

Portable cell-based biosensor system for toxin detection

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Abstract

A portable cell-based biosensor has been developed and characterized. The prototype system relies on extracellular recording from excitable cells cultured over an array of platinized gold microelectrodes. Extracellular potentials were bandpass filtered between 80 Hz to 2.8 kHz and amplified with a selectable gain of either 1000 or 5000. The input-referred noise level of the system was only 8.7 μV_{RMS} in the laboratory setting, reaching only 10.6 μV_{RMS} in an outdoor environment, more than sufficient for measurement of extracellular potentials from excitable cells. The system also incorporates a feedback control system for temperature regulation and a 36-channel multiplexer for selection of up to four output channels for simultaneous display. Wherever possible, low-cost ‘off-the-shelf’ components were utilized in this prototype biosensor design. Using this system, extracellular recordings from chick myocardiocytes were performed under both laboratory and outdoor conditions. © 1998 Elsevier Science S.A. All rights reserved.

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1. Introduction

Portable biosensors utilizing immobilized antibodies have been developed for environmental detection of biological analytes [1,2]. While immunosensors provide a means for identification of a biological threat or toxin, binding assays cannot offer insight into the functional consequences of an analyte–tissue interaction. To address the need for physiologically relevant, function-based assays, several groups have explored the utility of tissues [3] and cells [4,5] as sensor elements. One means of acquiring cellular functional information for biosensor applications involves extracellular recording from excitable cells [6–8]. Our long-term goal is the development of a portable cell-based biosensor for detection of an array of chemical/biological agents. The purpose of this paper is to describe and characterize a prototype extracellular recording system suitable for field use. The major hardware features of this modular

system include a planar array with 36 microelectrodes and supporting hardware incorporating AC-coupled buffer and filter stages constructed from ‘off-the-shelf’ components. To demonstrate the utility of this system, extracellular recordings from chick cardiac myocytes have been performed.

2. Methods

2.1. Microelectrode array fabrication and platinization

Planar microelectrode arrays were designed and fabricated at the Center for Integrated Systems at Stanford University. Each array has 36 microelectrode sites that are 10 μm in diameter (Fig. 1). For array fabrication, a (100) orientation, p-type silicon wafer was thermally oxidized to provide an insulating 500 nm SiO_2 layer. Gold electrodes, interconnecting traces and bond pads were patterned using a bilayer lift-off structure consisting of sputter-deposited silicon (500 nm) and Shipley 1813 photoresist (1 μm). The photoresist was patterned

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using standard photolithographic techniques followed by an isotropic silicon plasma etch (CF_4/O_2) to undercut the sputtered silicon layer. A 500 nm layer of gold was then sputter-deposited using chromium (25 nm) as an adhesion layer, resulting in a Cr/Au bilayer on the exposed SiO_2 surfaces and on top of the photoresist. Acetone was used to dissolve the photoresist and thereby remove the Cr/Au bilayer not adhering to the SiO_2 substrate. The remaining sputtered silicon was then plasma stripped ($\text{C}_2\text{ClF}_5/\text{SF}_6$) to leave the desired gold microelectrodes, interconnecting traces and bond pads. To provide chemical and electrical insulation, the interconnecting traces and electrodes were then passivated with 1 μm of plasma-enhanced chemical vapor-deposited silicon nitride; this process was performed at low temperature (300°C) to limit gold roughening. A mixed frequency process (rapidly cycling between high and low frequencies) was utilized to allow sub-monolayers of tensile and compressively stressed silicon nitride to be deposited, resulting in overall near zero-stress films. Following passivation, windows were cut through the film using an anisotropic plasma etch ($\text{C}_2\text{ClF}_5/\text{SF}_6$) to define the electrode geometries and open the bond pads. The wafer was then diced and cleaned. Arrays were mounted in a ceramic 40-pin dual inline package (DIP; Spectrum Semiconductor Materials, San Jose CA) and bond-wire connections were made between the die and DIP. The bond-wires were encapsulated in a non-cytotoxic epoxy suitable for insulation (EpoTek 87GT, Epoxy Technology, Billerica, MA). For use in our cell culture facility, 35 mm polystyrene culture dish with a hole drilled in the center were adhered with the epoxy to the microelectrode arrays.

