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A functional system for high-content screening of neuromuscular junctions *in vitro*

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Abstract

High-content phenotypic screening systems are the logical extension of the current efficient, yet low information content, pre-clinical screens for drug discovery. A physiologically accurate *in vitro* neuromuscular junction (NMJ) screening system would therefore be of tremendous benefit to the study of peripheral neuropathies as well as for basic and applied neuromuscular research. To date, no fully-defined, selective assay system has been developed which would allow investigators to determine the functional output of cultured muscle fibers (myotubes) when stimulated via the NMJ in real time for both acute and chronic applications. Here we present the development of such a phenotypic screening model, along with evidence of NMJ formation and motoneuron initiated neuromuscular transmission in an automated system. Myotubes assembled on silicon cantilevers allowed for measurement of substrate deflection in response to contraction and provided the basis for monitoring the effect of controlled motoneuron stimulation on the contractile behavior. The effect was blocked by treatment with D-tubocurarine, confirming NMJ functionality in this highly multiplexed assay system.

INNOVATION

This selective assay system facilitates the interrogation of multiplexed cantilevers in real-time for NMJ presence and functionality. As such it provides a high-content test bed for the assessment of novel therapeutics and disease models. To date, assessment of *in vitro* NMJs has been limited to visual inspection, which lacks a high degree of accuracy and repeatability, or dual patch clamp electrophysiology, which requires significant operator training and is not easily scalable for industrial protocols. Furthermore, neither system permits any functional readout of muscle contractile force in response to stimulation via the NMJ. Consequently, the amount of data pertaining to functional output of such synapses is limited. The system described in this manuscript represents a relatively simple and yet reliable method for assessing NMJ functionality, as well as the criteria necessary for

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AUTHOR CONTRIBUTIONS

A.S. designed and performed the experiments, analyzed data and wrote the majority of the manuscript. C.L. designed the scanning software used in these experiments, analyzed data and contributed to the manuscript. K.P. performed all surface treatments to cantilevers and glass coverslips used in this study. J.H. conceived the cantilever technique, supervised the project and contributed to the manuscript as well as performed the final edits.

applying this technology to drug discovery protocols. Focusing on measurement of contractile force as the means to identify successful motoneuron-myotube synaptic transmission further advances this model by providing direct measurement of functional output, thereby enabling analysis of time-dependent alteration of the cultured cells in response to chemical or pathological challenge. The ability to measure such functional responses in real-time in a multiplexed system should produce more rapid and higher information content preclinical screens for drug development, thereby streamlining current inefficient and costly methodologies.

INTRODUCTION

Neuromuscular junctions (NMJs) are peripheral synapses essential for conveying efferent signals from the motoneurons of the central nervous system to their proximal skeletal muscle fibers¹. A significant body of work has therefore focused on the possibility of generating and maintaining *in vitro* NMJs, in order to better study the chemical and pathological properties of peripheral neuropathic conditions, characterized by the breakdown of these structures²⁻⁸.

The capacity for both human and rodent cells to form NMJs *in vitro* has been studied extensively. Immunocytochemical data from a number of papers highlights the close apposition of pre- and post-synaptic markers in co-cultures of muscle cells and motoneurons, indicating successful cellular contact and potential sites for NMJ development⁴⁻⁷. Electrophysiological recordings from such cultures have demonstrated the existence of effective neuromuscular transmission between the 2 cell types⁶⁻⁸. In such cultures, electrically stimulated action potentials elicited in the neuron are followed by a depolarizing potential recorded in the associated muscle-fiber (myotube) membrane^{7,8}. Furthermore, myotube contraction has been achieved via stimulation of the associated neurons through treatment with exogenous, neuron-specific, excitatory neurotransmitters such as glutamate^{6,7,9}. Conversely, the contractile ability of myotubes in close association with co-cultured motoneurons has been shown to be blocked by treatment with the acetylcholine receptor inhibitor D-tubocurarine⁷⁻⁹.

