Reports

Stimulation with a low-amplitude, digitized synaptic signal to invoke robust activity within neuronal networks on multielectrode arrays

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Multielectrode arrays (MEAs) are used for analysis of neuronal activity. Here we report two variations on commonly accepted techniques that increase the precision of extracellular electrical stimulation: (i) the use of a low-amplitude recorded spontaneous synaptic signal as a stimulus waveform and (ii) the use of a specific electrode within the array adjacent to the stimulus electrode as a hard-grounded stimulus signal return path. Both modifications remained compatible with manipulation of neuronal networks. In addition, localized stimulation with the low-amplitude synaptic signal allowed selective stimulation or inhibition of otherwise spontaneous signals. These findings indicate that minimizing the area of the culture impacted by external stimulation allows modulation of signaling patterns within subpopulations of neurons in culture. The simple modifications described herein may be useful for precise monitoring and manipulation of neuronal networks.

Commerically available MEAs generally consist of a culture chamber with electrodes etched into the base. MEAs are often used for the analysis of neuronal signaling during formation and manipulation of networks. In addition to monitoring and capturing spontaneous activity, any of the array electrodes can be used to stimulate a neuronal network by applying an external signal. For example, a biphasic square pulse of ± 700 millivolts (mV) amplitude and 800 milliseconds (msec) duration is often applied to a single electrode ("monopolar stimulation") or to two electrodes ("bipolar stimulation") within the MEA (1-11). More recently, region-specific excitation has been attempted by employing these types of signals at multiple electrodes, referred to as "multipolar stimulation" (12). Results are generally reported as the total number of spikes or as changes in the inter-spike or inter-burst interval as a consequence of stimulation. The latter reporting method relies upon statistics of modulated spike timing as the determinant of an invoked response.

In addition to the 59 array electrodes, MEAs also have a single so-called "bath

ground" electrode etched onto the glass substrate that is required to keep electrical noise throughout the culture sufficiently low enough to record signals reproducibly above background. The bath ground electrode is relatively large, is situated distant from the 59 electrode array, and is often utilized as the reference to complete the stimulus circuit. This configuration is commonly referred to as "monopolar" (7,13). Unfortunately, stimulation via an array electrode and coupled with the bath ground electrode results in dispersion of the applied electric field throughout a large area of the culture (Fig. 1A) and consequently can impact a large portion of the neuronal network.

Related factors that complicate the use of this relatively large electrical field include stimulator driving impedances and the effects of displacement current densities along the insulated conductive traces required to connect the electrodes to external electronics. These traces, also etched onto the glass, transfer time-varying charges to and from electrodes during stimulation that radiate electro-

magnetic energy across the electrical insulator and into the culture (14). The problem becomes more complicated in the presence of a monolayer of cells, since the effective propagation medium becomes non-uniform and generates inflections in the field (15). These factors can collectively exert non-uniform effects on neurons within the stimulated region, which can compromise stimulation and confound interpretation of results. Herein, we examined the impact of using one of the array electrodes, rather than the bath electrode, as a ground to complete the stimulus circuit, to form a localized electrical field.

Along with minimizing electrical input, we also considered the potential benefit of stimulation with a low amplitude signal. The rationale for use of a biphasic square pulse of large amplitude as a neuronal stimulus was to bring the amplifier out of saturation as quickly as possible following a monophasic stimulus event of the same magnitude (7,12). It has further been suggested that the first half of a biphasic pulse serves as a pre-pulse, lowering the

Figure 1. The applied signal field and response signals induced by different stimulation patterns. Panel A presents a representative recording from a mature culture in the absence of stimulation ("No stimulus"), the single and triple biphasic square wave of ± 700 mV, and the derivation of the Biological Signal. Representative response signals for each stimulation regimen are presented. Panel B presents the approximate electric field distribution resulting from the bath ground electrode and the localized stimulation configuration, utilized herein with the configuration in panel A, representative responses as indicated. The stimulus pulse is reflected in recorded signals due to its high amplitude. Panel C presents representative response signals following localized stimulation with the square wave or Biological Signal as indicated. The accompanying graph quantifies the relative distribution of individual spikes or bursts elicited by each signal over 30sec after stimulation. Values were derived from 3 separate MEAs stimulated with each signal. Panel D presents two representative responses resulting from stimulation across 2 electrodes with the Biological Signal or the inverted Biological Signal (see text).

