Focus on ALS:

Building better *in vitro* ALS models with human iPSC technology

2024 Disease Focus Area

FOCUS on ALS

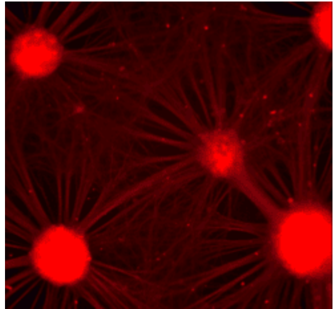
ALS (amyotrophic lateral sclerosis) is the **most common form** of motor neuron disease, where the progressive destruction of motor neurons leads to loss of muscular functions including walking, talking, swallowing and breathing. **There is currently no cure**, with treatment aimed at symptomatic relief and prolonging survival; most patients live only 3-5 years from the onset of symptoms[1].

With **limited treatment options** and a predicted **69% increase** in cases by 2040[2], attention has turned to *in vitro* ALS models that use human iPSCs from healthy or ALS patient donors. The cells generated from these iPSCs **retain the characteristics** of their donors, enabling researchers to generate *in vitro* ALS models to **improve knowledge** of the disease or to **screen potential therapies** on a more human-relevant platform.

1 Xu L et al. doi: https://doi.org/10.1007/s00415-019-09652-y
2 Arthur, K et al. doi: https://doi.org/10.1038/ncomms12408

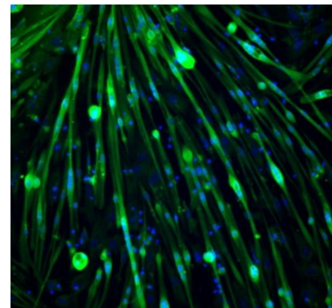
Key cell types involved in ALS:

- **Motor neurons:** Central to ALS pathophysiology is the progressive destruction of motor neurons, preventing communication between the brain/spinal cord and muscles.
    - axoCells motor neurons are mature in 10 days and demonstrate key marker expression (including HB9 and ChAT) and functional relevance in assays including electrophysiology and calcium imaging. Our ALS-derived motor neurons (ax0074, C9ORF72 mutation) demonstrate phenotypic and functional differences compared to healthy control-derived motor neurons, including more fibrous neurites and higher firing frequency.



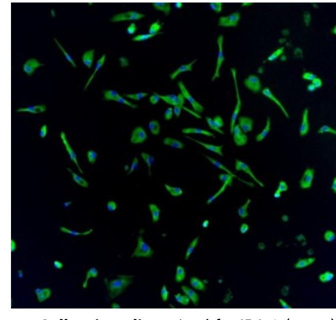
axoCells motor neurons stained for TUJ-1 (red) at day 21. X20 magnification

- **Muscle cells:** ALS is characterized by the progressive loss of muscle function driven by motor neuron destruction (although a specific role for muscle cells has also been suggested[1]). Motor neurons and muscle cells can be combined to form an ALS neuromuscular junction (NMJ) model for research and drug discovery.
    - Our axoCells myotubes are assay-ready in 5 days and demonstrate mature morphology and key protein expression (including desmin, dystrophin, titin and myosin heavy chain). They can be used together with axoCells motor neurons to build a robust neuromuscular junction model.



axoCells myotubes stained for titin (green) at day 5. X20 magnification

- **Microglia:** There is an overlap between ALS and frontotemporal dementia (FTD) with several genes implicated including C9ORF72, the most common cause of familial ALS. As the main neuroinflammatory cell of the brain, microglia can be used to model the ALS-FTD overlap
    - Our 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 IBA-1 (green) at day 7.

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

The phenotype of ALS iPSC-derived cells

We have investigated **phenotypic characterization** and **functional relevance** with ALS-derived motor neurons. Below is data from motor neurons bearing the key mutations associated with ALS (**C9ORF72, SOD1 and TARDBP (TDP43)**), exhibiting characteristic morphological phenotypes.