Despite this wealth of data, the functionality of *in vitro* NMJs has so far only been measured visually via microscope analysis^{6,9}, or using patch-clamp recordings^{7,8}. While such data provides important information relating to the transmission of signals across neuromuscular synapses, it does not allow investigators to examine the functional capacity of the cultured myotubes in response to activation via the NMJ. Moreover, such systems are only capable of measuring acute responses to chemical or pathological challenge and do not permit investigation of long-term effects on cellular function. An automated system able to record physiological data, such as peak force, time to half relaxation and recovery following myotube exhaustion, in response to stimulation via neuromuscular synapses would be of great benefit to the study of muscle and NMJ pathology. Furthermore, such a system would provide a useful, high-content phenotypic test-bed for investigating novel therapeutic strategies.

This study presents the development of a fully-defined co-culture model for primary myotubes and motoneurons, and highlights its ability to record functional myotube contraction data in response to neuronal stimulation, via measurement of cantilever deflection, in real-time. The developed model extends the complexity of a previously published^{10,11} skeletal muscle only model which was adapted to include primary embryonic rat motoneurons. Co-cultures of primary rat myotubes and motoneurons were maintained on cantilever chips and analyzed for evidence of NMJ formation. To further improve on the previous single myotube model, which only allowed for individual cantilever examination, hardware and software were installed to produce a scanning laser and photo-detector system. This improvement effectively facilitated simultaneous analysis of an array of 32 cantilevers, thereby enabling the multiplexed analysis of all potential myotube-neuron pairings, and hence provided greater power to the analysis of successful transmission events. Cantilever deflection was recorded in response to exogenous treatment with the neuron-specific neurotransmitter glutamate and the neuro-muscular blocker D-tubocurarine, in order to interrogate cantilevers for evidence of functional neuromuscular transmission in this system.

METHODS

Cell culture

Muscle cells and motoneurons were isolated from E18 and E15 Sprague-Dawley rat fetuses, respectively. Cells were plated and maintained on cantilevers and coverslips using standard cell culture techniques. Detailed protocols are provided in the Methods Summary.

Cantilever fabrication

Chips containing an array of individual cantilevers were produced from silicon-on-insulator (SOI) wafers fabricated using previously published methods^{10,11}. Briefly, 100 mm SOI wafers with a 4 μm thick device layer and buried oxide layer of 1 μm were used to produce the devices. The cantilever structures were produced in the device layer by patterning with photolithography methods using S1818 photoresist and etching using deep reactive ion etching (DRIE), with the buried oxide layer acting as an etch stop. A 1 μm thick layer of silicon dioxide was deposited on top of the cantilevers using plasma enhanced chemical vapor deposition (PECVD) to protect the cantilevers during processing. The backside of the wafer was similarly patterned and etched using a second mask, so that the silicon beneath the cantilevers was removed, producing a large window underneath an array of cantilevers. The buried oxide layer and oxide layer protecting the cantilevers were removed using a buffered oxide etch solution. The resulting structures were freestanding, bare silicon cantilevers that could be imaged from above and interrogated with a laser from below. The chips were separated by cleaving along perforated edge lines produced during the backside etch. Dimensions of the cantilevers were verified using scanning electron microscopy.

DETA surface modification

To promote cell adhesion to the cantilevers and control glass cover-slips, the surfaces were coated with an amine-terminated alkylsilane, (3-Trimethoxysilyl propyl) diethylenetriamine (DETA) (United Chemical Technologies, Bristol, PA) using methods published previously^{4,9,11}. DETA is an analog of spermidine; a natural polyamine known to promote

long term survival of cells *in vitro*^{12,13}. This surface coating has been used to modify surfaces for the culture of a variety of cell types^{4,9–11,14–16}, validating its use for *in vitro* experimentation.

Briefly, the cantilevers and glass coverslips were acid washed in baths of concentrated HCl in methanol (1:1) and concentrated H₂SO₄, followed by rinsing in boiling de-ionized water and oven drying. The surfaces were silanized using a solution of 0.1% DETA-silane in toluene, heated to 70°C for 30 minutes. This was followed by a series of toluene rinses and reheating to 70°C for 30 minutes in fresh toluene to remove any unreacted silane. The surfaces were oven cured at 110°C for 2 hours and stored in a desiccator until use. The surface coatings were verified using X-ray photoelectron spectroscopy and contact angle goniometry.

Electrophysiological recordings

Motoneurons were assessed using standard patch clamp techniques, as described in the Methods Summary.