threshold for activation of response signaling (16). We examined herein the relative efficacy of utilizing a synaptic burst, recorded from a neuronal network maintained on an MEA for >1 month. We also examined the effect of stimulation with a square wave and a recorded synaptic signal using a localized stimulation circuit, which allowed us to monitor the effects of stimulation on selected regions within the network.

Materials and methods

Cultures

Cortical neurons harvested from day 15 C57BL/6 mouse embryos were dissociated and plated at 100 cells/mm² in

Laminin-coated, 250mm² glass multi-electrode arrays containing 59 titanium nitride electrodes in a 1.4mm² area on a 200µm grid at the center of the chamber from Multi Channel Systems (Reutlingen, Germany) and maintained in B27-supplemented Neurobasal medium (Invitrogen, Carlsbad, CA) as described (17). Sacrifice of the pregnant female was carried out under procedures approved by our Institutional Animal Care and Use Committee. Spontaneous neuronal signals were discernable within ten days of plating. However, cultures were maintained for ≥ 1 month prior to recording in order to allow maturation and stabilization of the neuronal network (17-19).

Recording

Established neuronal networks on MEAs were placed in a MEA-1060-INV amplifier (Multi Channel Systems) with a fixed gain of 1200 on all 59 channels as described (17). Groups of 16 amplified channels were recorded with our custom interface (17) connected to the DT9814 data acquisition system (Data Translation; Marlborough, MA). Data was captured at 3.125 kHz simultaneously accessing 16 channels and 12.5 kHz accessing 4 channels; the latter was used to validate the use of undersampling for the purpose of observing more channels (20). Data files consisting of 30sec recordings were independently generated for each channel which were

analyzed both manually and with a spike detection algorithm that adapts to the local baseline noise level in order to distinguish bona-fide neuronal activity from baseline electrical disturbances (21). Signals were scored if they displayed a $\geq 10\mu\text{V}$ deviation from baseline (17). Data presented herein were derived from multiple cultures from ≥ 3 independent platings. Images present representative signal profiles for all recorded channels. Prior studies using combinations of excitatory and inhibitory neuronal antagonists have confirmed synaptic origin of signal in these cultures (17,19,22).

Results and discussion

As described previously (17,23-26), long-term ("mature") cultures develop spontaneous signals that radiate across large areas of the culture, as evidenced by their simultaneous appearance at multiple electrodes. When recorded, spontaneous signals in our mature cultures exhibited a regular amplitude ($40.0 \pm 5.0 \mu\text{V}$) and duration ($0.8 \pm 0.2\text{sec}$, mean $\pm$ standard deviation, $n = 20$ individual signals from ≥ 3 cultures from separate experiments).

Notably, spontaneous signals of these mature cultures displayed a remarkably regular periodicity, with intervals of $4.2 \pm 0.3\text{sec}$ between events ($n = 7$ inter-spike intervals quantified from ≥ 3 cultures from separate experiments).

We next examined the responsiveness of cultures to external stimulation. Stimuli were applied through the Real-Time All Channel Stimulator (RACS) with the maximum dynamic range set to $\pm 700\text{mV}$ (1,3,7,12,13) to electrodes within regions already displaying spontaneous signals. We first applied a biphasic "square wave" signal ($+350\text{mV}$ for $400\mu\text{sec}$ followed immediately by -350mV for $400\mu\text{sec}$ for a total of $800\mu\text{sec}$), utilizing the monopolar electrode configuration (i.e., with the bath ground electrode as the distal grounding electrode) (7,13; Fig. 1A). Stimulation with this biphasic signal elicited a 2.7-fold increase in the number of signals over those generated spontaneously (Fig. 1B). Consistent with prior studies (26,27), the amplitude of response signals was approximately half that of spontaneous signalling (to 20.5 ± 2.4 versus the $40 \pm 5\mu\text{V}$ amplitude of spontaneous signals). Spontaneous

signals displayed a periodicity of $3.9 \pm 0.2\text{sec}$ following stimulation, which was virtually identical to that of spontaneous signals prior to stimulation.

