsic properties that contribute to their hyperexcitability. These include changes in the expression and function of ion channels and alterations in other cellular components, such as the cytoskeleton, which may affect the structural and functional integrity of the neurons.

For example, ALS motor neurons may show a reduction in the expression of small conductance calcium-activated potassium (SK) channels, which normally act to hyperpolarize the membrane following an action potential and prevent excessive firing. The downregulation of SK channels can therefore lead to prolonged depolarization and increased excitability. Additionally, mutations in ALS-linked genes, such as SOD1, TDP-43, and FUS, can directly or indirectly affect the expression and function of these channels, further contributing to the hyperexcitability phenotype.

Motor neurons in ALS may also exhibit changes in the expression of synaptic proteins, which can alter synaptic strength and plasticity. These alterations can lead to increased synaptic input onto motor neurons, further enhancing their excitability. The loss of inhibitory inputs, such as those mediated by GABAergic or glycinergic interneurons, can also contribute to the hyperexcitability phenotype, as the balance between excitation and inhibition in the motor neuron circuitry becomes disrupted.

Genetic and environmental contributions

The hyperexcitability of motor neurons in ALS is influenced by both genetic and environmental factors. Mutations in several genes associated with familial forms of ALS, such as SOD1, C9orf72, TARDBP (encoding TDP-43), and FUS, have been linked to hyperexcitability in motor neurons. For example, SOD1 mutations are known to cause oxidative stress and mitochondrial dysfunction, which can lead to the dysregulation of ion channels and calcium homeostasis, contributing to hyperexcitability.

The expansion of GGGGCC repeats in the C9orf72 gene, the most common genetic cause of ALS, has been associated with the formation of toxic RNA foci and dipeptide repeat proteins, which can disrupt normal cellular function and contribute to neuronal hyperexcitability. Similarly, mutations in TARDBP and FUS, which encode RNA-binding proteins involved in RNA processing and transport, can lead to the accumulation of toxic protein aggregates and dysregulation of gene expression, further exacerbating the hyperexcitability of motor neurons.

Environmental factors, such as exposure to toxins, oxidative stress, and inflammation, can also contribute to the development of hyperexcitability in ALS motor neurons. For instance, chronic exposure to environmental toxins like pesticides and heavy metals has been linked to an increased risk of ALS, potentially through their effects on neuronal excitability and synaptic function. Oxidative stress, which is a common feature in ALS, can lead to the modification of ion channels and other proteins involved in maintaining neuronal excitability, further promoting hyperexcitability.

Observations from the ALS iPSC-derived motor neuron toolbox

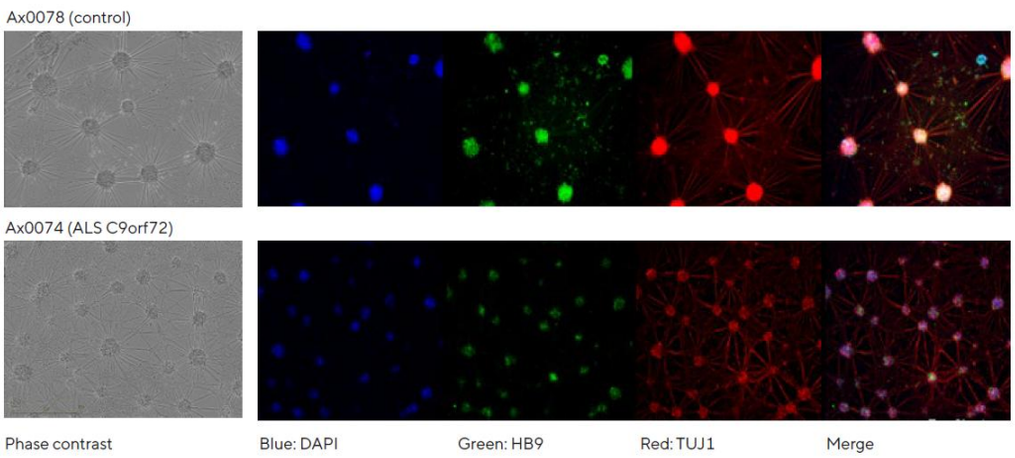
The reproducible characteristics of ALS patient iPSC-derived motor neurons observed using MEA, SNA and imaging have been used to establish functional quality control for the motor neuron differentiation process, allowing us to generate for the first time a panel of comparative iPSC-derived motor neurons for use in preclinical drug discovery and basic research.

In both unaffected donor lines, we observed synchronization, short burst events and constant signal amplitude. In comparison, motor neurons from the ALS lines and the unaffected sibling control motor neuron carrying the C9orf72 mutation, demonstrated a reproducible loss of regular synchronous firing and different hyperexcitability phenotypes, recapitulating clinical observations.