Automated myotube contraction detection system

Myotube contraction was recorded and characterized via measurement of cantilever deflection using an automated system that was based on a previously described¹¹ single-unit laser and photo-detector system. In this system, cantilevers supporting co-cultured myotubes and motoneurons were inserted into a transparent culture dish fitted into a modified upright Olympus BX51WI electrophysiology microscope. The culture dish was filled with NBActiv 4 medium (+10 mM HEPES) to maintain the cells during the analysis. A heated culture dish system (Delta T, Biopetech, Butler, PA) was incorporated into the stage to maintain the culture at 37°C throughout the analysis.

The automated system consisted of a Helium Neon laser beam that was automatically scanned across the tips of each cantilever at a 30° angle relative to the plane of the cantilever, and a quadrant photo-detector module was moved to detect the reflected beam. Four stepper motor-driven linear actuators attached to XY translational stages controlled the positions of the laser and photo-detector, with each unit mounted to an XY stage and requiring an actuator for the X and Y translation directions. Stainless steel electrodes were mounted inside the stage dish at a separation distance of 15 mm and connected to a pulse generator (A–M systems, Sequim, WA), capable of producing field stimulation pulses of varying intensity, frequency, and waveform, to allow the system to produce field stimulation of myotubes when appropriate.

Software was written in National Instruments LabVIEW to control the linear actuators that scanned across the cantilevers. To calibrate the system, the laser and photo-detector positions were automatically set to the approximate locations for each of four characteristic cantilevers that defined the array: the two end cantilevers on each of the two rows. Minor manual adjustment was required to precisely position the laser beam at the tip of the cantilever and to position the detector such that the reflected beam hit the center of the photo-detector. After these four positions were established for a particular cantilever chip,

the LabVIEW program linearly interpolated the positions of the remaining cantilevers. The written software allowed for only minor manual adjustment of four positions to define all thirty-two cantilever positions. Slight modifications to the software to extend this system to many more cantilevers in the array would be trivial.

After automatically determining the position of each cantilever in the array, the LabVIEW program scanned the laser and detector across the entire array of cantilevers, stopping the laser and detector at each cantilever tip for a user-defined period of time, set in the graphical user interface. Functions were written in the program to allow for the interrogation of a user-specified subset of cantilevers to maximize the collection of pertinent data.

The photo-detector and pulse stimulator were connected through an Axon Instruments 1440 digitizer (Molecular Devices, Union City, CA) to a computer running AxoScope 10.0. The change in position of the reflected laser beam on the photo-detector was recorded in AxoScope, along with the timing of any electrical field pulses produced by the pulse generator.

Measurement of myotube contraction in response to neuronal stimulation

Broad field electrical stimulation was first used to verify the contractile ability of the cultured myotubes. Cultures were subjected to a 3 V, 40 ms pulse at a frequency of 1 Hz, and the cantilevers were scanned for 5 seconds each to identify those with active myotubes. A representative trace demonstrating the response of cultured myo-tubes to this stimulation is provided in Fig. 1. Across all experimental conditions, a successful contractile response was taken as any peak equal to or larger than 0.1 V.

The electrical stimulus was then switched off and the active cantilevers scanned again in order to observe rates of spontaneous contraction. This condition was followed by bath application of 200 μ M glutamate, to stimulate motoneuron firing, and the cantilevers were again scanned for evidence of contractile activity. A second application of glutamate was made following addition of 12.5 μ M D-tubocurarine (Sigma-Aldrich) to block neuromuscular transmission; the cantilevers were again scanned for contractile activity following this treatment.

Calculation of force generation

Conversion of photo-detector readings to cantilever deflection and myotube force were performed according to previously published methods using a modified Stoney's equation¹¹. The photo-detector measured the changes in cantilever bending-induced laser deflection (in Volts), from which the deflection of the cantilever tip was calculated. Equations (1) and (2) are restated versions of those from Wilson *et al.*¹¹ for cantilever tip deflection (δ) and stress produced by the myotube, assuming a uniform thick film the full width of the cantilever (σ_c). The system parameters used in these equations are the system-specific coefficient relating voltage to laser position on the photo-detector (C_{detector}), the angle of the laser and detector relative to the plane of the cantilever (γ), the elastic modulus of silicon (E_{Si}), the thicknesses of the cantilever (t_{Si}) and myotube (t_f), poison's ratio of silicon (ν_{Si}), cantilever

length (L), path length of laser from cantilever tip to detector (P), and the width of the cantilever (w_{Si}).

