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A multiplexed chip-based assay system for investigating the functional development of human skeletal myotubes *in vitro*

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Abstract

This report details the development of a non-invasive *in vitro* assay system for investigating the functional maturation and performance of human skeletal myotubes. Data is presented demonstrating the survival and differentiation of human myotubes on microscale silicon cantilevers in a defined, serum-free system. These cultures can be stimulated electrically and the resulting contraction quantified using modified atomic force microscopy technology. This system provides a higher degree of sensitivity for investigating contractile waveforms than video-based analysis, and represents the first system capable of measuring the contractile activity of individual human muscle myotubes in a reliable, high-throughput and non-invasive manner. The development of such a technique is critical for the advancement of body-on-a-chip platforms towards application in future pre-clinical drug development screens.

Keywords

Human; skeletal muscle; cantilevers; functional assay; body-on-a-chip

Development of novel body-on-a-chip technologies for eventual application in improved pre-clinical screening protocols has the potential to revolutionize the drug development process (Sung et al., 2013). Of particular interest, is the establishment of defined and

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Contributions

A. Smith designed the experiments, assisted with data analysis and wrote the majority of the manuscript. C. Long designed the hardware necessary to perform the described experiments. K. Pirozzi and S. Najjar cultured the cells and collected the majority of the data. C. McAleer assisted with experimental design and manuscript preparation. H. Vandenburg provided the human cells used in these experiments and provided guidance on their maintenance *in vitro*. J. Hickman conceived of the cantilever technology, oversaw the project and assisted with manuscript development.

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controllable human *in vitro* systems as a means to curtail or even eliminate animal testing from toxicity/efficacy studies of novel therapeutics.

Skeletal muscle cells from mammalian sources have been studied *in vitro* for over 30 years (Bischoff, 1974; Yasin et al., 1977). However, an ability to maintain such cells in configurations which facilitate assessment of their contractile output has proved more difficult. While the seeding of myocytes within three-dimensional scaffolds has alleviated this issue to a degree (Dennis and Kosnik, 2000; Dennis et al., 2001; Langelaan et al., 2011; Rhim et al., 2010; Rhim et al., 2007; Sakar et al., 2012; Smith et al., 2012; van der Schaft et al., 2013; Vandenburg, 2010; Weist et al., 2013), the ability to investigate skeletal muscle contraction at the single myotube level in a reliable and reproducible manner remains problematic. Moreover, many 3D models rely on visual interrogation in order to measure contractile activity, which can make accurate assessment of contraction profiles challenging (Agarwal et al., 2013; Sakar et al., 2012; Vandenburg et al., 2008). Those systems which perform direct measurement of functional output (Rhim et al., 2010), do so with the use of a force transducer coupled to the muscle model which is invasive and difficult to integrate with more complex multi-organ platforms. Three dimensional systems are also difficult to integrate effectively with supporting and interacting cell types, limiting their applicability to body-on-a-chip platforms.

Here we present the development of a culture system for maintaining functional human myotubes on microscale silicon cantilever arrays *in vitro*. Individual cantilevers were interrogated using a non-invasive laser and photo-detector system which recorded substrate deflection in response to myotube contraction (Wilson et al., 2007). Although cantilever technology has been studied previously with rat tissue as a means to investigate muscle contractile behavior *in vitro* (Pirozzi et al., 2013; Wilson et al., 2010; Wilson et al., 2007), adaptation of these systems to promote the long-term survival and functional maturation of human myotubes is critical in the development of such technology towards pre-clinical drug development applications.