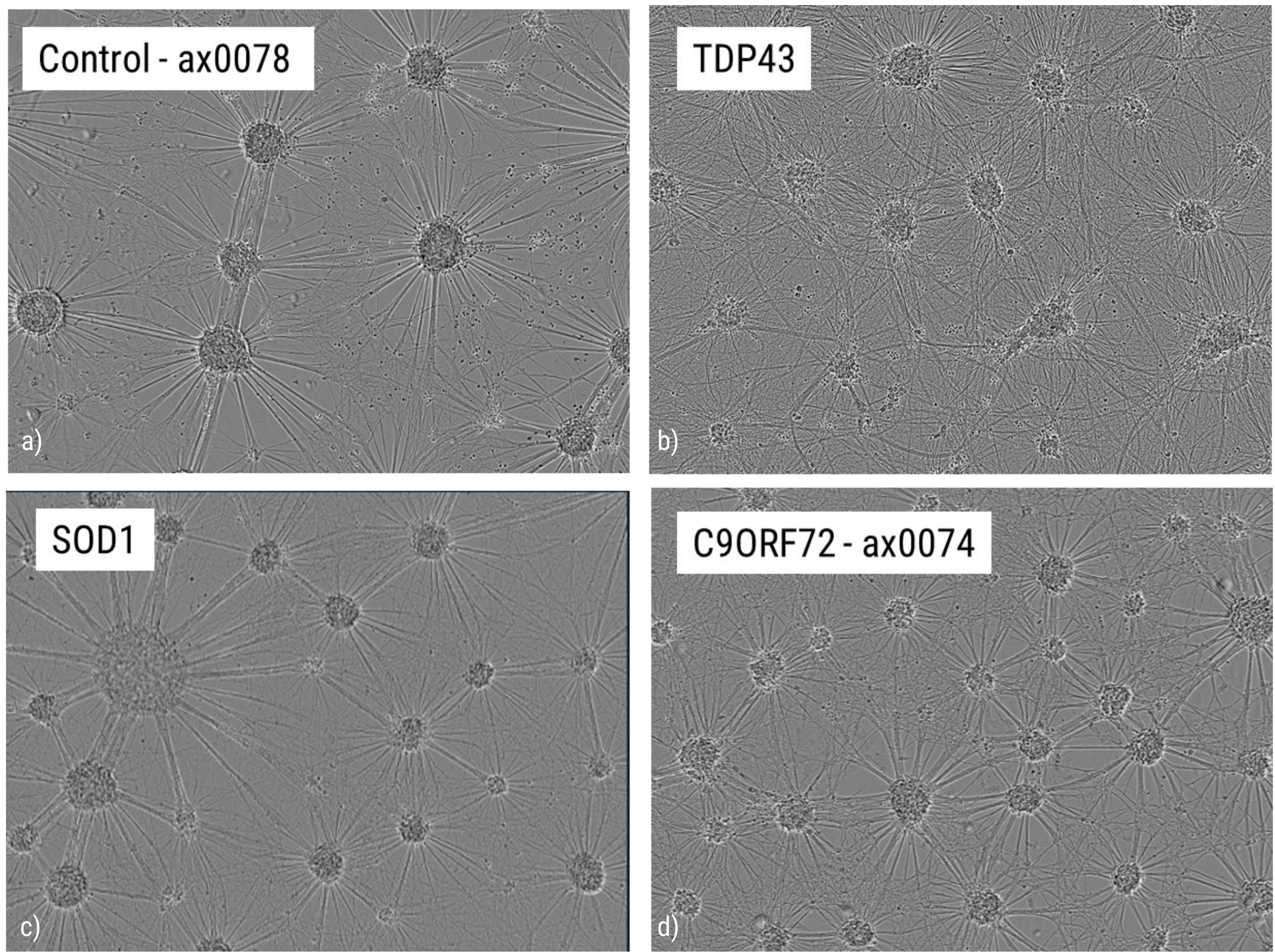


Figure 1. Phase contrast images of iPSC-derived motor neurons demonstrating phenotypic changes based on specific ALS-associated mutations.

