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Skeletal myotube integration with planar microelectrode arrays *in vitro* for spatially selective recording and stimulation: A comparison of neuronal and myotube extracellular action potentials

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Abstract

Microelectrode array (MEA) technology holds tremendous potential in the fields of bio-detection, lab-on-a-chip applications, and tissue engineering by facilitating non-invasive electrical interaction with cells *in vitro*. To date, significant efforts at integrating the cellular component with this detection technology have worked exclusively with neurons or cardiac myocytes. We investigate the feasibility of using MEAs to record from skeletal myotubes derived from primary myoblasts as a way of introducing a third electrogenic cell type and expanding the potential end applications for MEA-based biosensors. We find that the extracellular action potentials (EAPs) produced by spontaneously contractile myotubes have similar amplitudes to neuronal EAPs. It is possible to classify myotube EAPs by biological signal source using a shape-based spike sorting process similar to that used to analyze neural spike trains. Successful spike-sorting is indicated by a low within-unit variability of myotube EAPs. Additionally, myotube activity can cause simultaneous activation of multiple electrodes, in a similar fashion to the activation of electrodes by networks of neurons. The existence of multiple electrode activation patterns indicates the presence of several large, independent myotubes. The ability to identify these patterns suggests that MEAs may provide an electrophysiological basis for examining the process by which myotube independence is maintained despite rapid myoblast fusion during differentiation. Finally, it is possible to use the underlying electrodes to selectively stimulate individual myotubes without stimulating others nearby. Potential uses of skeletal myotubes grown on MEA substrates include lab-on-a-chip applications, tissue engineering, co-cultures with motor neurons, and neural interfaces.

Keywords

Microelectrode array; skeletal muscle; extracellular action potential; myoblast fusion

Introduction

Future generations of biomedical devices rely increasingly on the integration of a cellular component for sensing or actuation. In such hybrid bio-sensors, living cells convert some stimulus into a signal that is measured by a secondary sensor, taking advantage of highly sensitive and selective sensing mechanisms and signal amplification cascades [1]. These new hybrid sensors allow scientists to study the behavior of individual cell types to environmental or experimental stimuli as well as the behavior of multiple cells or cell types acting in concert [1-3]. Substrate-embedded microelectrode arrays (MEAs) are a particularly convenient tool for this application because they are capable of noninvasive and selective stimulation and recording. Because they detect local changes in voltage at their contact sites, however, they are most effectively used with electrogenic cell types that generate rapidly changing bio-electric potentials. Much of the work done to date interfacing MEAs with cells *in vitro* has been done with two cell systems [1, 3]: 1) neurons, where the ability to identify the activity of single cells is used to identify patterns of population activity and network dynamics, and 2) cardiac myocytes, where spatial and temporal resolution allow the measurement of transduction velocity through sheets of linked cardiomyocytes.

Here we demonstrate the feasibility of integrating a third cell type, skeletal muscle cells, with MEAs *in vitro* for recording their bioelectric activity and optically detecting their contractions. While myotubes have been shown to develop on an MEA surface using the mouse myoblast C2C12 cell line [4], very little has been done to quantitatively examine the extracellular action potentials (EAPs) of myotubes that have been similarly differentiated from myoblasts *in vitro*. In this study, myotube EAPs are quantitatively compared to EAPs from cortical neurons *in vitro* in order to provide a performance benchmark. Availability of skeletal myotubes for lab-on-a-chip applications opens another door in the development of high-throughput screening technologies, provides an improved test-bed for research into neuromuscular disorders [5], and may have implications in skeletal muscle tissue engineering where the selective activation of fibers is required for controlled muscle construct contraction [6, 7]. Taken a step farther, it opens the door for co-culture of myotubes and additional cell types such as motor neurons, which would allow investigators to look into the complexities of nerve-muscle interactions [8], or the formation of neural circuits like the spinal reflex arc [4, 9], with a new degree of clarity.