Case study: iPSC-derived motor neurons and microglia from ALS backgrounds display disease phenotype

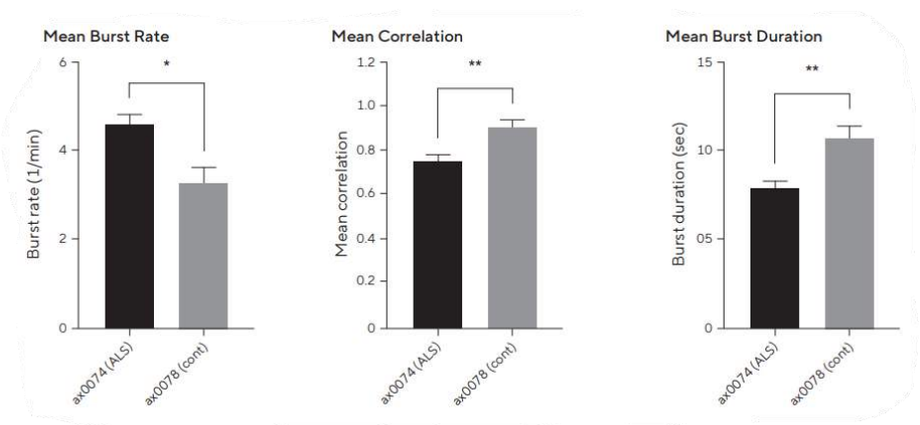
The overlap between ALS and FTD (frontotemporal dementia) has led researchers to investigate the role of neuroinflammation in ALS development. This has led to increasing interest in the role of microglia, the main neuroinflammatory cell, in the development of ALS. In collaboration with Sartorius, we investigated the morphology and functional performance of ALS-derived motor neurons and microglia compared to healthy control cells. Here we present key highlights from the project.

Immunocytochemistry of axoCells motor neurons and microglia



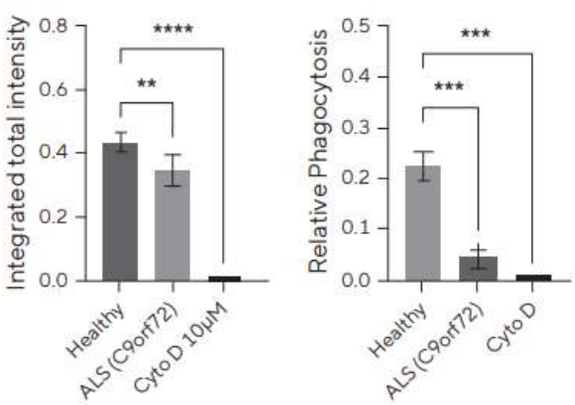
Mature axoCells™ iPSC-derived motor neurons (control and ALS) immunocytochemistry. Cells were matured for 20 days and stained for markers of motor neurons. DAPI was used as a nuclear dye.

Spontaneous neuronal activity analysis on day 18 axoCells motor neurons



The IncuCyte® Neuronal Activity Software Module was used to monitor and analyze calcium signaling. Statistical significance was assessed using a non-parametric T-test, *p<0.05, **p<0.01. ALS motor neurons exhibited significantly higher burst rates compared to the control line, along with lower burst duration and lower mean correlation (hence less synchronized burst firing).

Phagocytosis of myelin basic protein (MBP) by axoCells microglia



Phagocytosis of pHrodo® for IncuCyte® labeled MBP was assessed on an Incucyte® Live-Cell Analysis System up to 24h using fresh and cryopreserved microglia. Cytochalasin D (10 µM) was used as a control. Statistical significance was assessed using a one-way ANOVA, **p<0.01, ***p<0.001, ****p<0.001

This indicates that ALS microglia affected by the C9orf72 expansion in ALS exhibit decreased phagocytosis, suggesting utility in ALS studies.

ALS Toolbox for drug discovery and research

Extensively characterised iPSC-derived motor neurons from multiple ALS patient donors and unaffected controls available immediately, custom differentiation service provided for isogenic cell types including microglia and astrocytes. We also supply complete media solutions for ease of adoption and extensive user guides. Our scientific team are always available to support new users and workflow development.