Healthy control-derived motor neurons (**fig. 1a**) form uniform cultures with cabling between large cell bundles.

Motor neurons derived from iPSCs with a **TDP43** mutation (**fig. 1b**) exhibit heterogenous morphology, with different size clusters and shorter, curved neurites.

Motor neurons with a **SOD1** mutation (**fig. 1c**) also appear to have a heterogenous morphology, with large and small clusters, almost as if a mix of control-derived and C9ORF72 mutation-derived motor neurons.

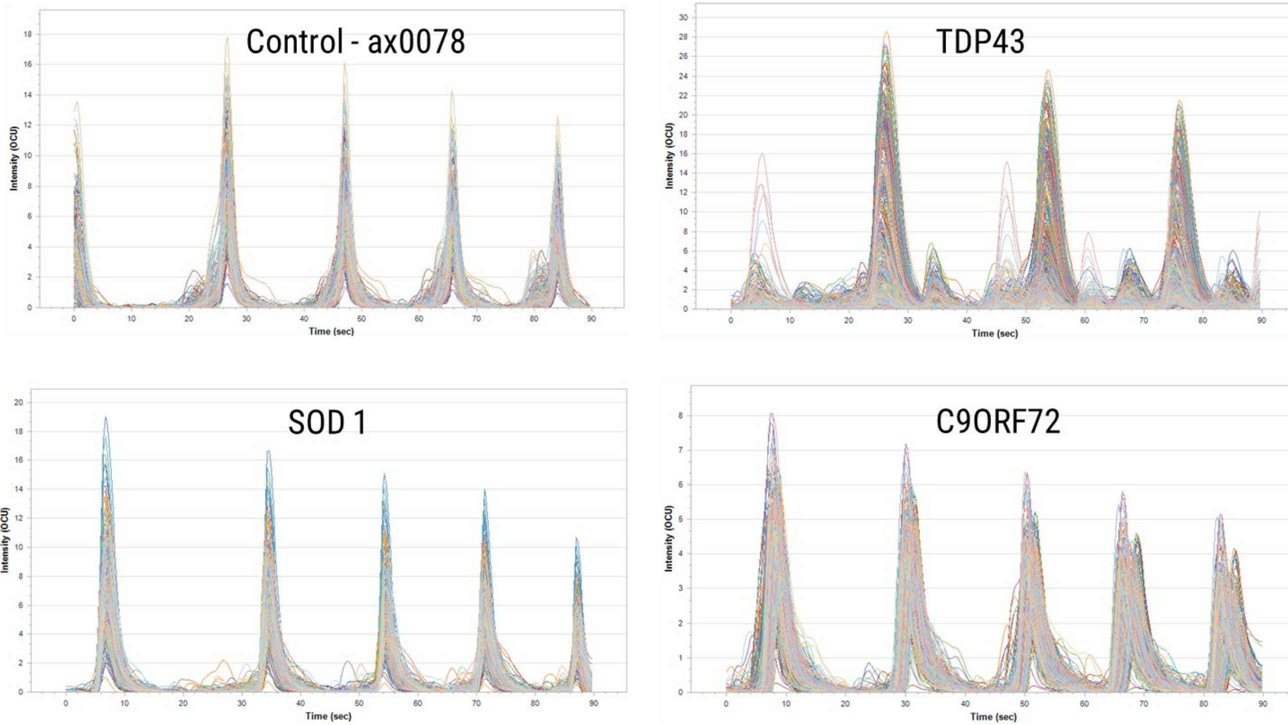
iPSC-derived motor neurons with a **C9ORF72** mutation **(fig. 1d)** form less uniform cultures, with smaller bundles and an increased number of smaller, fibrous neurites.

This demonstrates the utility of morphological characterization to assess for ALS disease phenotype.

