Extracellular Recordings From Patterned Neuronal Networks Using Planar Microelectrode Arrays

Conrad D. James*, Andrew J. H. Spence, Natalie M. Dowell-Mesfin, Rifat J. Hussain, Karen L. Smith, Harold G. Craighead, *Member, IEEE*, Michael S. Isaacson, *Member, IEEE*, William Shain, and James N. Turner

***Abstract*—Neuronal cell networks have been reconstructed on planar microelectrode arrays (MEAs) from dissociated hippocampal pyramidal neurons. Microcontact printing (μCP) and a photoresist-liftoff method were used to selectively localize poly-L-lysine (PLL) on the surface of MEAs. Haptotaxis led to the organization of the neurons into networks localized adjacent to microelectrodes. Various grids of PLL with 2–25-μm-wide lines spaced by 50–200 μm with 15–25-μm nodes at intersection points were used to guide cell body attachment and neurite outgrowth. Bursting activity with spike amplitude attenuation was observed, and multichannel recordings detected instances of coincident firing activity. Finally, we present here an extracellular recording from a $\sim$2 μm bundle of guided neurites.**

***Index Terms*—Hippocampal pyramidal neuron, microcontact printing, microelectrode array, neuron networks.**

I. Introduction

THE MAMMALIAN brain is a complex structure whose massive parallelism, adaptability, and superior pattern recognition capabilities have drawn great interest from neuroscientists and computer engineers. Specifically, the hippocampus has been a region of interest to researchers due to its implication in learning and memory [1]. To investigate the mechanisms of computation in *in vivo* neuronal networks, electrical activity needs to be monitored not only at multiple cells within a cell network, but also at multiple locations along single cells. In this way, both individual cells and cell networks can be scrutinized in order to understand how changes in single-unit activity shapes network operation and development. Single cell, multisite studies with conventional micropipette electrodes have provided considerable insight into computation in this respect, however micropipette recordings have serious drawbacks in terms of their invasiveness and their use in monitoring large populations of cells. Microelectrode arrays (MEAs) have been used for more than two decades to stimulate cells and record extracellular electrical activity from electrogenic cell networks [2]–[9] and hippocampal slices [10]–[12]. Although extracellular recordings suffer from lower signal-to-noise ratios (SNRs), the minimal invasiveness allows for long-term recordings and the multimicroelectrode capabilities permits studies on large networks [13]. Early work in this area was devoid of chemical or topographical patterning for influencing cell network organization on the surface of the MEA, but more recently, labs have moved toward directed organization of cell networks on recording devices [3], [8], [14], [15]. For chemical patterning, investigators have used direct photolithography [16]–[20] as well as soft lithography techniques such as microcontact printing (μCP) [21]–[26].

A major obstacle in probing the role of network architecture on computational function is the difficulty in producing highly organized networks at the level of individual neurites and cell bodies. We have used two chemical patterning techniques to produce high resolution (minimum feature size on the order of 2 μm) neuronal cell networks on the surfaces of MEAs using poly-L-lysine (PLL) as a cell-attachment and neurite guidance cue. These methods have been previously described, but often using large cell-guidance features ($>$10 μm) where cells are not organized individually at the level of neurites. Previous work showing high-resolution (features $<$5 μm) patterning of cells was not coupled with extracellular recordings on MEAs [27]–[29]. Here, we present data showing spontaneous bursting activity, coincident activity on multiple microelectrodes, and a controlled extracellular recording from a 2-μm-wide bundle of haptotactically guided mammalian neurites.

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*C. D. James was with Cornell University, Ithaca, NY 14850 USA. He is now with Sandia National Laboratories, Albuquerque, NM 87185 USA (e-mail: cdjame@sandia.gov).

A. J. H. Spence was with Cornell University, Ithaca, NY 14850 USA. He is now with the University of California at Berkeley, Berkeley, CA 94720 USA.

N. M. Dowell-Mesfin, R. J. Hussain, K. L. Smith, W. Shain, and J. N. Turner are with the Wadsworth Center and School of Public Health, Albany, NY 12201 USA.

H. G. Craighead is with Cornell University, Ithaca, NY 14850 USA.

M. S. Isaacson was with Cornell University, Ithaca, NY 14850 USA. He is now with the University of California at Santa Cruz, Santa Cruz, CA 95064 USA.