Product name	Relevant details	Cells (kit)
axoCells™ human iPSC-derived motor neurons (unaffected), male donor, aged 40-50, ≥2 million cells		ax0076 (ax0186)
axoCells™ human iPSC-derived motor neurons (unaffected, C9orf72 carrier), male donor, aged 62, ≥2 million cells	Sibling of donor for ax0074. Caucasian / Ashkenazi with family history of ALS and dementia.	ax0073 (ax0183)
axoCells™ human iPSC-derived motor neurons (ALS, SOD1), female donor, aged 61, ≥2 million cells	SOD1 Het D109Y (G>T)	ax0735 (ax01835)
axoCells™ human iPSC-derived motor neurons (sLS, TDP-43), female donor, aged 62, ≥2 million cells	TDP-43 A382T	ax0079 (ax0189)
axoCells™ human iPSC-derived motor neurons (sLS, C9orf72), female donor, aged 64, ≥2 million cells	Sibling of ax0073. Caucasian / Ashkenazi with family history of ALS and dementia. Onset of ALS symptoms aged 60, death at 65.	ax0074 (ax0184)

Media & supplements

Product name	Product code
axoCells™ human CNTF supplement, 20 µg	ax139888
axoCells™ human GDNF supplement, 10µg	ax139855
axoCells™ human BDNF supplement, 10 µg	ax139800
axoCells™ motor neuron maintenance media, 200 ml	ax0072
axoCells™ motor neuron accelerator supplement, 1 ml	ax0179

Additional third-party components may be required. Please refer to protocol for full list.

User guide

Specialized services for ALS preclinical research and drug discovery

We apply our expertise in patient iPSC line generation and curation, along with our deep expertise in iPSC-derived *in vitro* models of neurodegeneration to support every stage of preclinical discovery from novel target validation to candidate selection. Our scientific and project management teams work collaboratively with clients to scope and deliver bespoke projects on a standalone or dedicated resource (FTE) basis.

Patient line derivation, banking and iPSC quality management
We work with patient advocacy groups and private and public research organisations to support line sourcing, ethics and consent review, primary material management, iPSC reprogramming, iPSC quality control, cell bank creation, expansion and custom differentiation. We currently maintain a library of >70 fully quality controlled iPSC lines and are actively building diversity and additional disease-relevance into the collection.

iPSC technical services
Services include large scale reprogramming projects, iPSC generation from primary material, iPSC quality control and gene-editing. Following generation of iPSC, or using client or commercially available lines, we provide manufacturing-scale differentiation to a range of cells including cortical excitatory neurons, interneurons, sensory neurons, striatal neurons, motor neurons, microglia, astrocytes, ventricular and atrial cardiomyocytes, cryopreservation where appropriate and functional quality control.

***In vitro* model and assay development and validation**
Services include complex model development in monoculture and co-culture, custom protocol design and optimization, model characterization, phenotypic analysis, treatment titration and endpoint assays, integration with MPS and other platforms and collaborative scale-up for transfer to higher throughput platforms and CRO, or in-house workflows.

Candidate drug testing in iPSC models
Services include compound, viral delivery vectors or novel mechanism drug toxicity and effect screening using optimised in-house assays including multi-electrode array (MEA), flow cytometry, cellular imaging, real-time imaging, plate-based assays and 'omics.

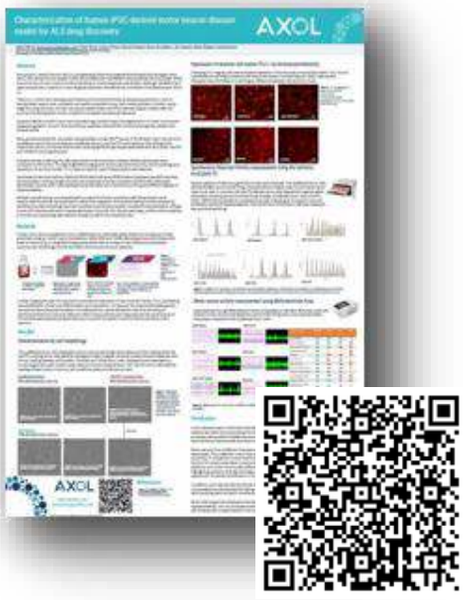
Assays commonly utilized in ALS-related projects
We are actively collaborating with multiple large organisations pursuing new therapeutic avenues for ALS/MND and provide reprogramming, custom banking and distribution or licensing. Service project provision is available for the evaluation of new compounds, viral vectors or combinations and these frequently include assessing functionality of neurons by MEA or IncuCyte based spontaneous neuronal activity measurements. In addition, effects on cell toxicity and morphology are assessed by tracking neurite outgrowth and utilising ATP/LDH based cell health assays on both motor neurons and microglia derived from ALS patients. To support new developments in the field, we are developing further reproducible assays, including Neurofilament light (NFL) quantification, TDP-43 aggregation (imaging and HTRF based), cryptic exons and developing functional tri-culture models with motor neurons, astrocytes and microglia to evaluate neuroinflammatory aspects.

We are always happy to discuss and help scope your project requirements. Please email operations@axolbio.com to arrange a technical call with our scientific leads.