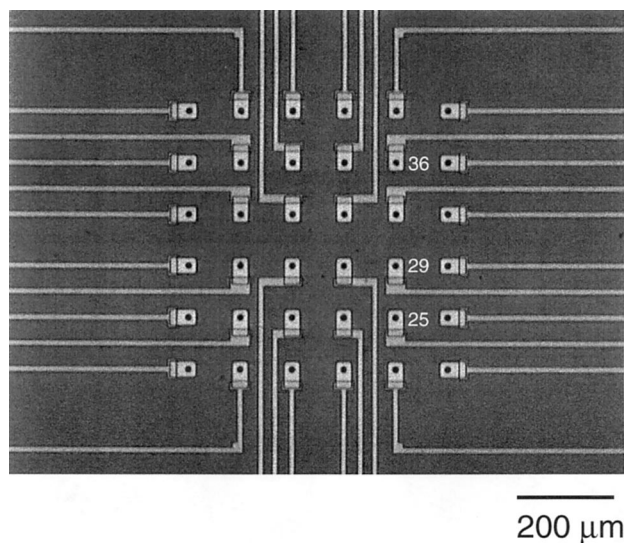


Fig. 1. Planar microelectrode array detailing individual platinumized, 10 μm diameter recording microelectrode sites. Microelectrodes 25, 29, and 36 are identified as sites for the extracellular recording example in Fig. 5.

Microelectrodes were electroplated with platinum black to decrease impedance values for adequate recording of biopotentials. Electrode impedance was measured in 0.9% saline with a LCR meter (model 4284A, Hewlett Packard, Rockville, MD) with an applied signal of 5 mV_{RMS} . The microelectrode impedance at 1 kHz was ~ 1 to 3 $\text{M}\Omega$ prior to platinization. To deposit platinum black, 1 V dc was applied at the microelectrode site for 30 s in a solution containing 5% chloroplatinic acid and 1% lead acetate (YSI, Yellow Springs, CO) where the current was limited to 5 nA μm^{-2} . After electroplating, the microelectrode impedance decreased to less than 300 $\text{k}\Omega$ at 1 kHz.

2.2. Data acquisition

In the cell experiments, the system output was recorded using a digital audio tape recorder (model RD-135T, TEAC, Montebello, CA). The RD-135T has a preamplifier stage with its gain set to maximize the 14-bit A/D conversion with a full scale input voltage range of ± 2 V yielding a sampling error of ± 244 μV with a sampling rate of 10 kHz per channel. For off-line analysis, the RD-135T output was digitized using a Digidata 1200B data acquisition board and Axotape version 2.0 (Axon Instruments, Foster City, CA) at a rate of 25 kHz and stored to the hard disk of an Intel Pentium-based PC.

2.3. System design considerations

The prototype biosensor was designed using ‘off-the-shelf’ components wherever possible. The biosensor is contained within an aluminum ‘carrying case’ 42 cm long, 23 cm wide, 21 cm high which weighs 12.4 kg with battery packs installed (Fig. 2A). The buffer and amplifier boards were designed to be stacked. The power supply, temperature control, and multiplexer are isolated from the buffer/amplifier stack by internally shielding each subsystem. For biosensor remote operation, three rechargeable nickel–cadmium (Ni–Cd) battery packs (model 1800_{MAX}, SR Batteries, Bellport, NY) were used, each delivering 18 V at a maximum current of 1.8 A. A standard 40-pin DIP containing the microelectrode array is held in a zero-insertion-force (ZIF) socket mounted on the buffer board (Fig. 2B). Between the ZIF and the DIP, a heating foil (model HK5221R13.0L12B, Minco, Minneapolis, MN) was installed to provide temperature stability which is a necessity for the cultured cells. A resistive heater controller (model RHM-4000, Wavelength Electronics, Bozeman, MT) featuring PID feedback and excellent stability was used to drive the thermal heating foil. Extracellular signals from the microelectrode array are ac-coupled to the followers (LMC6044, National Semiconductor, Santa Clara, CA) on the buffer stage (Fig. 3A). The ac