We also considered that more complex signals may evoke more complex or stronger responses. To examine this possibility, we first applied the biphasic square wave 3 times in rapid sequence with 358msec between pulses, at 2.8sec intervals (1; Fig. 1B) using the electrode configuration described above. This "triplet" signal induced a 1.5 fold increase in the number of signals when compared to the single square wave signal ($p < 0.05$; $n = 5$ independent recordings from multiple cultures).

We considered that an actual neuronal signal may invoke even more complex signaling. To examine this possibility, we extracted a 1 sec clip of a recording of a spontaneous signal from a mature culture, which we termed the "Biological Signal." (Fig. 1A). This Biological Signal had a recorded amplitude of approximately $70\mu\text{V}$. We first applied the Biological Signal with only slight amplification (to $100\mu\text{V}$); we received no apparent response, and spontaneous signals continued at their regular interval (not

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shown). We next amplified the Biological Signal to 1mV, which invoked response signals within the same time interval. To enable accurate reproduction of the recorded Biological Signal, the playback sample rate was configured to match that of the original recording and the dynamic range of the digital-to-analog converter was set to $\pm 1\text{mV}$; this setting was accomplished by feeding a biphasic square pulse into an MEA containing media only (no cells) and adjustment of the RACS circuit board potentiometers while monitoring this biphasic signal through the amplifier. This signal induced a robust, biphasic response (Fig. 1A).

As anticipated, all channels where neurons were located over electrodes responded to the bath ground. We considered a regional application relying on interconnectivity for signal radiation. To accomplish this regional signaling, we utilized a series of 3 adjacent electrodes for the entire circuit, including one of the array electrodes as the proximal ground, rather than relying on the bath ground (Fig. 1B). In the example presented in Fig. 1B, we stimulated electrode 12, recorded from adjacent electrode 11 and utilized a local electrode 10 as the ground

electrode. This setup allowed regional stimulation and analyses, and could be situated anywhere within the array. In preliminary studies, we observed that disconnecting the bath ground resulted in excessive noise that precluded reproducible signal monitoring. Therefore, we retained the bath ground, but minimized the extent of current sinking at the bath ground by selecting an array electrode for hard-grounding that was situated between the stimulus and bath ground electrodes (Fig. 1B). Hard-grounding was required to minimize the circuit impedance of the reference electrode and insure that it would not exceed that of the bath ground. The appropriate stimulus access pin on the amplifier (in this case the pin for electrode 10; Fig. 1B) was connected to signal ground using a wire lead comprised of a mini-test clip and an alligator clip.

We then applied the square wave and Biological Signal using this local arrangement. (Fig. 1C). Both the square wave and the Biological Signal invoked similar overall numbers of response signals (37 ± 3 vs 34 ± 1 , respectively in a 30 sec interval, $n = 10$ each), which, rather than radiating throughout the culture, were

confined to those electrodes connected via synaptic connections to the localized stimulation and response electrodes (not shown; see reference 13). Notably, the Biological Signal induced relatively more bursts (defined as continuous responses that remained above baseline for $\geq 3\text{sec}$; 28-30) than individual signals ($p < 0.03$; Student's t test; Fig. 1C).

Since prior studies indicated that improved response was observed by sequential stimulation at 2 adjacent electrodes (31), we also applied the Biological Signal at 2 sets of adjacent electrodes (electrodes 15 and 12; and electrodes 12 and 7), and recorded responses from a third electrode situated midway between these pairs (electrode 11). Since the Biological Signal was not a simple biphasic signal, this also afforded us the opportunity to apply it in an inverted form. Application of the Biological Signal at electrodes 15 and 12 did not invoke a response, but did evoke a response when applied at electrodes 12-7. By contrast, the inverted Biological Signal invoked a stimulus when applied at electrodes 15-2 but not at electrodes 12-7 (Fig. 1D). These differential responses from the same electrodes rule out any under-

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lying electrical artifact, since inversion of the signal determined whether or not a response was observed.