$$\delta = \frac{2L}{3} \tan \left[\frac{\theta}{2} - \frac{1}{2} \arctan \left(\tan \theta - \frac{\text{Voltage}}{C_{\text{detector}} \times P \times \cos \theta} \right) \right] \quad (1)$$

$$\sigma_c = \frac{E_{Si} t_{Si}^3}{6 t_f (1 - \nu_{Si}) (t_f + t_{Si})} \frac{3\delta}{2L^2} \times \frac{1}{1 + \frac{t_f}{t_{Si}}} \quad (2)$$

In Eq. (2), the myotube is approximated as a uniform film. Therefore, the force in the myotube is equal to the force in the film, leading to Eq. (3), by equating the calculation of force from stress and the assumed cross sectional area that was used for the application of Stoney's equation.

$$F_{\text{myotube}} = \sigma_c \times t_f \times w_{Si} \quad (3)$$

The aspect ratio of the cantilevers in the system was designed such that the force component in the longitudinal direction of a myotube was greater than 99% of the total force in the myotube, regardless of alignment along the cantilever. For most cases, the longitudinal force component was greater than 99.6% of the total force in the myotube. Therefore, the effect of the alignment of the myotube on the measured force was extremely small due to the cantilever design. For this reason, although the hardware and software designed for use in these experiments is capable of measuring the torsional strain generated by the contracting cells (Fig. 1c), this data was not included in the final force determination since its contribution to total force output was negligible.

Immunocytochemistry

Cells maintained on cantilevers were assessed for expression of Myosin Heavy Chain (MyHC), β -III-Tubulin, Synaptic Vesicle Protein 2 (SV2) and Acetylcholine Receptors (AChR) using standard immunostaining techniques which are detailed in the Methods Summary.

Statistical analysis

Differences in force per contraction and in contraction frequency among the three conditions ("Spontaneous activity", "200 μ M glutamate", "12.5 μ M D-tubocurarine following glutamate") were evaluated statistically using one-way repeated measures ANOVA ($\alpha = 0.05$), both for the muscle-only controls and for the moto-neuron-myotube co-cultures. Since the same set of myotubes were tested in all three conditions, the repeated measures ANOVA blocked for the variation among the myotubes and provided better power for the detection of Differences caused by the testing conditions. The assumptions for ANOVA, quality of variances and normality, were tested using Bartlett's test and QQ-plots, respectively. Following the repeated measures ANOVA with a statistically significant F-statistic, means were statistically compared using Tukey's HSD test for multiple comparisons ($\alpha = 0.05$). For the case of the contraction force, a logarithmic transformation was applied to the data to

satisfy the ANOVA assumptions prior to ANOVA and Tukey's HSD tests. All values stated in the text are the mean standard error of the mean.

RESULTS

Co-cultures of primary rat muscle cells and motoneurons were maintained on arrays of silicon cantilevers for 13 days *in vitro* (DIV) prior to analysis. Measurement of myotube contraction on each cantilever was achieved using a scanning laser and photo-detector system which measured the deflection of the cantilever tip (**Fig. 1**). Myotube contraction in response to the neuronal stimulant glutamate, and cessation of contractions following addition of D-tubocurarine, were examined as evidence of functional neuromuscular transmission, and therefore NMJ formation, in this system.

Stimulation of motoneurons through bath application of glutamate

Patch-clamp recording controls verified the ability of the described culture protocol to promote the maturation of electrically-active motoneurons after 11–13 days in culture. Such cells exhibited characteristic inward and outward ionic currents and depolarization-evoked action potentials as well as the ability to fire action potentials repetitively (**Fig. 2a,b**). Gap-free, current-clamp recordings also demonstrated the response of these cells to bath application of glutamate (**Fig. 2c**). Such treatment resulted in the depolarization of the membrane and elicited action potentials in all motoneurons examined ($n = 17$), indicating the suitability of this treatment for stimulating these cells *in vitro*. Motoneuron activity in response to glutamate lasted roughly 2.5 minutes. This result dictated the need for a second application of glutamate prior to D-tubocurarine treatment, during neurotransmission assessment, to ensure a lack of contractile response was due to blocked AChR receptors rather than a lack of motoneuron firing. A second application of glutamate to patched cells was capable of again depolarizing the motoneuron membrane in all cells examined ($n = 3$, data not shown).

