Development Of 2D And 3D Functional Platforms To Study Peripheral Nervous System Physiology For Innervated Skin And Neuromuscular Studies

AXOL
Discovery Stems From Here

Stuart Prime, Signe Spring, Amaia Paredes, David Wallbank - Axol Bioscience Ltd, Cambridge, UK

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OVERVIEW AND INTRODUCTION

There is an increasing demand for researchers to use functional 2D and 3D platforms for *in vitro* studies as they provide more phenotypically relevant models.

Axol Bioscience is developing two such functional platforms to model the Peripheral Nervous System using human iPSC-generated cell types. The two platforms being developed aim to recreate the relationship between skin and sensory neurons, and muscles cells and motor neurons within the neuromuscular junction. This work is supported by the EU Horizon 2020 funding program.

- **Human Skin Equivalent Module -** A first-in-class industrial innervated human skin tissue model. Obtaining a equilibrium between skin cells and sensory neurons is a challenge that has never been achieved before owing to the level of expertise needed in culturing both cell types. The model incorporates a specialized scaffold structure ensures the monitoring of functional connectivity. The Sensory-skin model has the potential to mimic neurogenic inflammation (itch), pain and well-being and will make it possible to study the interaction between sensory neurons and cutaneous cells, as well as perform efficacy testing and study drug candidates.

- **Muscle Motor-neuron Module** - A highly novel and cutting-edge model targeting both industrial and academic institutions. Currently, no physiologically relevant model capable of mimicking the processes of neuronal control over muscle tissue has been produced at a scalable level. This PLATFORMA neuromuscular junction model will be a superior tool for investigating disease mechanisms, new drug targets and screening compounds to treat ALS and other peripheral neuropathies.

This poster focuses on the development of an iPSC muscle and motor neuron module. Our objective is to create the World's first human neuromuscular tissue-on-chip model. Current animal models of neuromuscular junctions do not represent human physiology, gene expression or morphology. We aim to develop a model that closely resemble human muscle tissue where functional neuromuscular junctions can be recapitulated, enabling better disease modeling (e.g. ALS) and assays for toxicity and efficacy of new therapeutics.

Development and Optimization

1. We first established a faster maturation process for motor neuron development. Cells are assay ready within 10-20 days
2. Established a protocol for the generation of iPSC-derived skeletal muscle that is phenotypically similar to primary skeletal muscle cells
3. Developed microfluidic devices for 2D models to improve cell type and marker expression analysis for studying synapse development and cellular innervation.
4. Developed co-culture supportive media for human iPSC-derived motor neurons and skeletal muscle cells

2D Models

Assessment and validation of cellular innervation and synapse development within a 2D environment was achieved using a PDMS microfluidic chamber. The cellular separation enabled clear distinction between cell types and markers, facilitating better insights into cellular innervation and synapse development.

2D models enabled the development and trials of a functional co-culture media to support muscle and motor neuron iPSC cell types both within this microfluidic system and 3D scaffold system on the Multi Electrode Array.

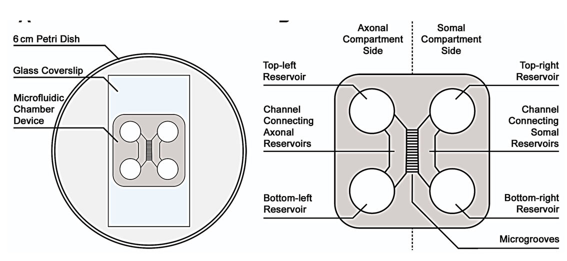


Figure 1. Microfluidic Chamber.
Typical layout of a PDMS microfluidic chamber

3D Scaffold and MEA Interface

The 3D scaffold used for the platform development is a custom-made innovation within the Platforma program.

An Axion Maestro Pro MEA platform, capable of 6 to 96 well formats, was used for all MEA investigations. These plate have electrodes mounted onto the well floor.

For the NMJ studies, a 48 well format was utilized, with 16 active electrodes per well, with a 50µm separation between each electrode. The neuronal cells were then spotted onto the array in a 10µl volume of media.

Neuronal and muscle cells develop within the MEA array and 3D scaffold which supports the motor neuron complex to functionally innervate the Skeletal muscle layer. Cultured motor neuron progenitor or skeletal muscle progenitor cells are seeded into the 48 well plate and on to the 3D scaffold – depending on the configuration required. These are then differentiated in-situ to form their mature cell types, using Axol Bioscience proprietary methods, media and reagents.