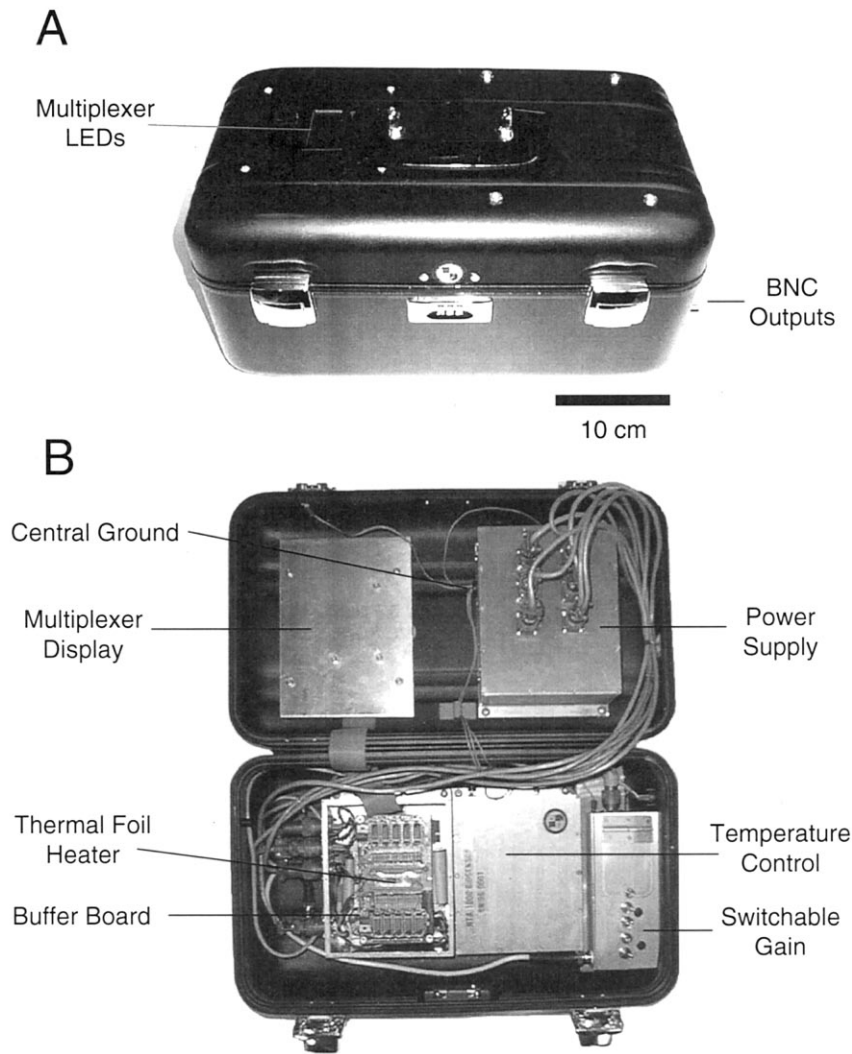


Fig. 2. Portable biosensor system. A, closed case view of the biosensor. B, Open case view showing several internal subsystems.