Analysis of functional neuromuscular transmission

An increase in contraction frequency of 2 Hz in response to glutamate treatment, compared to baseline spontaneous activity, was taken as an initial indicator of functional neuromuscular transmission in this system. This increase in frequency criterion was selected as a means to exclude small increases in frequency due to random variation, thereby ensuring the exclusion of false positives. Cantilevers displaying such increases were further investigated through the addition of the neuromuscular blocking agent, D-tubocurarine. In all cases of glutamate increasing contraction frequency by at least 2 Hz, treatment with D-tubocurarine was capable of returning the contraction frequency to spontaneous levels.

Approximately 12% of cantilevers examined provided such responses ($n = 10$ out of 83 electrically active myotubes from 6 independent cultures) indicating that a sufficient number of successful neuromuscular transmissions was achieved in this system and could be recorded using the apparatus described (**Fig. 3**). Among these cantilevers, the average frequency increased significantly ($p = 0.002$) from 1.4 Hz spontaneously to 4.9 Hz with glutamate treatment, and returned to 1.4 Hz with the addition of D-tubocurarine (**Fig. 4a**) (p

= 0:002). No significant difference was observed between spontaneous frequency and frequency after the combined additions of glutamate and D-tubocurarine (p 0:99).

Controls behaved as expected; glutamate treatment on electrically active myotubes from muscle-only cultures did not significantly alter contraction frequency across all cantilevers examined (n = 10). The largest increase in frequency observed in the muscle only controls in response to glutamate was 0.85 Hz (**Fig. 4b**), roughly two and a half times smaller than the selection criterion used to indicate neuro-muscular transmission. Additionally, D-tubocurarine had no inhibitory effect on myotube contraction in all control cases. A significant increase in contraction frequency was observed in D-tubocurarine treated, muscle-only controls when compared with the frequency these myotubes exhibited in response to glutamate treatment (p = 0:04). However, early investigations on denervated rat tissue had demonstrated that D-tubocurarine can have a significant fibrillary effect on skeletal muscle, even when using doses capable of producing complete neuromuscular blockage in innervated tissue¹⁷. The fact that this behavior was likewise observed in this *in vitro* model, while unexpected, provides further evidence for accurate pharmacological responses from cells maintained in this system.

Analysis of the contractile peaks demonstrated that glutamate treatment had no significant effect on the force generated by the contracting myotubes in co-culture with motoneurons (p = 0:60) as well as in muscle-only controls (p = 0:24) (**Fig. 5**). In co-cultures, an apparent, non-significant decrease in force between spontaneous contractions (106.0 nN ± 70.0) and glutamate-evoked contractions (67.4 nN ± 19.4) was observed. Spontaneous contractions elicited once neuromuscular transmission was blocked through addition of D-tubocurarine to the culture medium (45.1 nN ± 15.0) were likewise not significantly different from the contractile force produced in response to glutamate treatment.

Immunocytochemical characterization of co-cultures

Cultures stained for Myosin Heavy Chain (MyHC) and β -III-Tubulin illustrated the close association of myotubes and neuritic extensions on cantilevers (**Fig. 1b**). The highly striated nature of the examined myotubes indicated development of organized contractile machinery within these cells and supported evidence from the laser and photo-detector system for functional maturity of the analyzed myotubes.

Extensive β -III-Tubulin staining not only verified the survival of neurons in this co-culture model but also highlighted substantial levels of neurite outgrowth from cells in this system. Co-localization of the presynaptic marker, Synaptic Vesicle Protein 2 (SV2), with acetylcholine receptors (AChRs), stained with Alexa-Fluor-594-conjugated α -bungarotoxin, indicated the close association of pre- and post-synaptic markers in the examined cultures, highlighting likely locations for neuromuscular cellular contact and synaptic transmission. **Figure 6** provides representative composite images of the appositions observed on examined cantilevers. Such co-localization events were observed at a frequency of 1.5 ± 0.5 per cantilever supporting active motoneuron-myotube pairings (n = 4).