Here we present **calcium imaging data** comparing healthy control-derived motor neurons and ALS-derived motor neurons (**fig. 2**). We use the Sartorius IncuCyte® S3 to measure **spontaneous neural activity (SNA)** as a useful assay to identify ALS-like phenotypes.

Calcium imaging data of iPSC-derived motor neurons (day 21 of maturation from progenitors)

2a) Spontaneous neural activity (SNA) data



2b) Mean correlation for SNA data

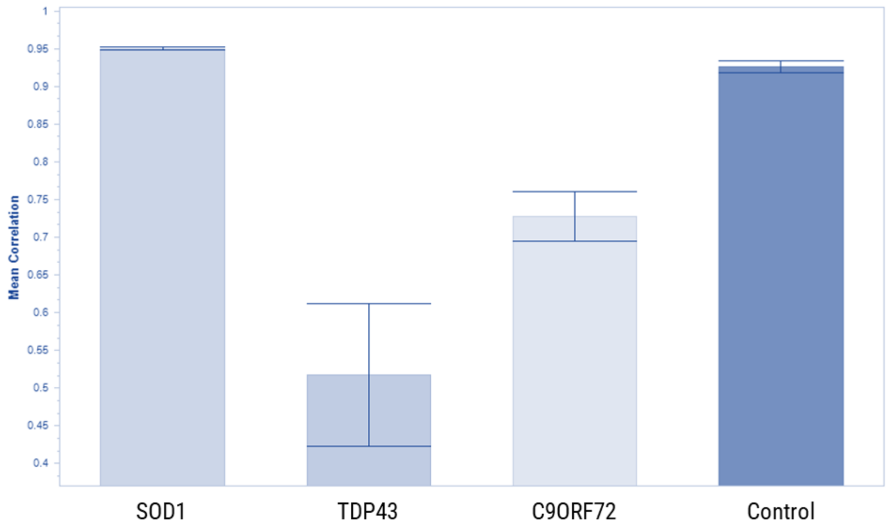


Figure 2: Calcium imaging data on day 21 iPSC-derived motor neurons (data generated from the Sartorius IncuCyte® S3 real-time imaging platform)

2a) Spontaneous neural activity (SNA) data for healthy control-derived motor neurons and ALS-derived motor neurons (SOD1, TDP43 and C9ORF72) over 90 seconds.
2b) Mean correlation for SNA data as a measure of synchronicity (where 1 indicates complete synchronicity and 0 indicates random firing).

SOD1 motor neurons exhibit similar synchronicity and firing frequency to healthy control motor neurons, whereas **TDP43** motor neurons exhibit more random firing patterns, as demonstrated by the multiple peaks of different intensities (**fig. 2a**) and the lower mean correlation (**fig. 2b**). **C9ORF72** motor neurons also exhibit less synchronous firing patterns. Overall, this demonstrates SNA as a useful assay to identify phenotypic differences between the three main ALS mutations.

Assay capabilities

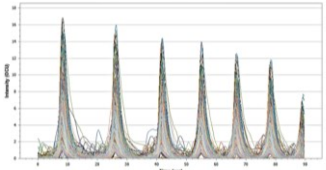
To assess ALS-like phenotypes, we regularly employ **real-time imaging of spontaneous neural activity (SNA)** and **multi-electrode array (MEA) of neuron firing.**

Spontaneous neural activity

We use the Sartorius IncuCyte® S3 Neuronal Activity Assay to measure SNA with real-time imaging. We transduce motor neurons with a lentivirus expressing a calcium-sensitive reporter for use on this platform.

