Phenotypically characterized iPSC biological components to generate next generation NMJ models for enhancing breakthroughs in ALS treatment

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Introduction

Human iPSC neuromuscular junction modelling seeks to establish an *in vitro* system which supports two cell types - skeletal muscle cells and motor neurons cells - in the same environment.

iPSC technology enables the generation of multiple relevant cell types, including skeletal muscle and motor neurons, from people affected by ALS patients and people not affected. In this study we derived cells from multiple ALS and unaffected iPSCs. Cells were characterized in monoculture then also in a 2D microfluidic device where they were seen to form neuromuscular junctions. Identifiable ALS relevant phenotypic differences were noted pointing towards the utility of such systems for research and drug discovery.

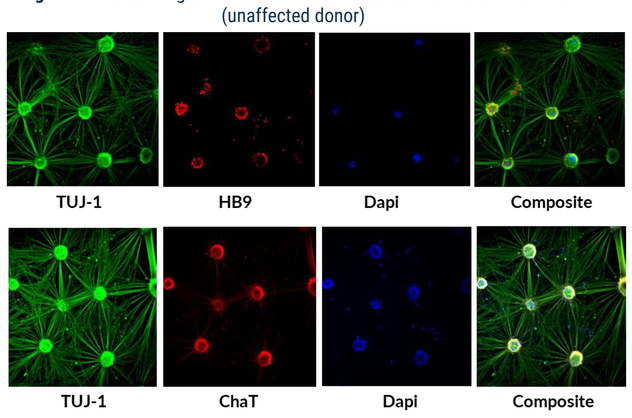
iPSCs from ALS donors and unaffected donors

Cell type progenitor	axoCells™ product code	Status at time of sampling	Gender	Age at sampling	Source material	Mutation
Motor neuron	ax0076	Healthy	Male	50	Pulmonary fibroblast	N/A
Motor neuron	ax0074	Patient	Female	64	Dermal fibroblast	C9orf72: >145 G4C2
Motor neuron	ax0735	Patient	Female	61	Dermal fibroblast	SOD1
Motor neuron	ax0079	Patient	Female	62	Dermal fibroblast	TDP43

Human iPSC-derived motor neuron characterization

- Motor neuron precursors were generated from ALS and unaffected donor iPSCs according to Axol's proprietary methodology.
- Cells were assessed by immunohistochemistry (ICC) for motor neuron relevant markers day 10 post thaw / maturation. Signals were quantified using a Leica imaging system and FIJI software for quantification of cellular markers:
- **Tuj-1 = 100%, HB9 ≥ 80%, Chat ≥ 90%, Isl-1 ≥ 80%**
- Cells were further characterized using multi-electrode array (MEA) where showed spontaneous and synchronized burst-firing.

Figure 1. ICC staining of axoCells™ Human iPSC-derived Motor Neurons (unaffected donor)