Below outlines the system layout for the NMJ model:

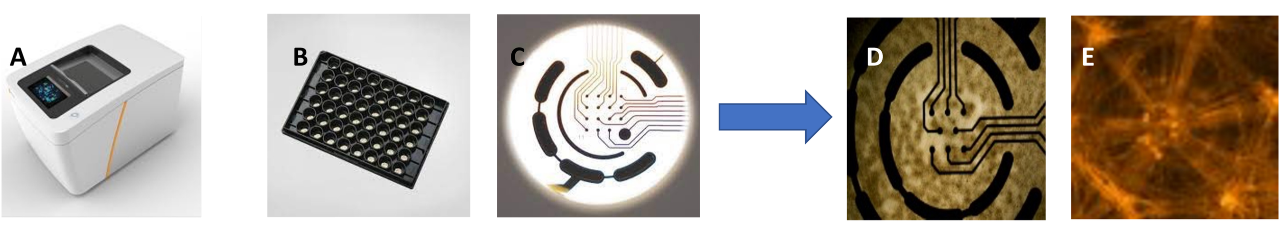


Figure 2. MEA + 3D Scaffold Configuration.
Figures A, B and C show the axion MEA system, well plate and MEA array that resides in wells of the plate.
Figures D (phase contrast) of MEA coated motor neuron culture and Figure E 3D configuration of the motor neurons (SIR-tubulin marker) .

METHODS

Cell culture of iPSC progenitor cells to mature cell types was performed using Axol Bioscience user protocols and media for motor neurons. Muscle derivation is in development using proprietary media and methods, along with co-culture media which supports both motor neuron and muscle cell types.

Immunostaining:

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

Scaffold staining uses SIR –Tubulin live cell staining for 1hr at 37^{0}C, 5% CO_2.

MEA Recordings:

Extracellular field potentials were acquired at 37^{o}C, 5% CO_2using a high-throughput MEA system, here we simultaneously recorded extracellular potentials from 16 electrodes per well across 48-wells plates (Axion Maestro Pro) at a sampling rate of 20kHz/channel.

Muscle contraction measurement utilized the neuronal module for spike analysis and the Cardio contractility module for muscle contraction.

Motor Neuron And Skeletal Muscle Characterization

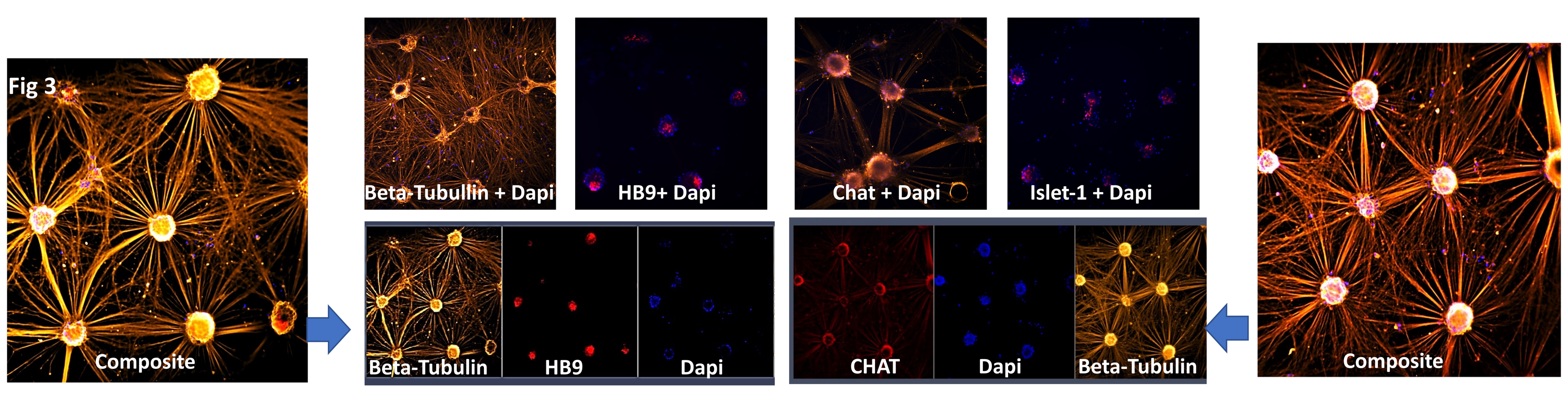


Figure 3. Mature Motor Neuron Characterization.
Motor neuron characterization – ICC Markers at 20X magnification in 96 well imaging plates. All cells are Day 18 post plating and stained with neuronal marker Beta-Tubulin(Yellow), mature motor neuron markers HB9 (red) and CHAT (orange), Islet-1 (red) and Dapi (nuclei marker, blue).