Primary human myoblasts were isolated as described previously (Guo et al., 2014; Hennessey et al., 1997; Powell et al., 1999), and seeded at a density of 75 cells/mm² on microscale silicon cantilever chips. Cantilevers were first coated with a defined self-assembled monolayer, DETA, which is a spermidine analogue and has been shown to aid the long-term attachment and survival of cells in culture (Eisenberg et al., 2009; Kaeberlein, 2009). The use of silicon based substrates in the development of these chips allowed for a high degree of repeatability and normalization of fabrication parameters. This feature is of vital importance when developing assays for integration with complex, multi-organ systems, giving this model a significant advantage over the use of others utilizing less highly regulated fabrication procedures. The cultured cells were maintained in a commercially available growth medium (Lonza, Allendale, NJ, USA, Cat # CC-3160) until confluent. They were then switched to a serum-free, differentiation medium for a further 4 days to induce myotube formation (Figure 1). Following differentiation, the medium was slowly replaced with a neuronal base medium, NBActiv 4 (Brain Bits LLC, Springfield, IL, USA), by replacing half the medium every 2 days. Using this protocol, cells were routinely

maintained on cantilevers for at least 3 weeks ($n = 6$), and were assessed following 14 to 21 days *in vitro*.

The contractile activity of human myotubes cultured on silicon cantilever chips was assessed as described previously (Pirozzi et al., 2013; Wilson et al., 2010). Briefly, cantilever chips were transferred to a transparent culture well mounted on the stage of a modified electrophysiology microscope. The culture well was filled with NBActiv 4 medium (+ 10 mM HEPES) to maintain the cells during the analysis. A Helium Neon laser beam was scanned through the underside of the culture well and across the tips of each cantilever. A quadrant photo-detector module was moved simultaneously to detect the reflected beam. Software written in LabView (National Instruments) facilitated automatic scanning from one cantilever to the next, effectively enabling simultaneous interrogation of entire cantilever arrays in real-time. Stainless steel electrodes were mounted inside the stage dish and connected to a pulse generator (A—M systems, Sequim, WA, USA), capable of producing field stimulation pulses of varying intensity, frequency, and waveform, which allowed the system to produce field stimulation of myotubes to induce controlled contraction events.

The photo-detector was connected through an Axon Instruments 1440 digitizer (Molecular Devices, Union City, CA) to a computer running AxoScope 10.0 and the change in position of the reflected laser beam on the photo-detector was recorded using this software. Recorded cantilever deflections (in Volts) were then converted to measurements of force (in nN) using Stoney's equations as described previously (Pirozzi et al., 2013) (Figure 1c).

Data collected from human myotubes using this system suggests that human skeletal muscle myotubes undergo functional maturation in culture. A significant increase in contractile force was observed between cultures examined following 14 days *in vitro* ($n = 19$), and those assessed after 21 days in culture ($n = 6$, $p = 0.045$, one-tailed student *t* test) (Figure 2a). No significant change was observed in time to half relaxation measurements between the 2 time-points examined ($p = 0.11$, two-tailed student *t* test) (Figure 2b). Time to half relaxation is a well-established measure of muscle fiber functional properties (Itoh et al., 2013), and changes in fiber phenotypes are known to correlate with alterations in such characteristics (Irintchev et al., 2002). The fact that no change in half relaxation time was recorded following longer culture periods indicated that the added time did not alter the functional phenotype of the cultured cells with regard to fast and slow isoform switching, but rather allowed time for greater development of cellular contractile machinery, leading to an improved functional output *in vitro*.

This model facilitates the functional assessment of human muscle fibers at the single cell level, providing a greater degree of clarity with regard to culture variability and consistency than is available using densely seeded hydrogels. Use of the laser and photo-detector system to record substrate displacement enables the collection of data at a far higher rate (300 to 1000 points per second) than more conventional video analysis (roughly 30 frames per second), making highly sensitive assessment and comparisons of contraction waveforms from different experimental conditions possible. Furthermore, video analysis produces its own problems in correctly determining deflection. It requires either very slow analysis by the researcher, frame-by-frame, or use of very intensive computer image analysis

techniques, which must identify each cantilever when un-flexed and during maximal contraction. Such requirements increase the cost of the system, while simultaneously introducing variability into data acquisition based on operator interpretation of maximal contraction and relaxation points.

The ability of this system to scan across entire cantilever arrays ensures multiple data points are collected from individual culture chips, allowing analysis of functional variability between cells from a single culture (a capacity not possible in 3D myotube bundles encased in hydrogels). Furthermore, scanning through this multiplexed system to obtain multiple data points from single cultures greatly increases the statistical power of any observations derived from the subsequent data analysis. Current chips are designed to hold 32 cantilevers in an array. However, adaptation of this system to incorporate a greater number for higher throughput applications would be straightforward, requiring a simple redesign of the fabrication mask (Wilson et al., 2007).