coupling eliminates dc signal components and also ensures that input offset currents cannot produce electrochemical changes at the cell–electrode interface. The shunt resistance of 45 M Ω (Dale Electronics Inc., Columbus, NE) was chosen to ensure that for signals at 1 kHz, there would be less than 1% attenuation of the extracellular signal amplitude. Each of the buffered channels is passed to an amplifier stage (LT1014, Linear Technology, Milpitas, CA) designed to yield high-pass and lowpass corner frequencies of 80 Hz and 2.8 kHz, respectively, and a gain of 200. The amplifier outputs are passed to a 36 channel multiplexer allowing the user to identify active microelectrode sites. A momentary contact rocker switch mounted on the top of the biosensor is buffered with Schmidt trigger (74HC14, Harris Semiconductor, Melbourne, FL) to drive two 4-bit up/down binary counters (DM54LS193, National Semiconductor), the binary output of which serve as address lines AO–A5. These address lines serve as inputs to two BCD to 7-segment decoder/driver

(DM7447A, Fairchild Semiconductor, South Portland, ME) to control two LED displays indicating the number corresponding to a particular microelectrode site. A dual 2-to-4 line decoder provides enable lines EN₀–EN₂, which activate the appropriate analog multiplexer (8 channel: ADG408BN, 16 channel: ADG406BN, Analog Devices, Norwood, MA). Extracellular signals are then directed via the appropriate multiplexer to V_{OUT} . In addition to the multiplexer output, three additional channels can be selected for output via pin-to-pin assignment from the ribbon cable. The signals are low pass filtered with a 4-pole Bessel anti-aliasing filter (DP74L4L, Frequency Devices, Haverhill, MA) with a corner frequency of 5 kHz. Depending on the tissue preparation, extracellular potentials can range from 20 μ V to 1 mV [8]. Since typical 12-bit A/D converters with an input voltage range of ± 10 V have a sampling error of ± 4.88 mV, an overall system gain of at least 240 is required. To ensure that the extracellular signals can be distinguished, the four channel out-

determined by grounding all of the system inputs, measuring channel output using an oscilloscope with digitizing and math functions (model 7854, Tektronix, Wilsonville, OR), and dividing by the amplifier channel peak gain. For quantification of system characteristics, the overall system gain was set to 1000 (60 dB). Where appropriate, data are given as mean $\pm$ standard deviation.

2.5. Cell culture

Embryonic day 11 chick cardiac myocytes (Truslow Farms, Chestertown, MD) were obtained by enzymatic dissociation using trypsin (Gibco-Life Technologies, Rockville, MD) and DNase I (Gibco-Life Technologies) of embryonic cardiac tissue as described earlier [9,10]. For a typical experiment, cells were plated at a

Fig. 3. Biosensor circuit and logic diagrams. A, extracellular potentials detected at the microelectrode sites are buffered via an ac-coupled follower with a corner frequency of 80 Hz, amplified by ~ 200 . The LMC6044 is a CMOS quad operational amplifier characterized by extremely low input leakage currents, making it ideal for portable, high source impedance amplifier applications. The buffered extracellular signal is amplified by the next stage using a LT1014, a quad precision operational amplifier exhibiting low offset voltages and high common-mode and power-supply rejection ratios. Amplifier outputs can be selected using a multiplexer (MUX) or directly from a ribbon cable connector. A selected channel is passed through an anti-aliasing, low-pass Bessel filter (LPF) with a corner frequency of 5 kHz. The filtered signal is amplified by 5 and 25 using LM6171 operational amplifiers which are well-suited for driving capacitive loads characteristic of ribbon cables. The filtered extracellular signal is amplified by a gain of either 1000 or 5000 and directed to the output BNC by a switch as shown. Resistors and capacitors were bin-sorted to be within 0.1 and 0.5% of design values, respectively. B, logic diagram for the multiplexer. A SPDT rocker switch drives two 4-bit up/down binary counters (DM54LS193, National Semiconductor), the binary output of which serve as address lines A0–A5. These address lines serve as inputs to two BCD to 7-segment decoder/driver (DM7447A, Fairchild Semiconductor) to control two LED displays indicating the number corresponding to a particular microelectrode site. A dual 2-to-4 line decoder provides enable lines EN_0 – EN_2 , which activate the appropriate analog multiplexer (8 channel: ADG408BN, 16 channel: ADG406BN, Analog Devices). Extracellular signals are then directed via the appropriate multiplexer to V_{OUT} .