STD 2D Culture And Motor Neuron Activity Measurement Via MEA Detection

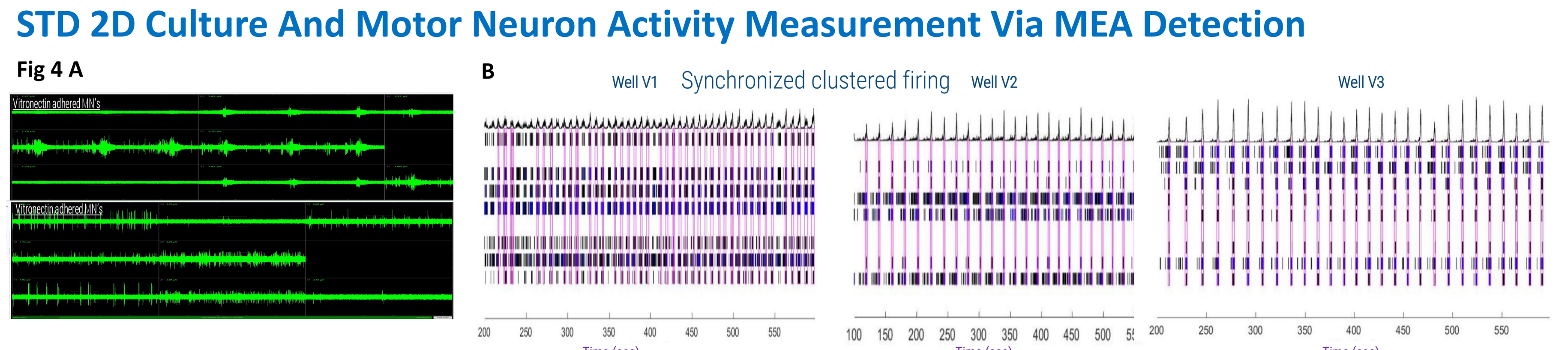


Figure 4. Motor Neuron Activity In 48 Well MEA Axion Plates.
Day 15 post plating – Data generated using Axol Motor neuron accelerator supplement.
Figure 4 A, typical firing activity patterns of motor neurons in 2D culture on an MEA system. B, graphical display of the synchronized network burst firing activity of the motor neurons