Resources

iPSC-derived motor neurons and microglia from ALS background display disease phenotype

Fibroblasts taken from a healthy individual and an ALS patient carrying a C9orf72 hexanucleotide expansion (GGGGCC >145) were reprogrammed to induced pluripotent stem cells (iPSCs), and then differentiated to motor neurons and microglia alongside controls. The cells were then assessed for differences in morphology and functional activity, measured via electrophysiology, spontaneous neural activity, and microglial phagocytosis. Diseased iPSC-derived motor neurons exhibited less organized morphology compared to control, as well as less synchronized firing patterns and hyperexcitability. ALS patient-derived microglia demonstrated a decreased capability for phagocytosis, particularly when cryopreserved. Taken together, these results demonstrate clear morphological and functional differences in differentiated cells derived from a C9orf72-positive ALS patient, compared to healthy control cells which can therefore be used as a robust model for C9orf72 ALS research and drug discovery.



Characterization of human iPSC-derived motor neuron disease model for ALS drug discovery

In this poster, we have successfully characterized axoCells human iPSC-derived motor neurons from six different donor lines including two unaffected donors, one C9orf72 carrying donor (the sibling to the C9orf72 ALS donor), and three donors each carrying significant genotypes associated with ALS: SOD1, C9orf72 and TDP-43 for ALS drug discovery.

Using functional screening, the cells were shown to demonstrate a disease relevant phenotype when compared to the control. Through Brightfield imaging and Immunocytochemistry (ICC), the expected morphology and expression of neuronal marker TUJ1 (Neuron-specific class III beta-tubulin) was observed.

Spontaneous Neuronal activity (SNA) and Multi-electrode array (MEA) analysis displayed overall firing rates, synchronization of firing, length of bursts and amplitude of bursts consistent with the cells' phenotype. Specifically, the ALS donor cells displayed a reproducible loss of synchronous firing and different degrees of hyperexcitability.

Multiple manufacturing runs showed batch-to-batch functional consistency with these results. Such successful characterization of these human iPSC-derived cells which display attributes in line with ALS clinical pathology, confirms their suitability to function as a disease model for ALS drug discovery.

Resources

Publications featuring axoCells™ motor neurons

1. McCallister, T.X., Lim, C.K.W., Terpstra, W.M., Zeballos C, M.A., Zhang, S., Powell, J.E. and Gaj, T. (2023) A high-fidelity CRISPR-Cas13 system improves abnormalities associated with C9ORF72-linked ALS/FTD. bioRxiv. Available at: https://doi.org/10.1101/2023.12.12.571328
2. Spijkers, X.M., Pasteuning-Vuhman, S., Dorleijn, J.C., Vulto, P., Wevers, N.R. and Pasterkamp, R.J. (2021) A directional 3D neurite outgrowth model for studying motor axon biology and disease. Scientific Reports. Available at: https://doi.org/10.1038/s41598-021-81335-z
3. Rimington, R.P., Fleming, J.W., Capel, A.J., Wheeler, P.C. and Lewis, M.P. (2021) Bioengineered model of the human motor unit with physiologically functional neuromuscular junctions. Scientific Reports. Available at: https://doi.org/10.1038/s41598-021-91203-5

References and further reading

1. *G Lorenzo Odierna, Steve Vucic, Marcus Dyer, Tracey Dickson, Adele Woodhouse, Catherine Blizzard, How do we get from hyperexcitability to excitotoxicity in amyotrophic lateral sclerosis?, Brain, Volume 147, Issue 5, May 2024, Pages 1610–1621, https://doi.org/10.1093/brain/awae039*

2. *Nathan Pavey, Andrew Hannaford, Mehdi van den Bos, Matthew C Kiernan, Parvathi Menon, Steve Vucic, Distinct neuronal circuits mediate cortical hyperexcitability in amyotrophic lateral sclerosis, Brain, Volume 147, Issue 7, July 2024, Pages 2344–2356, https://doi.org/10.1093/brain/awae049*

3. *1 Badu-Mensah, A., Guo, X., McAleer, C.W. et al. Functional skeletal muscle model derived from SOD1-mutant ALS patient iPSCs recapitulates hallmarks of disease progression. Sci Rep 10, 14302 (2020). https://doi.org/10.1038/s41598-020-70510-3*

4. *Xu, L., Liu, T., Liu, L., Yao, X., Chen, L., Fan, D., Zhan, S. and Wang, S. (2019) Global variation in prevalence and incidence of amyotrophic lateral sclerosis: a systematic review and meta-analysis. Journal of Neurology. Available at: https://doi.org/10.1007/s00415-019-09652-y*

Quality manufacturing
We manufacture iPSC-derived cell types from our ISO9001:2015 production facility.

Global logistics
We supply our products worldwide to pharma, biotech, research and technology providers.

Dedicated team
With a highly skilled, science-rich team, we work collaboratively to realize better human disease models.

If you would like to discuss the adoption of iPSC-derived motor neurons into your workflow, or our specialist services provision, please contact us at operations@axolbio.com

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