This enables us to assess phenotypic differences in SNA between healthy control and ALS-derived motor neurons (**fig. 3**)

a) Healthy control-derived motor neurons



b) ALS-derived motor neurons

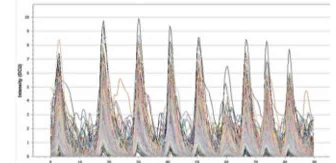
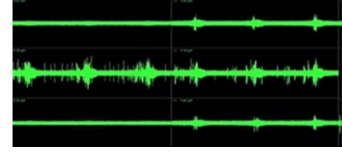


Figure 3. Comparison of healthy control-derived (a) and ALS-derived motor neurons (b), measured via SNA. ALS-derived (**C9ORF72**) motor neurons exhibit less synchronous firing, with multiple peaks of variable intensity, compared to the synchronous firing pattern of healthy control-derived motor neurons. This highlights the utility of SNA to identify ALS-like phenotypes.

Multi-electrode array

We use the Axion BioSystems Maestro Pro MEA system for electrophysiology assays in up to a 48-well plate format. This enables us to assess for differences in sodium spikes to assess firing patterns between healthy control and ALS-derived motor neurons (**fig. 4**)

a) Healthy control-derived motor neurons



b) ALS-derived motor neurons

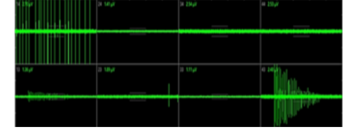


Figure 4. Comparison of healthy control-derived (a) and ALS-derived motor neurons (b), measured via MEA. Healthy control-derived motor neurons exhibit synchronous firing patterns, whereas ALS-derived motor neurons (C9ORF72) demonstrate hyperexcitability and loss of synchronicity. This highlights the utility of MEA to identify ALS-like phenotypes.

Building an *in vitro* neuromuscular junction (NMJ)

We participated in the **Horizon PLATFORMA project** which explored the challenges of building an *in vitro* NMJ model for ALS research and drug discovery, using iPSC-derived motor neurons and muscle cells.

Key challenges and outputs of the project were:

Challenge	Outcome
"It currently takes 56 days to produce mature iPSC-derived motor neurons"	Motor neuron maturation time reduce to just **10 days**
"ALS lines are prone to excitotoxicity, making them difficult to culture long-term"	Development of functional cells in **7 days**
"We need functional ALS motor neuron data"	ALS motor neurons produced and **functional performance** measured in assays including electrophysiology
"We need to develop skeletal muscle cells for the NMJ"	Human iPSC-derived skeletal muscle cells **developed**
"To facilitate co-culture of motor neurons and muscle cells, we need media that works on both cell types"	Creation of a **single maturation media** that works on **both** motor neurons and muscle cells
"Motor neuron/muscle cell co-culture is technically challenging with a complex protocol"	Co-culture protocol **simplified** making it **accessible** to a wider community

In this PLATFORMA project, we explored three different ALS model formats:

The PLATFORMA Project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No. 951890

Formats explored	Example outcomes
Co-culture model: Motor neurons and muscle cell were plated at opposite sides of a 10cm dish and developed into a central mixed innervated population at the center. This was facilitated by a maturation media that works on both cell types.	Co-culture of motor neurons and muscle cells.
2D NMJ model: A microfluidics device was used with motor neurons and muscle cells in opposite chambers. Neurite extensions grew through the grooves to innervate the muscle cells (shown by bungarotoxin staining).	2D NMJ model built on a microfluidics device.
3D NMJ model: Using a 3D scaffold, motor neurons were placed on top of skeletal muscle. We then measured muscle activity, spike profiles and contractility at day 15 to assess for an ALS phenotype.	Motor neurons growing on a 3D scaffold.

Scan the QR code for more about the maturation and characterization of ALS motor neurons.

We are continuing research in this area and welcome any feedback or suggestions at operations@axolbio.com

Case study: iPSC-Derived Motor Neurons and Microglia From ALS Background 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.