RESULTS

Fast Maturation Of Motor Neurons

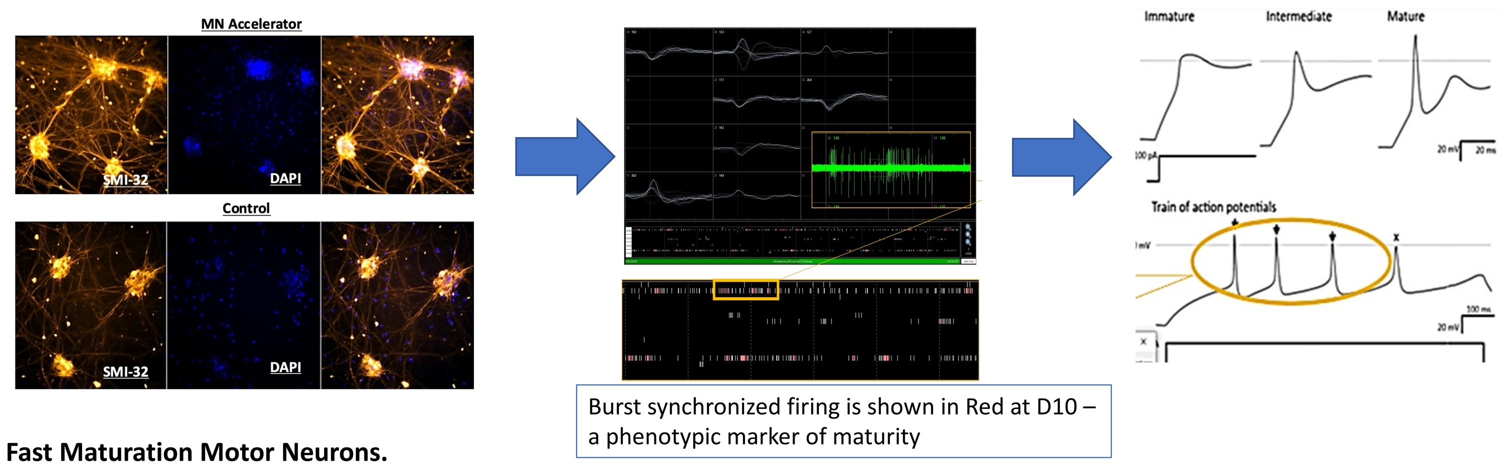


Figure 5. Fast Maturation Motor Neurons.
The development of fast maturing motor neurons was achieved by developing a media supplement whose components mimic the cellular environment. This supplement is **Axol's - motor neuron maturation accelerator media -** ax0179

Within 10 days the cellular neuraxis is highly developed when compared to standard culture conditions. Neurons will display multiple train burst firing responses when measured upon an MEA system, these will be synchronized responses indicating a mature neuron response.