DISCUSSION

The ability to measure and record physiologically-relevant, functional outputs from neuromuscular synaptic contacts *in vitro* holds exciting possibilities for the establishment of a phenotypic assay system for the study of both peripheral neuropathies, such as Amyotrophic Lateral Sclerosis (ALS), and muscle wasting conditions such as muscular dystrophy and sarcopenia. Stimulation of cultured muscle through robust synaptic connections would drastically improve the biomimicry, and therefore the biological relevance, of nerve-muscle *in vitro* co-cultures by enabling the elucidation of new targets for drug screening. Furthermore, the development of a rodent-based neuromuscular model, should also allow for the development of co-cultures derived from transgenic or knock-out animals. This would pave the way for *in vitro* models of diseased synapses, even in cases where the mutation is lethal in late fetal development².

To the best of our knowledge, only one study published to date has detailed the development of a co-culture model potentially capable of measuring the functional output of myotubes synapsed to neurons *in vitro*. Larkin *et al.*¹⁸ demonstrated the ability for myo-tubes cultured in a 3D matrix to respond to neuron presence and measured contractile force through attachment of the matrix anchors to a force transducer. Although this work focused on the ability of the cultured neurons to promote muscular maturation, and did not specifically characterize the formation of NMJs, it remains a potentially viable system for measuring NMJ transmission. However, the use of animal sera in the culture protocol and the inclusion of neuronal slices, rather than isolated neurons, greatly reduce the defined parameters of the culture system. Moreover, the use of densely seeded 3D constructs prevents the analysis of individual synapses *in vitro* as is theoretically possible using the cantilever system presented in this manuscript.

The laser and photo-detector system described here adapts previously published technology for single unit evaluation of myotubes on cantilevers^{10,11} and demonstrates how it can be automated for use in testing the functionality of *in vitro* neuromuscular junctions in real time. Glutamate is an excitatory neurotransmitter^{7,19} capable of eliciting action potentials in moto-neurons as demonstrated in this study through patch-clamp recording analysis. Glutamate receptors are present in mammalian NMJs but their role is believed to be coupled to Nitric Oxide signaling and modulatory in nature²⁰. Stimulation of the skeletal muscle fiber membranes is exclusively achieved via AChRs in mammals²¹, meaning that successful stimulation of myotube contraction in these co-cultures, through addition of glutamate, was most likely achieved through stimulated release of acetylcholine from the cultured motoneurons. This statement is further supported by analysis of muscle-only control cultures. In such cultures, glutamate was found to have little or no effect on the contraction frequency of the cultured myotubes, indicating that glutamate stimulated myotube activity was indirect, via activation of the motoneurons present in the co-cultures.

A significant increase in contraction frequency was recorded from glutamate stimulated co-cultures, compared with spontaneous firing controls. Similarly, glutamate treatment was separately shown to elicit action potentials in cultured neurons (**Fig. 2c**). Combined, these data strongly suggest the presence of functional neuromuscular transmission in this system.

Furthermore, the ability of D-tubocurarine to reduce glutamate-stimulated contraction frequencies to baseline levels, as demonstrated, confirms the correct physiological nature of the neurotransmission observed in this system. Since increased rates of neuronal stimulation have been shown to increase muscle force output *in vivo*²², the lack of statistically significant changes in contraction force across all conditions highlights the potential variability of myotube development *in vitro* when compared with mature mammalian motor units. Variability in myotube number and maturation between cantilevers may explain the lack of a statistically significant increase in myotube force output in response to increased neuronal activity. Methods to understand and promote neuronal control of contractile force are currently being investigated as a means to further improve the relevance of this system for drug development protocols. However, it should be noted that modeling of NMJ breakdown in disease or measuring blockade with drug treatment is possible using the current system through analysis of contraction frequency data as described above.

The collected data provides evidence for neuromuscular transmission on 10 cantilevers across 6 independent cultures. Such a success rate is sufficient for the effective modeling of diseased synapses, or for *in vitro* drug studies, and hence validates the use of this model for such applications. The relatively rare occurrence of functional NMJs *in vitro* with standard coverslip preparation predicated the development of a culture model with a high level of redundancy to ensure functional pairings formed correctly in positions where the force output could be measured (i.e. on a cantilever). The use of a 32 cantilever array on each chip was therefore employed, and necessitated the use of a scanning system to interrogate multiple cantilevers simultaneously for evidence of neuro-muscular activity. Successful implementation of this multiplexed array allowed for identification of active neuron-muscle pairings in sufficient numbers to facilitate pharmacological testing. Furthermore, in order to ensure the greatest degree of certainty regarding effective synaptic transmission events in this system, stringent selection criteria were used which discounted cantilevers with high spontaneous contractile activity. Consequently, the level of NMJ formation in the reported study was probably higher than stated. The generation of uniform co-cultures with functional pairings controlled for spontaneous activity on each cantilever across entire arrays, would increase the high-throughput nature of the culture model for drug screening applications as well as lower the number of cantilevers required for the system. As such, integration of this system with chemical surface patterning techniques²³ to control cell positioning and alignment is currently being investigated, along with methods to reduce or account for spontaneous contractile activity. Second generation iterations of this model should therefore represent strong candidates for further commercial development towards a viable product for the drug development industry.