ALS relevant functional phenotypes in iPSC-derived motor neurons

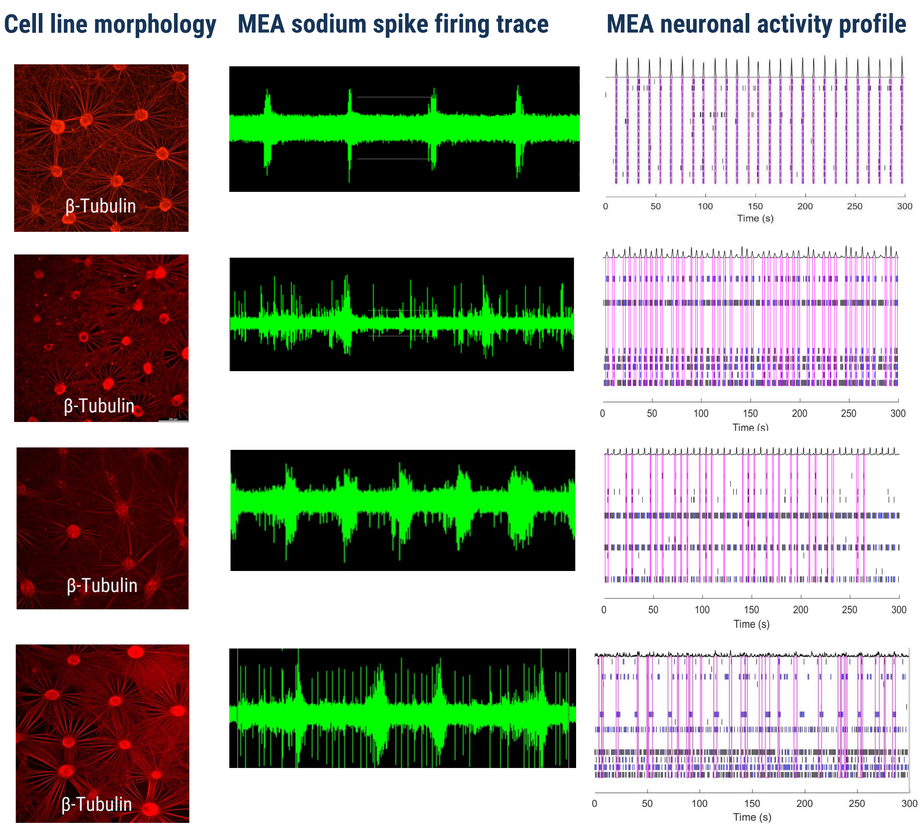


Figure 2. Imaging and MEA (Axion) analysis of iPSC-derived motor neurons from day 10 (post thaw) onwards, display distinct functional and morphological phenotypes

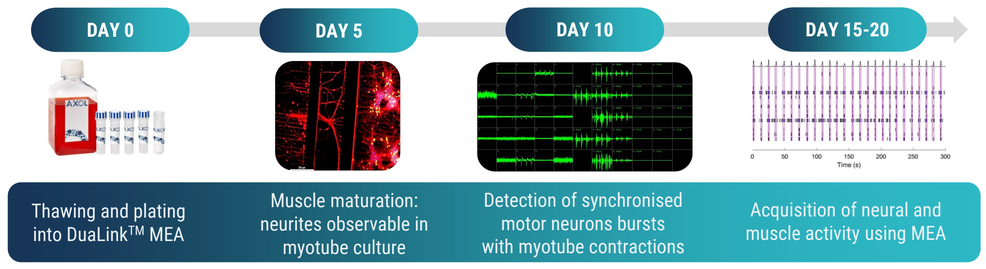
Human iPSC-derived myotube characterization

- Myotubes are generated from human iPSCs as myoblast progenitors.
- Expression of specific markers for Skeletal muscle cells at day 5: using ICC and measured using a Leica imaging system and FIJI software for quantification of cellular markers:
- **Actin ≥ 80%, Titin ≥ 80%, MyoD1 ≤ 10%, Myosin-HC ≥ 50%,, Myogenin ≥ 50%, Dystrophin ≥ 80%**
- Myotubes are multinucleated (4-5 per nuclei) and show a clear sarcomere structure indicative of myotube / skeletal muscle morphology.

Figure 3. Human iPSC-derived myotubes (unaffected donor)

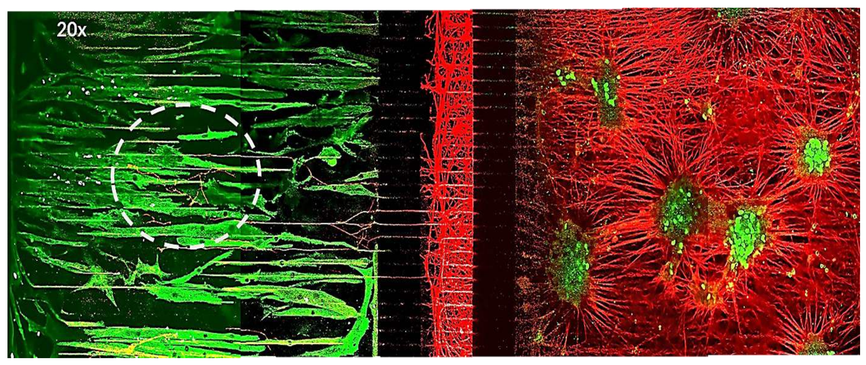
Modelling NMJ in a two-chamber MEA compatible microfluidic platform

Figure 4. iPSC-derived motor neurons and myotubes were successfully established in a two-chamber microfluidic system which also enabled imaging and MEA analysis.



Confocal images at D15 in NETRI DuaLink™

6.