Materials and Methods

Myotube and neuronal isolation and culture

Myoblasts were isolated and cultured as previously described [10]. Briefly, tissue from hind limb muscles of gestational day 21 Sprague Dawley rat pups was minced, digested, separated from debris, pelleted by centrifugation, and resuspended in growth media. Cells were plated into 75 cm² flasks and incubated for 24 hrs. Adherent cells were resuspended

and plated onto MEAs (Multichannel Systems) in Neurobasal medium plus 2% B27 supplement, 1% penicillin/streptomycin, and 1% Glutamax (all ingredients from Invitrogen) at a surface density of 300,000 or 200,000 cells/cm² for recording and stimulation experiments, respectively. Prior to seeding, surfaces were coated overnight with 40 µg/ml laminin (Sigma, St. Louis, MO).

Cortical neurons were isolated from gestational day 18 Sprague Dawley rat pups as previously described except that the cortex was removed instead of the hippocampus [11]. After the cortex was isolated, the meninges were removed, and a single-cell suspension was prepared. Cortical cells were then seeded onto MEAs in MEM medium plus 1% penicillin/streptomycin, 10% horse serum, and 3% v/v of 20% glucose solution (all ingredients from Invitrogen) at a surface density of 225,000 cells/cm². Prior to use, the medium was glutamate-depleted by a 24 hr exposure to astrocytes in culture, and the MEA surface was coated overnight with 0.3% polyethyleneimine dissolved in borate buffer.

Acquisition and analysis of electrophysiological data

Recordings of spontaneous cellular activity were made on a heat-controlled stage at 37° C in room atmosphere using a MCS recording array, sampling extracellular voltage from 60 contact pads at 20,000 Hz. Recordings were limited to 5 min in order to reduce the effects of room atmosphere on medium pH, and were performed in culture medium in order to avoid subjecting cultures to medium changes which can affect cellular activity. Medium pH and cellular activity were not observed to change over the 5 min recording sessions. Contact pad spacing was 200 µm, and diameter was 10 µm. Data analysis was performed on recordings taken during the period of most frequent spontaneous activity for each cell type (day *in vitro* (DIV) 12 for myocytes and DIV 21 for cortical neurons).

Spike sorting was performed using custom algorithms composed in MATLAB and based in part on the algorithms discussed in [12]. Briefly, potential spikes were identified using a voltage threshold of 5x the RMS noise for each channel. Spikes were aligned to their point of maximal deflection and principal component analysis (PCA) was performed on the resulting set of vectors. Using their position in a 2D space based on the first two PCs, likely spike events were then clustered using a K-means algorithm. Each of the clusters represents the activity of a single cell (or “unit”) as its activity is recorded in the extracellular voltage trace (EVT) from a single electrode. Metrics of spike characteristics were then calculated based on the morphology of the average spike shape for each unit.

Analysis of contractile activity

An unordered myotube culture was grown on a commercially available MEA. On DIV 7, after maturation of intracellular contractile machinery but preceding the onset of significant amounts of spontaneous activity, stimulation at selected electrode sites was delivered using an MCS signal generator and associated software. 10 Cycles of a 2V biphasic pulse with a 40 µs duration repeated at a frequency of 1Hz was delivered across sets of 2 or 4 electrodes as indicated in Fig. 3. Videos of cells were acquired using the Qcapture-pro software at 25 frames per second. Automated localization of contractile activity was performed as discussed in [10]. Briefly, videos were analyzed using a series of image processing and

pattern recognition steps which make it possible to identify regions of synchronized contractility within videos of myotube cultures, returning the location of the contractile myotubes (Fig. 3, A - top) as well as their pattern of contractions (Fig. 3, A - bottom).

Results & Discussion

Myotube vs. neuronal extracellular action potentials

Consistent with prior findings [4], myoblast differentiation into myotubes occurred on MEA surfaces forming long, overlapping tubular structures, approximately 20 μm to 40 μm across and up to several millimeters in length (Fig. 1, A). In comparison, neurons are in the range of 20 μm in diameter, with farther reaching neuritic arbors (Fig. 1, B). Both cell types are electrogenic, creating transmembrane currents that propagate rapidly along their length through the activation of voltage-gated ion channels. The unique membrane composition, morphology, and behavior of each cell type, however, results in different EAP characteristics. Differences in EAP characteristics may additionally be due to slight differences in recording medium and surface treatment used for each cell type. Effects secondary to medium composition are likely small, however, based on the similar electrolyte profiles of MEM and NB medium. Similarly, differences in EAP shape due to the different surface treatments are likely minimal since surfaces in both culture systems are treated with a simple adsorption process. These small differences in culture conditions are unavoidable due to the different requirements for optimal cell growth and function for myocyte and cortical cell cultures. Using separate culture systems designed to foster growth of each cell type allows us to compare behavior when both cell types are functioning optimally.