The importance of the scanning system developed for use in this study cannot be overstated. Previous work¹¹ using the manually adjusted system has relied on analysis of single cantilevers, since the ability to interrogate an entire array was lacking. The use of the scanning model, with an array of 32 cantilevers, greatly reduces the number of cultures required for a study of this nature since all cantilevers could be interrogated simultaneously. The use of the scanning system allows investigators to potentially achieve 32 independent data points from a single chip or chip in a well, thereby providing far greater statistical

power for the subsequent analysis. The scanning system therefore allows the application of this culture model in paired analysis of sequential experimental conditions, while also permitting comparisons of unpaired sample groups, by enabling substantial sample sizes to be easily collected. Such technology will prove invaluable when using this system for any study requiring large numbers such as investigation of rare *in vitro* cellular events, small Differences in cellular responses or high-throughput drug studies.

The use of this model for pharmaceutical testing of *in vitro* NMJs is predicated on the functionality of these neuron-myotube contacts, precluding the use of D-tubocurarine in the identification of functional neuromuscular transmission. D-tubocurarine is a neuromuscular blocker that binds to and inactivates AChRs, thereby preventing the NMJ neurotransmitter acetylcholine from eliciting end-plate potentials in the underlying muscle fiber²⁴. However, the selection criterion of an increase in contraction frequency of at least 2 Hz when treated with glutamate proved sufficient for identifying cases of successful transmission. In all cases where such a change was observed, D-tubocurarine was shown to return contraction frequencies to the baseline levels. Such uniform responses suggest that glutamate treatment alone with this selection criterion is capable of identifying cantilevers capable of functional neuromuscular transmission without the use of D-tubocurarine or other neuromuscular blockers. This criterion is expected to exclude some cases of successful transmission, however the cantilevers selected by this criterion are likely those with the strongest coupling between neuron and myotube and therefore represent the most suitable targets for evaluation of pharmaceutical effects.

Immunocytochemical analysis highlighted the ability for this culture model to promote apposition of pre- and post-synaptic markers, indicative of motoneuron-myotube, cell-cell contact and the beginnings of synapse formation. Such structures were observed on cantilevers that yielded positive functional data, however, on these occasions they appeared as a part of a larger cellular mass, rather than between individual myotube-motoneuron pairings. Ventral horn digests, achieved using the described protocol, yield a heterogeneous population of cells, of which approximately 30% are motoneurons²⁵. Interneurons and supporting glia, make up the remainder²⁵, and it is possible the presence of these cells in these clusters aided the development of machinery capable of facilitating neuromuscular transmission and synapse formation^{26,27}.

Previous work using similar culture parameters as those defined in this study have allowed the maintenance of motoneuron-muscle co-cultures for at least 50 days⁴. The ability to sustain these co-cultures for such extensive periods would further increase the applicability of this model for drug efficacy and toxicity studies, as well as for modeling of disease states *in vitro*, since it facilitates the examination of more chronic, long-term treatments and conditions as well as acute behavior and responses.

Finally, the use of defined, serum-free media compositions for the maintenance and maturation of muscle-nerve co-cultures, as documented here, is vitally important to the development of more complex phenotypic systems for studying mammalian responses to chemical and pathological challenges *in vitro*. Current efforts towards developing integrative, body-on-a-chip type models^{28–32}, for use in high-throughput *in vitro* studies, is

reliant on the development of a common medium that can adequately support the growth and development of multiple and disparate cell-types. The ability of the medium detailed in this study to maintain and allow functional maturation of both skeletal muscle myotubes and motoneurons indicates that the development of such a common media system is possible. Furthermore, it provides a base medium for the assessment of further additives required to establish more complex models of the peripheral nervous system, including sensory neurons, glia and Schwann cells.