Human Ipsc-derived Skeletal Muscle

Primary human skeletal muscle

iPSC-derived human skeletal muscle

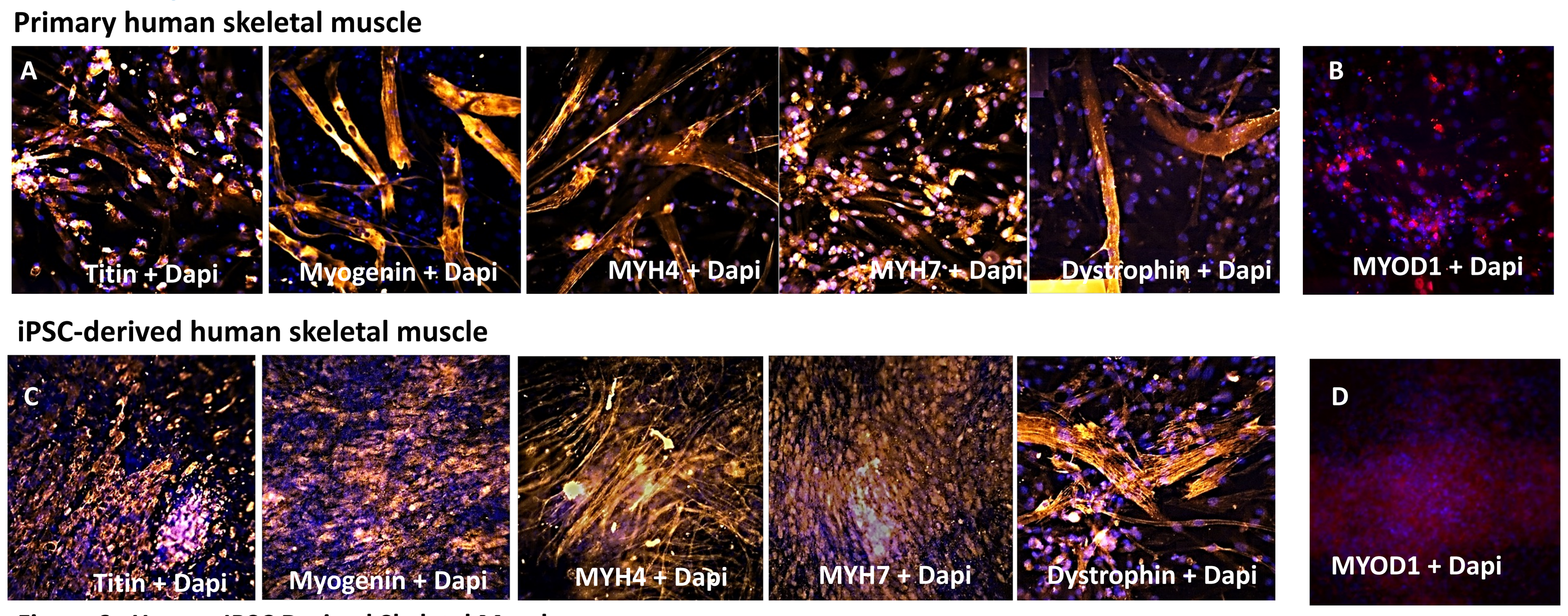


Figure 6. Human IPSC Derived Skeletal Muscle.
Figure 6A and 6C compare Axol iPSC-derived skeletal muscle to primary tissue skeletal muscle. Both mature muscle types demonstrate the same skeletal muscle markers when matured for 10 days from myotube progenitor cells. Figures 6B and 6D compare the expression of MyoD1, a myogenic progenitor marker which will decrease with maturity of the culture in a pure population. Axol Skeletal muscle cells have relatively low expression of the MyoD1 marker when compared to the Primary tissue.

Figure 7. Multi-nuclei assessment of Skeletal muscle.
Muscle strands containing 3 or more multi-nuclei bodies is phenotypically characteristic of mature muscle types. Axol iPSC-derived skeletal muscle cells contain 5 nuclei per strand, on average.

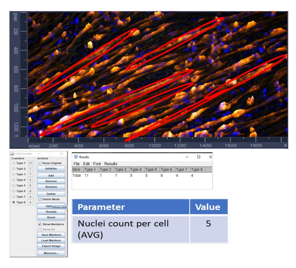
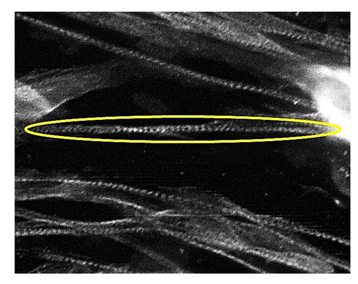


Figure 8. Sarcomere Structure.
A sarcomere is the basic functional contractile unit of skeletal muscle. The Actin / Myosin ladder structure can be clearly observed the plated iPSC skeletal muscle.



Motor Neuron / Skeletal Muscle Co-culture

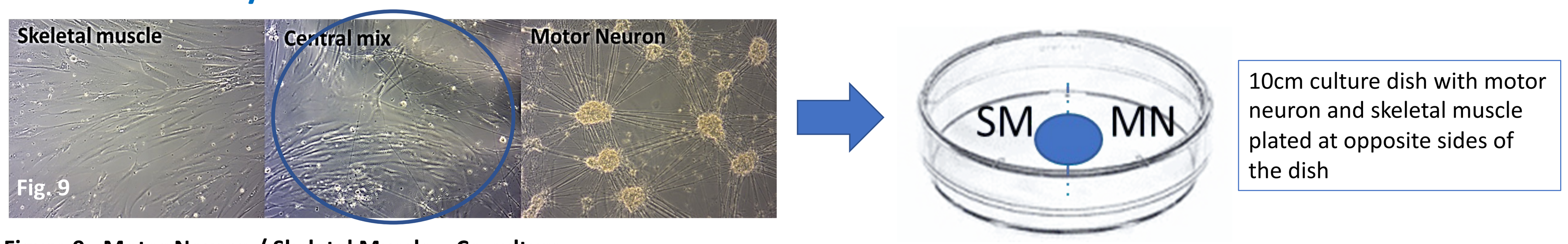


Figure 9. Motor Neuron / Skeletal Muscle – Co-culture:
The co-culture was enabled by the development of maturation media which is capable of supporting both iPSC cell types.
This figure demonstrates the supportive nature of the media where both cell types are plated at opposite sides of a 10cm dish and grow and develop into a central mixed innervated population at the center of the dish.