Channel 1: White– Alpha Bungarotoxin (NMJ synapse marker / Titin (Myotube)
Channel 2: Tuj1 (Neurite marker)
Channel 3: ChAt (Motor neuron marker)/ Tuj1

Figure 6. Neurite development crossing channels and microchannels into the skeletal muscle compartment *(Tuj1 expression in red)*

NMJ functional synapse as measured by positive alpha bungarotoxin

7.

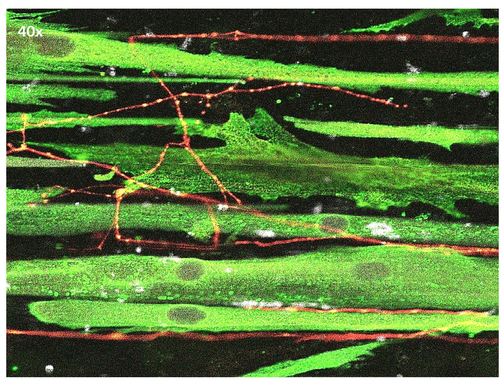
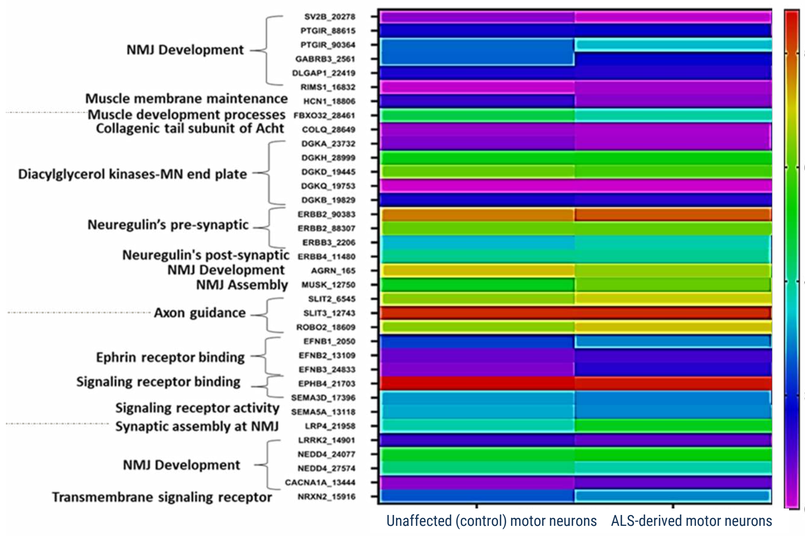


Figure 7. White punctations in 20X diagram with the post-synaptic acetylcholine receptor α-bungarotoxin displaying key functional innervation of the muscle and motor neurons with a functional acetylcholine NMJ synapse.

ALS and beyond - targets of known genetic interest within the model



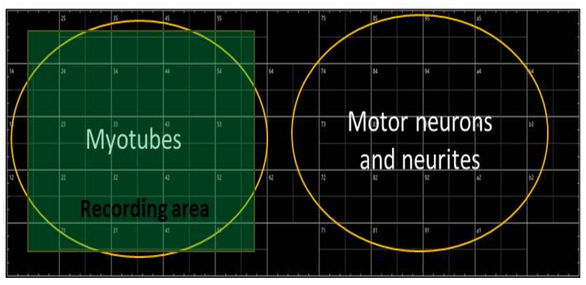
Disease type	Clinical drug target
Osteoporosis	SLIT3
Muscle Atrophy	FBX032
ALS	LARK2
Cancer	NEDD4, EPHB4
Diabetes	ERBB2
ALS Hyperexcitability	KCNQ 2/3 (KV7. 2/3)
Ataxia / ALS	ATNX2
ALS	FUS
Cancer / Autophagy & NI in ALS	TBK1
Immunodeficiency, Bowl Disease & ALS	TNFSF12
ALS, Ileocolitis & Keshan Disease	GPX3

Figure 8. TempoSeq or RNASeq analysis of the neuromuscular junction and its components for current clinical known drug targets against a variety of disease types. This highlights the model relevance for a variety of different disease targets including ALS.