density of 1×10^6 cells cm^{-2} . Culture media consisted of: 6% heat-inactivated bovine calf serum, 40% Medium 199 (M0650, Sigma), 0.1% Penicillin–Streptomycin (BRL 15140-122, Gibco), and 54% sterile-filtered low K^+ balanced salt solution (in mM): 116 NaCl, 0.8 NaH_2PO_4 , 1.18 KCl, 26.2 NaHCO_3 , 0.87 CaCl_2 , 5.5 dextrose. Myocardial cell viability based on trypan blue exclusion typically exceeded 90%. Cardiac cell monolayers were usually used within a week of dissection.

3. Experimental results

3.1. Extracellular recording system performance

The system can be operated either by battery or external power supply providing ± 16 V. Two battery packs provide power at the positive and negative rails of the main system comprising the buffer, amplifier, multiplexer, and anti-aliasing filter stages, whereas a separate battery pack is dedicated for thermal control. Under quiescent conditions, total power consumption reached 14 W yielding 2–3 h of system performance in an ambient temperature of 21–23°C.

Within the laboratory and the ac-coupled buffer inputs grounded, an output noise level of 8.8 ± 0.9 mV_{RMS} was measured, which corresponds to an input-referred noise of 8.7 ± 0.3 μV_{RMS} . Even with introduction of the microelectrode array bathed in 0.9% saline and the solution grounded through a gold wire, the noise level rose only slightly to 9.8 ± 0.3 μV_{RMS} . Furthermore, we assessed input referred noise in an outdoor environment which was only 10.6 ± 0.8 μV_{RMS} . Overall, the system noise levels compared well with the previously described amplifiers for extracellular recording; 15 μV [11], 10 μV [12,13].

The amplifier exhibited a bandpass frequency response over a frequency range suitable for extracellular recordings with low and high corner frequencies at 80 ± 2 Hz (mean $\pm$ STD) and 2.8 ± 0.1 kHz, respectively. At 1 kHz, the channel gain and phase shift were 59.9 ± 0.1 dB and $-36.4 \pm 0.8^\circ$, respectively. It should be noted that the low-pass Bessel filter anti-aliasing chip (Frequency Devices) can account for -24° phase shift at 1 kHz. A typical gain and phase shift profile is illustrated in Fig. 4A. To more accurately assess the impact of the microelectrode array on the system frequency response, a gold wire was introduced into the solution to drive the bath potential using the signal analyzer and the output was recorded to determine the frequency response. Addition of the microelectrode array resulted in very little change in the system response; low and high corner frequencies were 85 ± 3 Hz and 2.7 ± 0.1 kHz, respectively, and the gain at 1 kHz was 59.7 ± 0.2 dB.