Spontaneous neuronal activity analysis on day 18 axoCells motor neurons

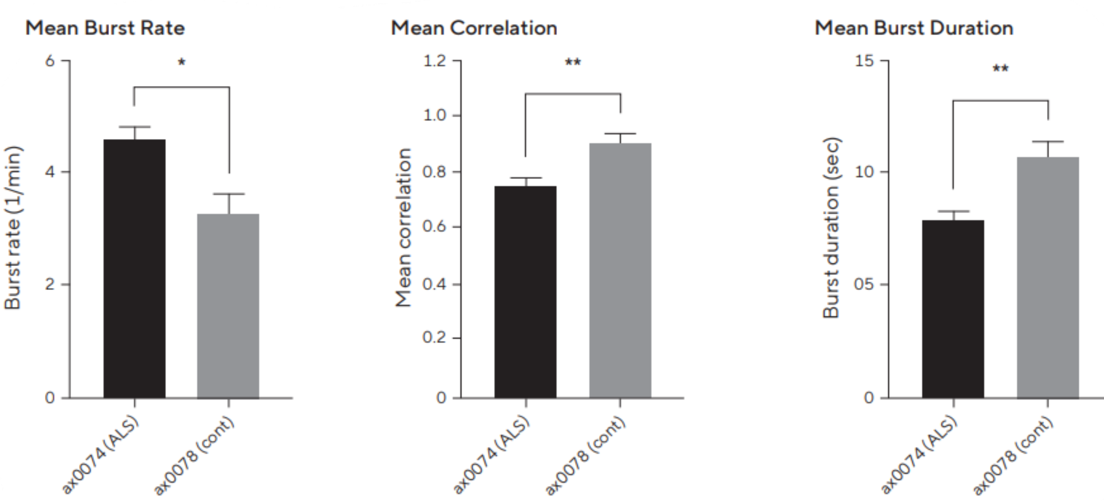


Figure 5. The IncuCyte® Neuronal Activity Software Module was used to monitor and analyze calcium signaling. Statistical significance was assessed using a non-parametric T-test, *p<0.05, **p<0.01. ALS motor neurons exhibited significantly higher burst rates compared to the control line, along with lower burst duration and lower mean correlation (hence less synchronized burst firing).

Phagocytosis of myelin basic protein (MBP) by axoCells microglia.

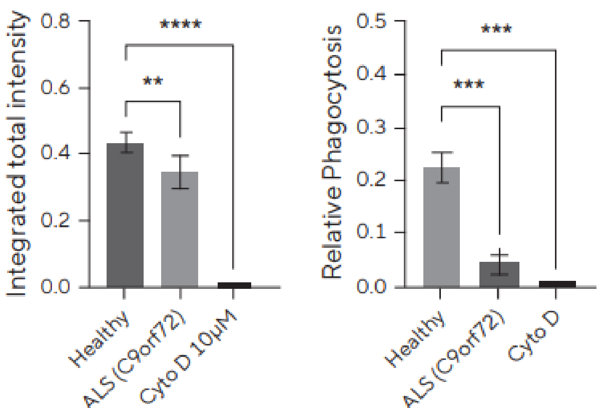
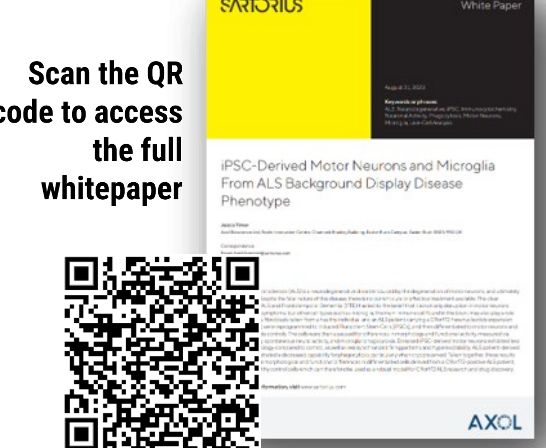


Figure 6. Phagocytosis of pHrodo® for IncuCyte® labeled MBP was assessed on an Incucyte® Live-Cell Analysis System up to 24h using fresh and cryopreserved microglia. Cytochalasin D (10 µM) was used as a control. Statistical significance was assessed using a one-way ANOVA, **p<0.01, ***p<0.001, ****p<0.001

This indicates that ALS microglia are affected by the C9ORF72 expansion in ALS with decreased phagocytosis, demonstrating a useful assay to identify an ALS-like phenotype.