The shape of the EAP is primarily a result of the spatial relationship between the cell and the electrode contact [13, 14]. Neurons have static surface interactions over short periods of time and recordable EAPs are primarily generated by the soma, which results in a predominance of negative EAP shapes like that pictured in Fig. 1, C.1. Bipolar (Fig. 1, C.2) and unipolar-positive EAP shapes may also be seen by recording from proximal neurites [14], but the vast majority of neurites are too small to produce a detectable signal [15]. In comparison, the EAPs generated by myotubes are far more variable. Standard unipolar and bipolar EAPs are possible (Fig. 1, D.1 – red and green), but arbitrary shapes are more frequent (Fig. 1, D.1 – blue and D.2). This is likely due to the longer period over which the depolarization occurs, the propagation of the intracellular action potential along the myotube, and the changing shape and location of the myotube during excitation and contraction [16-18]. Despite the variability in spike shape, however, spikes generated by a single biological unit are remarkably reproducible, as evidenced by the small within-unit variability (Fig. 1, E).

Myotubes and neurons produce EAPs with similar amplitudes. However, after normalizing the peak-to-valley amplitude of each unit by the noise associated with the electrode contact on which it is recorded, we find that myotube spikes tend to have a higher signal-to-noise ratio (SNR) than neuronal spikes (Fig. 1, F). This is likely a result of the larger size of myotubes relative to neurons [19], which results in an increased extracellular voltage change caused by the larger ionic movement accompanying depolarization and improved electrode sealing and coverage due to the larger surface area contacted by myotubes.

Myotube network vs. neuronal network activity patterns

Cultures of both myotubes and neurons show synchronous activation of multiple units, which appears as vertical bands of events in Fig. 2. This culture level behavior has different root causes for each cell type. Many myotubes are large enough to pass within recording distance of multiple electrodes (Fig. 1, A – red field). Therefore, when a single myotube depolarizes, multiple electrodes will record a single EAP event at the same time, causing the vertical banding in the raster plot of culture (Fig. 2, C). In contrast, single neurons will only ever be within recording distance of a single electrode (Fig. 1, B – red field). Because of their far-reaching processes, however, it is possible for neurons to be synaptically connected with other neurons. As waves of activity travel through these multicellular networks, they produce synchronous activation of multiple neurons, each one within the recording field of a different contact. As such, the precise activation pattern of each unit in the network is unique (Fig. 2, D).

It is important to note that multiple networks coexist in the same culture (both in myotube cultures and neuronal cultures). Each network's activity is observable as the synchronous activation of a different set of units (Fig. 2, B-C – rectangles 1 and 2). Coexisting networks in neuronal culture are expected due to the large number of neurons and the specific and structured way synapses are formed and pruned. The behavior is surprising in myotubes, however, where disordered culture tends to produce multipolar or branching myotubes that contract as single entities [10] and where the precedent set by cardiac myocytes is the formation of mass syncytia. If myotubes fused with all other contacted myotubes, the result would be a single contractile entity. The existence of multiple myotube networks within an unstructured culture supports the theory that myotube fusion is a directed process which proceeds with some specificity [20].

Selective stimulation of myotube networks

The discovery that multiple independent myotubes are identifiable by their pattern of spontaneous bio-electrical activity, and that their independence is conserved even when they may partially overlap, led to an investigation of whether these myotube networks can be selectively stimulated. Stimulation of a central region of the culture resulted in contraction of multiple myotubes within the culture (Fig. 3, A), while stimulation of more peripheral regions triggered contraction only of selected cells within the culture (Fig. 3, B-D). Stimulation of an intermediate area between two of these cells resulted in simultaneous activation of both (Fig. 3, E).