CONCLUSIONS

This work presents, for the first time, the development of an automated phenotypic system capable of evaluating the functionality of *in vitro* neuromuscular synapses through determining the ability of neuron stimulation to elicit muscle contraction in a multiplexed device. Further development of this system will allow for integration of more associated and supporting cell types such as sensory neurons and Schwann cells, thereby increasing the biological relevance of the model. Such a system may prove invaluable as a future test-bed for novel therapeutic agents, as well as their targets and improving our understanding of synapse development and function in both healthy and diseased states.

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GRANT INFORMATION

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Figure 1.

Overview of the system developed for use in this study. **(a)** Idealized schematic representation of the scanning system used to measure cantilever deflection in response to myotube contraction. Controlled movement of the laser and photo-detector was used to align the laser beam with the tip of each cantilever in turn. **(b)** Composite image of an example of a primary rat myotube co-cultured with primary rat motoneurons on a cantilever for 13 DIV and immunostained for Myosin Heavy Chain (green) and β -III-Tubulin (red). Cantilever edges in this image were added to the image to give an indication of their scale in relation to the cultured cells. Scale bar = 100 μ m. **(c)** Example of a trace recording from a myotube (illustrated in **(b)**) stimulated using broad field electrical pulses. Top trace = laser deflection (in Volts) in the x-axis, indicating lengthwise strain on the cantilever. Middle trace = laser deflection (in Volts) in the y-axis, indicating torsional strain across the cantilever. Bottom trace = Indication of the temporal position of electrical myotube contraction in this system.

Figure 2.

Electrophysiological recordings from a motoneuron following 13 DIV. **(a)** Voltage-clamp recording. Current flow in the patched cell was measured in response to a stepwise increase in voltage across 10×500 millisecond pulses. Stimulation began with a 10 mV increase from a −70 mV resting potential (−60 mV net) and increased to +30 mV over the course of the program. **(b)** Current-clamp recording. Voltage change (and therefore action potential presence) was measured in response to a stepwise increase in current. Current injection began with a 10 pA increase from baseline, and increased by a further 10 pA with each

subsequent pulse for 10×500 millisecond pulses. Action potentials were first observed during 40 pA current injection. (c) Continuous gap-free recording of a motoneuron maintained in current-clamp at −70 mV. 200 μM glutamate was added to the culture medium after 35 seconds recording.

Figure 3.

Representative traces from analysis of the muscle-motoneuron co-culture cantilever system, demonstrating the functional effects of motoneuron stimulation with and without addition of an NMJ blocker. Raw data (in Volts) was converted to a measurement of myotube force (in nano-Newtons) and replotted. **(a)** Measurement of spontaneous contractions by the cultured myotubes without neuronal stimulation. **(b)** Measurement of myotube contraction following neuronal stimulation via the addition of 200 μM glutamate. **(c)** Measurement of myotube contraction following glutamate and 12.5 μM curare treatment.

Figure 4.

Assessment of the functional effects of glutamate and curare treatment on myotube contraction frequency in primary rat skeletal muscle-motoneuron co-cultures and muscle-only controls maintained for 13 DIV. **(a)** Effects of treatments on myotube contraction frequency in co-culture. **(b)** Effects of treatments on myotube contraction frequency in muscle-only controls. In both a & b, $n = 10$ (individual cantilever recordings selected from 6 separate cultures), error bars standard error of the mean, * $p < 0.05$, ** $p < 0.01$.

Figure 5.

Assessment of the functional effects of glutamate and curare treatment on myotube contractile force in primary rat skeletal muscle-motoneuron co-cultures and muscle-only controls maintained for 13 DIV. **(a)** Effects of treatments on myotube contractile force in co-culture, n = 10, p = 0:60. **(b)** Effects of treatments on myotube contractile force in muscle-only controls, n = 7, p = 0:24. Error bars standard error of the mean.

Figure 6. Immunocytochemical evidence for synaptic contact between primary rat myotubes and motoneurons maintained in co-culture on cantilevers for 13 DIV. **(a)** Synaptic Vesicle Protein 2 (SV-2). **(b)** β -III Tubulin. **(c)** α -Bungarotoxin. **(d)** Composite image of ac. Scale bar = 50 μ m. Such co-localization events were observed at a frequency of 1.5 ± 0.5 per active cantilever ($n = 4$).