2D Microfluidic Neuromuscular Junction Model

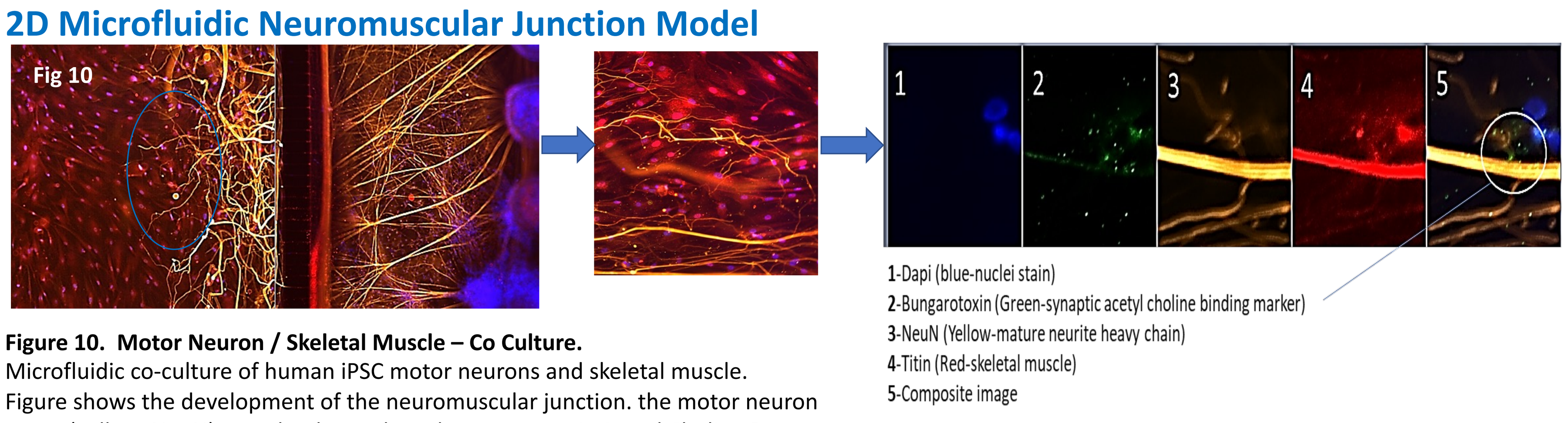


Figure 10. Motor Neuron / Skeletal Muscle – Co Culture.
Microfluidic co-culture of human iPSC motor neurons and skeletal muscle.
Figure shows the development of the neuromuscular junction. the motor neuron nerve (yellow, NeuN) completely overlaps the postsynaptic Acetyl Choline Receptors (green, fluorescent α-bungarotoxin conjugates) and muscle (red, Titin).

Multi-electrode Array Detection In 2D And 3D Scaffold NMJ Models

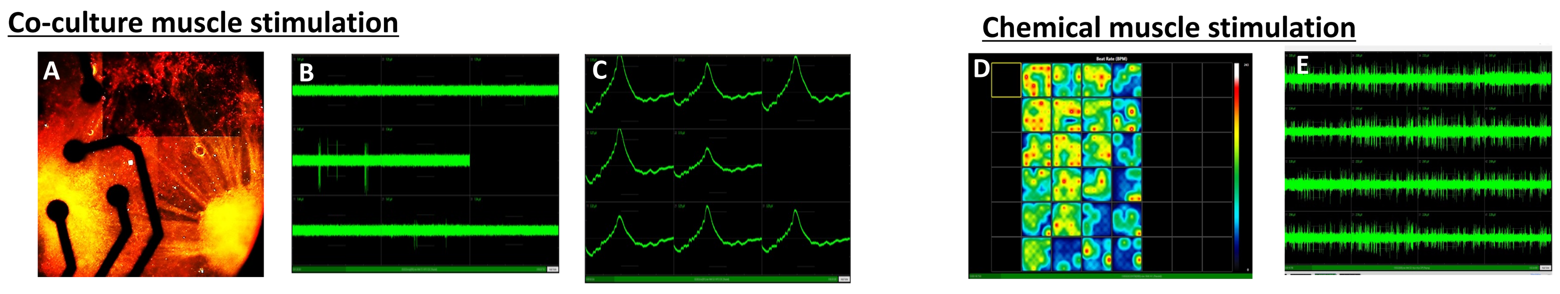
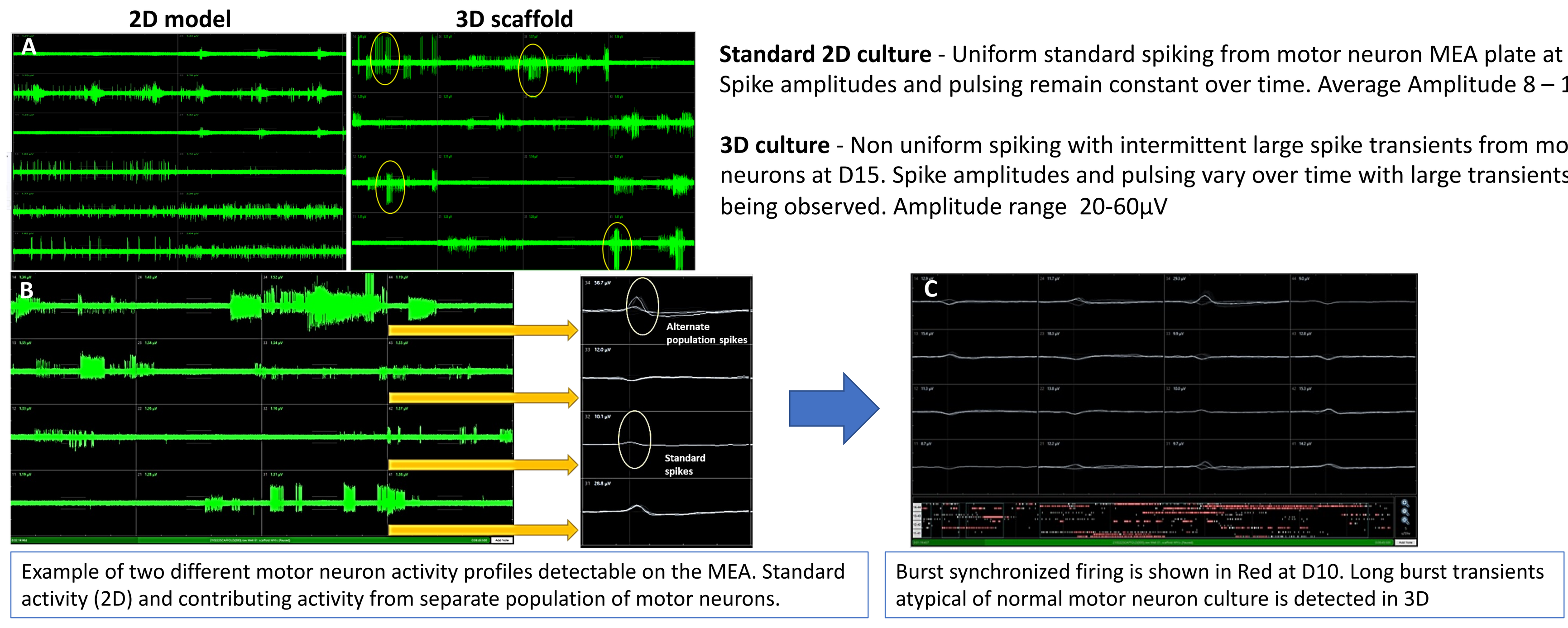