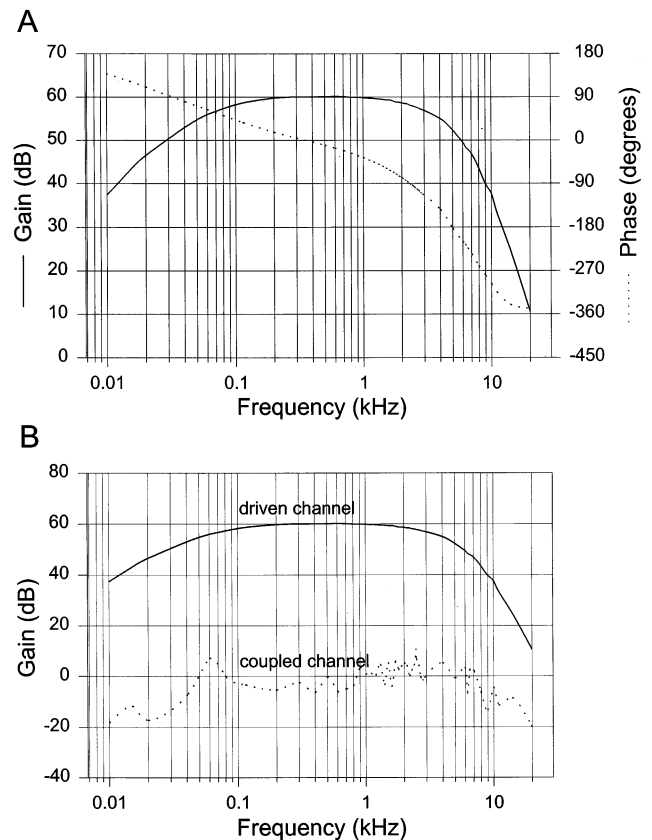


Fig. 4. Plots of the frequency response and crosstalk between channels. A, gain (solid) and phase (dotted) response of a typical amplifier channel. Over the frequency range of interest for extracellular recordings, 100 Hz to 2 kHz, the gain and phase vary from 57 to 60 dB and 45 to -80° , respectively. B, comparison of channel gain (solid) and crosstalk (dotted). Crosstalk between typical neighboring channels was assessed by measuring the output from channel A while driving the input to channel B and grounding the input of channel A.

For crosstalk measurements, we surveyed several different input channels. The input of one channel was driven with the signal analyzer while grounding the input of a second channel and recording the output of the second channel. As illustrated by the typical data in Fig. 4B, crosstalk gain was approximately 0 dB at 1 kHz reaching a maximum of approximately 10 dB at 3.0 to 3.7 kHz. For example, crosstalk for channel 3 was 2.03 ± 7.03 dB at 1 kHz reaching a maximum of 10.9 ± 3.1 dB over a range of frequencies, 3.4 ± 2.1 kHz. Relative to the driven channel, channel separation was -60 dB over the frequency range of interest. To evaluate the significance of the crosstalk measures, consider a maximum coupling of 11 dB and a biopotential of 1 mV at the input of a channel. One would expect a resulting 3.6 mV signal at the output of the coupled channel corresponding to a putative biopotential of only 3.6 μV . Therefore, any such crosstalk would fall below the input-referred noise of the amplifier.

3.2. Extracellular recordings of excitable cells

After 3–4 days in culture, chick cardiac myocytes exhibited a spontaneously pulsating monolayer. Consistent with earlier work [14–17], the extracellular cardiac potentials were multiphasic reminiscent of the second or third derivative of the action potential (Fig. 5A). Extracellular potentials were typically less than 1 mV in duration, occurring at a rate of 1–2 Hz with little variation in rate within a sample. For the experiment in Fig. 5A, propagation velocities between the microelectrode sites (shown in Fig. 1) ranged from 0.10 to 0.12 m s⁻¹. This range, which falls below that of a previous report [15], may be related to an irregular pattern of interconnectivity [16]. Nevertheless, delays between spikes at neighboring microelectrode sites were extremely consistent beat-to-beat. In five experiments, we observed complete cessation of extracellular activity with application of 300–500 μ M cadmium chloride or 30 μ M tetrodotoxin (TTX). Two further experiments conducted outdoors showed well-resolved extracellular potentials that were inhibited with the addition of TTX within 30 s (Fig. 5B). While these observations demon-

strate the physiological nature of the acquired signals, future work will address the subtle variations in extracellular waveforms, interspike intervals, and signal propagation in response to agents of interest.