In this project, we demonstrated distinct **ALS-like phenotypes** in axoCells motor neurons and microglia derived from ALS iPSCs, which can be quantified and used to inform future drug discovery purposes. As part of this project, we also looked into **morphology**, **immunocytochemistry** and **electrophysiology** (data available in the full whitepaper), demonstrating the wide range of useful assays for investigating ALS-like phenotypes in motor neurons and microglia.

Scan the QR code to access the full whitepaper



ALS-related iPSC lines and 'ready-to-ship' endpoint cells

ALS axoLines™

axoLines	Status at time of sampling	Gender	Age at sampling	Source material	Mutation	Variant
CENSOi035-B	Patient	Female	61	Fibroblasts	SOD1	Heterozygous D10 9Y (G>T) mutation
ax7073	Asymptomatic (sibling of ax7074)	Male	62	Dermal fibroblast	C9ORF72: >145 G4C2	
ax7074	Patient (sibling of ax7073)	Female	64	Dermal fibroblast	C9ORF72: >145 G4C2	
CENSOi027-D	Asymptomatic	Female	44	Fibroblasts	C9ORF72	Heterozyogous >1 00 expanded GGGGCC
CENSOi018-A	Patient (also with FTD)	Female	62	Fibroblasts	TARDBP (TDP43)	TARDBP: A382T

ALS axoCells™

axoCells Motor Neurons	Status at time of sampling	Gender	Age at sampling	Source material	Mutation	Variant
ax0073	Asymptomatic (sibling of ax7074)	Male	62	Dermal fibroblast	C9ORF72: >145 G4C2	
ax0074	Patient (sibling of ax7073)	Female	64	Dermal fibroblast	C9ORF72: >145 G4C2	
ax0078	Healthy	Male	74	Pulmonary fibroblast	N/A	N/A
axoCells Microglia	**Status at time of sampling**	**Gender**	**Age at sampling**	**Source material**	**Mutation**	**Variant**
ax0664	Healthy	Male	40-50	Dermal fibroblast	N/A	N/A
axoCells Myotubes	**Status at time of sampling**	**Gender**	**Age at sampling**	**Source material**	**Mutation**	**Variant**
ax3062	Healthy	Male	40-50	Dermal fibroblast	N/A	N/A

Our vision for the future: patient stratification model for ALS

ALS is a complex disease with **significant heterogeneity** in its genetics, progression rates, and clinical features, particularly for the sporadic form (which makes up 90% of cases) where there is no clear family history or risk factors[1]. This heterogeneity has **created challenges** for ALS drug discovery; despite **dozens** of phase 2 and phase 3 clinical trials, treatment options are still **severely limited[1]**.

We believe that human iPSCs can be used to tackle this problem, by developing and implementing a **"clinical trial in a dish" (CTIAD) model**. This would involve turning a large collection of sporadic and familial ALS patient samples into endpoint cells (including motor neurons and muscle cells), which could then be used to build an *in vitro* model that captures a **large, diverse patient cohort** with **strong statistical power**.

This CTIAD model could then be used to **test potential therapeutics** at the pre-clinical level to identify the best responders for subsequent clinical trials, known as **"patient stratification"**. Researchers could also use the CTIAD model to incorporate diverse patient demographics, probe disease mechanisms and identify key biomarkers.

We welcome your thoughts and comments on this CTIAD model at operations@axolbio.com.

1 Goyal NA, Berry JD, Windebank A, et al. Addressing heterogeneity in amyotrophic lateral sclerosis CLINICAL TRIALS. *Muscle Nerve*. 2020; 62: 156–166. https://doi.org/10.1002/mus.26801

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