This is the first demonstration of spatially selective stimulation of myotubes and the subsequent contraction of a selected subset of cells in the culture. This finding reinforces the notion that multiple independently active myotubes can coexist in the same culture, even when they may be in direct physical contact with one another at points along their length. Additionally, it confirms the ability to use MEA technology to selectively stimulate contraction in specific myotube networks without stimulating those around them.

Conclusion

By integrating a skeletal myotube culture with a substrate embedded MEA, we have shown that spontaneously contractile myotubes are capable of producing activity that is detectable both optically and electrically. Skeletal myotubes have EAPs with SNR similar to those recorded from neuronal somata cultured under similar conditions. However, myotube EAPs are longer in duration and can be multiphasic, while neuronal depolarizations are rapid, small, and predictably monophasic. The activity of each cell can be identified based on its unique EAP signature, and the activity of single myotubes spanning multiple electrode contacts can be observed as linked activity between these units. Additionally, multiple independently active myotubes exist in the same culture despite physical contact with other myotubes and can be distinguished optically based on contraction or on the network-like behavior of the underlying electrodes.

The integration of skeletal myotubes with MEAs has a wide range of implications. The ability to monitor myotube excitation and contraction during development and over extended periods may provide new insight into development and diseases of the neuromuscular system. The ability to study the means by which myotubes fuse or remain independent may fuel progress in the construction of skeletal tissue grafts capable of graded contraction. Additionally, the integration of motor neuron-myotube co-cultures with an MEA substrate would provide researchers with a sensitive means of observing the neuromuscular junction, unraveling the complicated trophic communication occurring between motor neurons and myotubes and observing simple neuronal circuit formation, such as the spinal reflex arc. This may open the door to new approaches to neural interfaces. One can conceptualize a myotube-integrated regenerative neural interface design that uses myotubes to provide neurotrophin-secreting targets for peripheral nervous system reinnervation, and takes advantage of the large myotube depolarizations to provide amplified signals derived from regenerated motor fibers [21]. Such an approach potentially increases the communication bandwidth of the targeted muscle re-innervation approach [22, 23] by reducing it to a single myotube scale. Future work will include the integration of an MEA with topographical guidance cues in order to create structured myotube cultures and ultimately facilitate the formation of structured motor neuron-myotube co-cultures.

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Bibliography

1. Kovacs GTA. Electronic sensors with living cellular components. *Proceedings of the Ieee*. 2003; 91(6):915–929.
2. Offenhausser A, Knoll W. Cell-transistor hybrid systems and their potential applications. *Trends in Biotechnology*. 2001; 19(2):62–66. [PubMed: 11164555]
3. Stett A, et al. Biological application of microelectrode arrays in drug discovery and basic research. *Analytical and Bioanalytical Chemistry*. 2003; 377(3):486–495. [PubMed: 12923608]