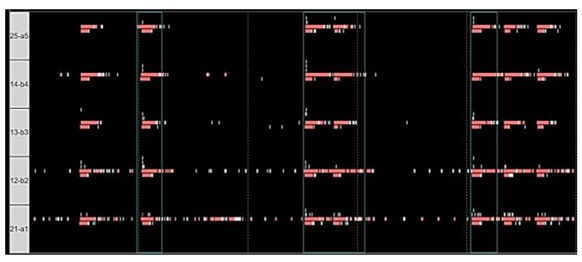
Hyperexcitability seen in ALS NMJ model

MEA analysis from the NMJ model demonstrated a recognizable 'hyperexcitable' phenotype from the C9orf72 motor neurons.

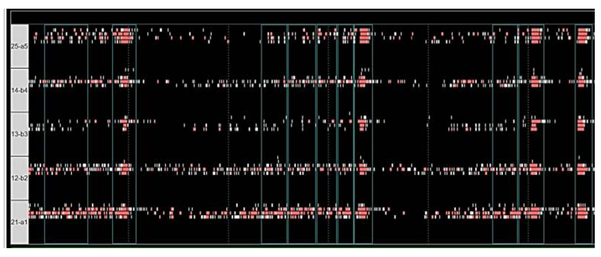
MEA readout from NMJ



9a. Unaffected motor neurons + unaffected muscle



9b. C9orf72 motor neurons + unaffected muscle



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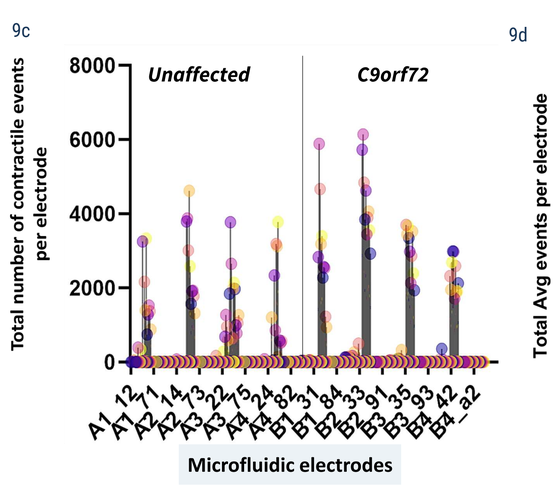

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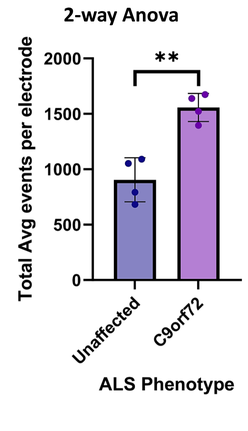

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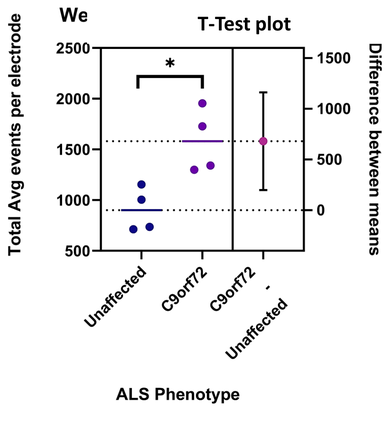

Figure 9 - A proof of concept model, data error bars indicate SEM with 24 biological independent replicates across 2 chips for the measurement of myotube induced contractility via motor neurons.
Figure 9b. Rasta plots depict myotube contractility driven by the motor neuron activity which is depicted in figure 2. Increased activity and long train contractility events are observed in the C9orf72 model against the unaffected model. 9c. Assessment of the myotube contractility at the individual electrode level, there is a clear separation in the number of events when comparing the unaffected against C9orf72. 9d. Statistical analysis via 2-way Anova with a P value of 0.537 for the ALS treatment, 9E. T-Test analysis to determining there is a difference in the means of the Unaffected and C9orf72 Als population data

Conclusion

- The combination of iPSC technology, microfluidics and MEA, offers the opportunity to create a functional NMJ model to support ALS research and drug discovery.
- iPSC-derived cells demonstrate expected marker expression and function both in mono-culture and this two-chamber environment.
- Quantification of motor neuron driven muscle contraction is enabled and by MEA, we report hyperexcitability in the C9orf72 line compared to unaffected donor line.
- Such models will have application in ALS but also a wider range of targets.

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