Figure 11. 2D Co-culture Of Motor Neurons And Skeletal Muscle In A MEA Plate. A, mature motor neurons (yellow, CHAT) and muscle cells (red, titin) in an MEA plate well. B and C, representative spontaneous activity of motor neurons, depicted by neuronal spiking (B) and muscle contractility (C) measurement. D, heat map displaying overall plate activity (beats per minute). E and F, representative acetylcholine induced activity, depicted by neuronal spiking (E).

3D Network activity with the 3D model



Standard 2D culture - Uniform standard spiking from motor neuron MEA plate at D15. Spike amplitudes and pulsing remain constant over time. Average Amplitude 8 – 12µV

3D culture - Non uniform spiking with intermittent large spike transients from motor neurons at D15. Spike amplitudes and pulsing vary over time with large transients being observed. Amplitude range 20-60µV

Figure 12. Measuring 3D Network Activity. A, Shows a comparison between a standard 2D Motor neuron network and a 3D network with a separated populations of neurons. B, comparison of the signal amplitude between 2D and 3D culture, with larger spike transients being driven by electrical input from the secondary neuron population. C, **p**rofile of synchronized firing within a well.

CONCLUSION

The results demonstrate the potential for generating human Peripheral nervous system models for neuromuscular junction studies an assay development for both drug discovery and research operations. The development of both the isolated 2D culture and 3D culture environments offer unique methodologies for measuring cell-to-cell interactions and neuromuscular junction development. These, coupled with human iPSC-derived motor neurons and skeletal muscle cell types, bring us one step closer to a true human *in vivo* like system.

Reagents already available from Axol Bioscience: iPSC-derived Motor neurons, both wild type and ALS disease type **(ax0078, a0073 & ax0074)**, motor neuron maintenance media **ax0078** and motor neuron Maturation Accelerator supplement **ax0179**.

Available Soon: iPSC-derived skeletal muscle, motor neuron / skeletal muscle co-culture media and 3D scaffolds.