4. Molnar P, et al. Photolithographic patterning of C2C12 myotubes using vitronectin as growth substrate in serum-free medium. *Biotechnology Progress*. 2007; 23(1):265–268. [PubMed: 17269697]
5. Rich MM. The control of neuromuscular transmission in health and disease. *Neuroscientist*. 2006; 12(2):134–142. [PubMed: 16514010]
6. Xi JZ, Schmidt JJ, Montemagno CD. Self-assembled microdevices driven by muscle. *Nature Materials*. 2005; 4(2):180–U67. [PubMed: 15654345]
7. Bian WN, Bursac N. Engineered skeletal muscle tissue networks with controllable architecture. *Biomaterials*. 2009; 30(7):1401–1412. [PubMed: 19070360]
8. Midrio M. The denervated muscle: facts and hypotheses. A historical review. *European Journal of Applied Physiology*. 2006; 98(1):1–21. [PubMed: 16896733]
9. Wilson K, Molnar P, Hickman J. Integration of functional myotubes with a Bio-MEMS device for non-invasive interrogation. *Lab on a Chip*. 2007; 7(7):920–922. [PubMed: 17594013]
10. Langhammer CG, Zahn JD, Firestein BL. Identification and Quantification of Skeletal Myotube Contraction and Association In Vitro by Video Microscopy. *Cytoskeleton*. 2010
11. Charych EI, et al. Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95. *Journal of Neuroscience*. 2006; 26(40):10164–10176. [PubMed: 17021172]
12. Adamos DA, Kosmidis EK, Neophilidis G. Performance evaluation of PCA-based spike sorting algorithms. *Computer Methods and Programs in Biomedicine*. 2008; 91(3):232–244. [PubMed: 18565614]
13. Bove M, et al. Interfacing Cultured Neurons to Planar Substrate Microelectrodes - Characterization of the Neuron-to-Microelectrode Junction. *Bioelectrochemistry and Bioenergetics*. 1995; 38(2): 255–265.
14. Gold C, et al. On the origin of the extracellular action potential waveform: A modeling study. *Journal of Neurophysiology*. 2006; 95(5):3113–3128. [PubMed: 16467426]
15. Dworak BJ, Wheeler BC. Novel MEA platform with PDMS microtunnels enables the detection of action potential propagation from isolated axons in culture. *Lab on a Chip*. 2009; 9(3):404–410. [PubMed: 19156289]
16. Jenerick H. An Analysis of the Striated Muscle Fiber Action Current. *Biophys J*. 1964; 4:77–91. [PubMed: 14130439]
17. Dimitrov GV, Dimitrova NA. Precise and fast calculation of the motor unit potentials detected by a point and rectangular plate electrode. *Medical Engineering & Physics*. 1998; 20(5):374–381. [PubMed: 9773690]
18. Hammarberg B, Stalberg E. Novel ideas for fast muscle action potential simulations using the line source model. *Ieee Transactions on Biomedical Engineering*. 2004; 51(11):1888–1897. [PubMed: 15543667]
19. Buitenweg J, Rutten W, Marani E. Geometry-Based Finite-Element Modeling of the Electrical Contact Between a Cultured Neuron and a Microelectrode. *Ieee Transactions on Biomedical Engineering*. 2003; 50(4):501–509. [PubMed: 12723062]
20. Clark P, et al. Alignment of myoblasts on ultrafine gratings inhibits fusion in vitro. *International Journal of Biochemistry & Cell Biology*. 2002; 34(7):816–825. [PubMed: 11950597]
21. Egeland BM, et al. Engineering and development of a stable, low-impedance, bioelectrical peripheral nerve interface. *Journal of the American College of Surgeons*. 2009; 209(3):S76–S76.
22. Kuiken TA. Consideration of nerve-muscle grafts to improve the control of artificial arms. *Journal of Technology and Disability*. 2003; 15(2):105–111.
23. Kuiken TA, et al. Targeted Muscle Reinnervation for Real-time Myoelectric Control of Multifunction Artificial Arms. *Jama-Journal of the American Medical Association*. 2009; 301(6): 619–628.

Figure 1.

Characteristic myotube-electrode and neuronal-electrode interaction. A) Myotube morphology on MEA at DIV 12. A single myotube is outlined in red dashes, and the area over which its EAP is likely detectable is shaded in red. B) Neuronal morphology on MEA at DIV 21. C1-C2) Representative myotube EAP shapes following spike sorting (6 ms shown, variable y-axis scale). D1-D2) Representative neuronal EAP shapes following spike sorting (6 ms shown, variable y-axis scale). E) Average signal to noise ratio (SNR) over all units. F) Average unit standard deviation over all units. All error bars represent the standard error of the mean.

Figure 2.

Synchronous multi-electrode activation in myotube cultures and neuronal cultures. Raster plot axes show the time (X-axis) and unit number (Y-axis) of each EAP as a point. The above raster plots show 30 s of data recorded from a myotube culture at DIV 12 (A), 30 s of data recorded from a neuronal culture at DIV 21 (B), and expanded view of 1 s data segments from within A and B. Two example networks are shown in each case (rectangles 1 and 2).

Figure 3.

Stimulation of myotube networks using a substrate-embedded MEA. Active myotubes are identified based on automated analysis of video-micrographs, and indicated by the colored meshwork, and the electrodes used for stimulation are indicated by the orange and yellow dots. A) Stimulation in a central region, triggering contraction in a large area of the overlying myotube layer. B-D) Stimulation at peripheral sites, showing activation of subsets of myotubes. D) Stimulation at an intermediate site, showing activation of the myotubes from C and D in red and blue hatchwork, respectively.