4. Conclusion

To our knowledge, this is the first demonstration of system capable of performing a cellular function-based assay in the field. The major features of this prototype portable biosensor capable of extracellular recording from excitable cells have been described and characterized. System components that include an ac-coupled buffer, an amplifier stage, a multiplexer, anti-aliasing filters, and a thermal control system were constructed using low-cost, off-the-shelf components. The system has several important attributes that make it suitable for extracellular recording applications including an appropriate bandwidth for measuring extracellular potentials from a variety of excitable cells and a low crosstalk between channels. The prototype system provides only 2–3 hours of operation with the Ni–Cd battery packs; lithium batteries would provide a substantially longer operational window, albeit at a higher cost.

The microelectrode array, which was designed to be manufacturable and conducive for cell adhesion and function, permitted cardiac culture over a duration similar to that achieved under control conditions in polystyrene culture dishes. Planar microelectrode arrays manufactured using photolithographic techniques have been previously shown to be useful [8,18–20], but typically employ a ‘zebra strip’, a flexible silicone strip striated with conductive carbon, to achieve electrical contact between the pads of the planar array and the amplifier stage. The integration of the present array into a standard 40-pin DIP enables the array to be rapidly and easily replaced.

For system evaluation with excitable cells, planar microelectrode arrays were modified by adhering a 35 mm polystyrene culture dish with a hole drilled in the center so that cells would be easily maintained in a cell culture facility. Clearly, future work must address the need for a more sophisticated and rugged chamber interfaced with the planar microelectrode array. An enhanced chamber design would incorporate fluidic ports for controlled cell media perfusion and sample injection. Recent efforts in microfabrication have resulted in the development of an integrated silicon-polydimethylsiloxane cell cartridge that permits fluidic connections and may offer a suitable microenvironment for cell culture [21].

In summary, we have described and characterized a prototype portable cell-based biosensor. Our approach utilizes extracellular potentials from excitable cells

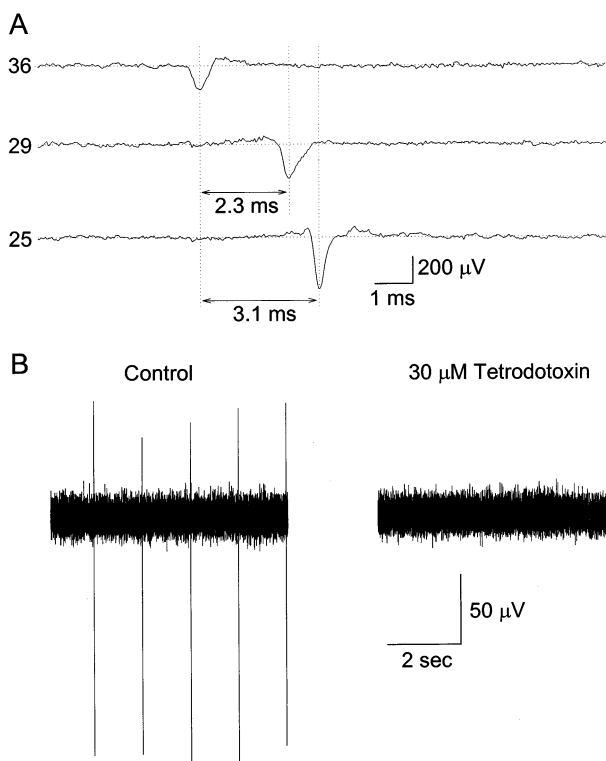


Fig. 5. Extracellular action potentials (APB) from a monolayer of cultured chick cardiac myocytes. A, simultaneous recordings from multiple microelectrode sites illustrating delay in AP propagation from microelectrode sites 25, 29, and 36. B, recording from a single representative microelectrode site before and after the administration of the sodium channel antagonist tetrodotoxin (TTX). TTX (30 μ M) induced a cessation of spontaneous firing after 30 s. Prior to application of TTX, the rate of spontaneous firing was 0.8 Hz.

recorded via a microelectrode array as a measure of cell functionality. The system incorporates temperature control, amplifier and filter stages to accommodate various excitable cell types, and a 36-channel multiplexer assembled from off-the-shelf components.

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