In those instances where the Biological Signal invoked a response, it occurred within a similar interval as observed with either single or triple square wave pulses. Spontaneous signaling resumed within a statistically-identical interval after stimulation with the Biological Signal when compared to square wave pulses (4.2 ± 0.6 sec). Notably, in those instances where the Biological Signal did not invoke a response, we observed a doubling of the delay in resumption of spontaneous signals; spontaneous signals resumed after 8.1 ± 1.2 sec. This is approximately twice the delay observed following all other stimulation conditions and approximately twice the normal interval between spontaneous signals (above). One interpretation of this phenomenon is that the Biological Signal generates an inhibitory signal under some conditions. These results further indicate that, depending upon signal orientation and location, the Biological Signal can invoke inhibitory or excitatory responses that alter downstream neuronal signaling.

The localized stimulation system described herein requires minimal modification of existing approaches and no hardware alterations. It provides localized stimulation with a specificity approaching that of microarrays using predefined tunnels that restrict the directionality of axonal outgrowth (and therefore restrict the directionality of signal propagation (32). Our altered signaling and circuit design is readily reversible to classical settings, which can allow localized versus global stimulation and monitoring of the same neuronal networks. Stimulation with a recorded synaptic signal may be more effective than classical square waves in attempting to strengthen connections within cultures or evoke activity in otherwise inactive subpopulations. Notably, the efficacy of a pre-recorded synaptic signal as described herein provides the novel opportunity for comparison of the effects of stimulation with normal versus aberrant synaptic signals (e.g., epileptiform signals or those elicited in response to excitotoxicity, etc.). An additional modification could include the utilization of an external reference electrode. These possibilities will be examined in future studies.

The Biological Signal as recorded was $70\mu\text{V}$. Neither this native amplitude nor slight amplification to $100\mu\text{V}$ induced

detectable network activity. However, amplification to 1mV induced reproducible activity. We did not attempt to determine the absolute minimum amplitude necessary for the Biological Signal to evoke network activity. We also did not attempt to alter the duration of the Biological Signal, which would likely impact its efficacy. The absolute value of these parameters would likely depend upon the particular Biological Signal utilized for stimulation. Notably, the 1mV Biological Signal as utilized herein was at least as effective as a substantially larger (700mV) square pulse. We have not determined whether or not the complexity of the Biological Signal is essential to evoke responses, or whether a biphasic square signal of similar low amplitude, or tetanic stimulation with such a signal (1), would achieve similar results.

The asymmetric profile of the Biological Signal allowed application in native or inverted orientation. We observed stimulation or suppression of spontaneous signaling using native or inverted Biological Signal, indicating that neuronal subpopulations were oppositely affected depending upon signal orientation. Inhibition of spontaneous signaling suggests a predominant impact on inhibitory neurons, consistent with the prior demonstration that GABAergic neurons are responsible for synchronized bursts in mature signal patterns (19,22).

These results elucidate inherent limitations of current MEA technology for use as extracellular neuronal stimulation platforms. At present, there is no ability to switch an electrode directly to ground (without resistance). This could be overcome by incorporating digitally controlled grounding switches into the stimulus equipment. In addition, the bath, reference and stimulus electrodes must all fall in a straight line in order to minimize current sinking at the bath ground electrode and generation of two electrical fields, which would preclude establishment of a local circuit. This inherent limitation cannot be overcome at present, since the bath must be grounded to achieve an acceptable signal-to-noise ratio. However, the use of a narrower bath electrode, possibly with a pointed end, would reduce field fringing around the signal reference electrode. Despite these limitations, the temporal accuracy of the responses to the Biological Signal and its ability to provoke or inhibit spontaneous signaling suggests that our localized

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electrode configuration provides the ability to monitor the activity of localized populations within networks.

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Competing interests

The authors declare no competing interests.

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