Incorporation of a heated culture dish (Delta T, Biopetech, Butler, PA, USA) into the recording stage enabled the maintenance of cells at 37°C throughout the analysis. This modification facilitated more long-term examination of functional performance and assessment of the rate of fatigue in cultured human myotubes using this system (Figure 3). All cultures examined ($n = 3$) demonstrated a substantial decrease in force production over extended stimulation periods. During this study, cultured myotubes took 49.01 minutes (standard error ± 9.97) under a consistent 3 V, 1 Hz, 40 msec pulse stimulus to reach a point where the elicited contraction had half the force of that initially measured. This data highlights the possibility to use this system as a means to model rates of fatigue in cultured skeletal muscle myotubes.

It is noteworthy, that this 2D system in no way inhibits preparation of cultured cells for standard molecular biology assays once functional recordings are completed. It is therefore possible to perform in-depth genetic and proteomic analysis of cells cultured within this system. Such data can then be married with functional output to provide a more complete understanding of cellular development *in vitro*. Furthermore, the use of a defined, serum-free medium for maintenance of differentiated human myotubes, as described here, is also advantageous for drug development studies where the use of undefined culture parameters may confound results (van der Valk et al., 2010).

The ability to measure peak force, time to half relaxation and rates of fatigue in cultured human muscle using a highly sensitive, non-invasive multiplexed assay system represents a strong candidate for application in future pre-clinical drug development studies, as well as for disease modeling and profiling applications. Such a model will facilitate in depth functional analysis of healthy and diseased human cells, providing the means to generate highly predictive estimations of *in vivo* tissue performance, thereby ensuring greater confidence in compounds progressing to clinical stages of testing. Finally, the utilization of silicon substrates for fabrication, and the non-invasive sampling technique employed makes this system highly amenable to integration with body-on-a-chip, multi-organ *in vitro* platforms currently under development (Sung et al., 2013).

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Abbreviations

DETA (3-Trimethoxysilyl propyl) diethylenetriamine

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Highlights

- An assay for measuring functional performance of human muscle fibers was developed
- The system provides multiple data points per chip in defined serum-free conditions
- The collected data characterizes the maturation of human muscle cells in vitro
- This system may prove useful for future pre-clinical drug studies
- This is the first chip-based assay for drug discovery using human skeletal muscle

Figure 1. Human myotubes survive on silicon cantilevers and contract, generating quantifiable substrate deflections which can be used to calculate force production *in vitro*

A. Low magnification bright field image of a human myotube cultured on a silicon microscale cantilever. Scale bar = 50 μm . **B.** High magnification image of a myotube cultured on a silicon cantilever and immunostained for myosin heavy chain presence. Scale bar = 50 μm . **C.** Representative recording of myotube force production in response to broad-field electrical pulses at a 1 Hz frequency, calculated from real time measurement of cantilever deflection using our laser and photo-detector system. Raw data (in Volts) was converted to a measurement of myotube force (in nano-Newtons), using a modified Stoney's equation, and re-plotted.

Figure 2. Myotube force production increases over time in culture while force profiles remain constant

A. Average myotube force produced following 16 and 23 days *in vitro*. $n = 3$, $*p = 0.045$. **B.** Average time to half relaxation in contracting myotubes analyzed following 16 ($n = 19$) and 23 ($n = 6$) days *in vitro*, $p = 0.11$.

Figure 3. Human myotubes fatigue in response to continuous electrical stimulation *in vitro*
 Representative recording of force produced by cultured human myotubes, calculated from real time measurements of cantilever deflection using our laser and photo-detector system. Recordings from a single cantilever at different experimental time-points are overlaid to illustrate the effect of chronic broad-field electrical stimulation on force production over time. Stimulation was started at time = 0 seconds and continued at a frequency of 1 Hz throughout the time course of the study. For both time-points, raw data (in Volts) was converted to a measurement of myotube force (in nano-Newtons), using a modified Stoney's equation, and re-plotted.