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II. Materials and Methods

A. Microelectrode Array Fabrication

The MEAs were fabricated as depicted in Fig. 1. Three-inch diameter fused silica wafers (CVD Electronics) were subjected to a full RCA clean and deionized water rinse. After drying, an adhesion layer of titanium (5–10 nm) and then gold (250–500 nm) was evaporated onto the cleaned substrates. Gold traces were defined using a patterned photoresist mask. An argon ion mill was used to remove gold and titanium from the unmasked regions of the wafer. After removing the resist, substrates were subjected to a one-minute O_2 plasma treatment to prepare the surface for insulation deposition, which consisted of a 1-μm-thick plasma enhanced chemical vapor deposition oxide/nitride/oxide triple stack. The multilayered insulation

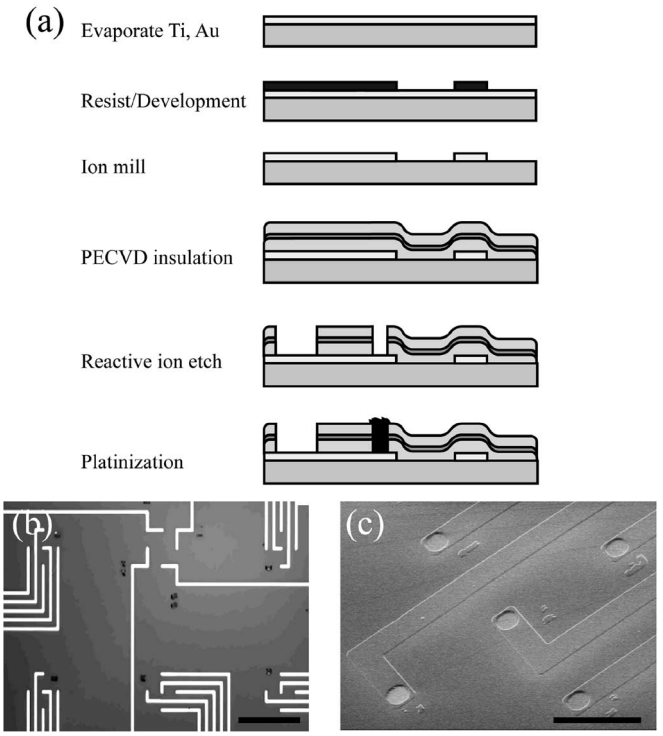

Fig. 1. (a) MEA fabrication process and (b) optical micrograph of six sets of microelectrodes on the device. Scale bar = 200 μm. (c) SEM micrograph of a set of microelectrodes. Scale bar = 25 μm.

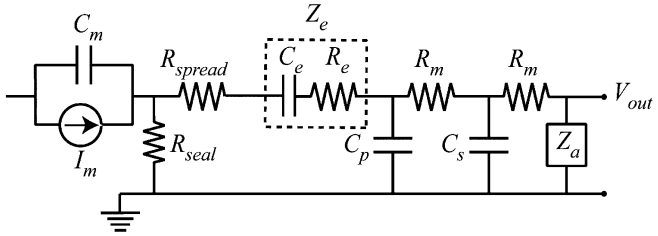

Fig. 2. Circuit diagram depicting important parameters for extracellular recording: I_m, membrane current from the cell; C_m, membrane capacitance; $R_{\rm spread}$, spreading resistance; $R_{\rm seal}$, sealing resistance; Z_e, microelectrode impedance; R_m, resistance of microelectrode traces; Z_a, amplifier input impedance; $V_{\rm out}$, detected signal. Capacitive losses are C_p, capacitance through the insulation, and C_s, shunt capacitance in cabling.

suffered less delamination than a single 1-μm-layer of nitride due to complementary compressive and tensile stresses in the layers. Contacts to both ends of the gold traces were made using a photoresist mask and reactive ion etches in CF_4 (nitride) and CHF_3/O_2 (oxide). The resulting MEA consisted of 124 individual microelectrode traces, grouped into 24 sets of five electrodes, and one set of four electrodes [Fig. 1(b)].

B. Circuit Model and Microelectrode Characterization

Fig. 2 shows the relevant circuit model parameters for extracellular recording [30], [31]. The primary unknown in the model, the membrane current (I_m), can be estimated using formulations found elsewhere [32], [33]. Platinum black was electrodeposited onto microelectrodes (LabChem Inc. #LC18680-7) to reduce their interfacial impedance, Z_e [34]. A platinizing current of 4–5 nA/μm^2 DC for 30–90 s was used. Before and after platinization, Z_e was measured over the range of 100 to

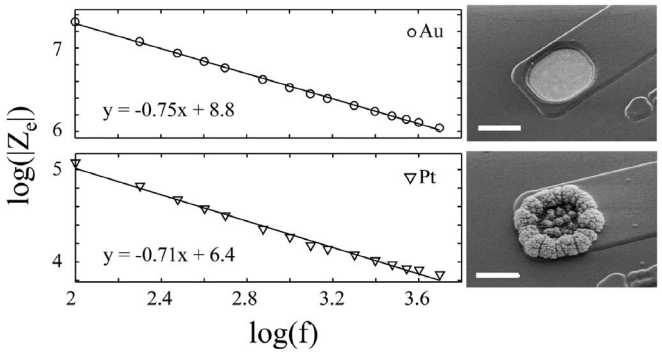

Fig. 3. The average impedance magnitudes and linear fits for gold $(n = 12, r^2 = 0.947)$ and platinized microelectrodes $(n = 9, r^2 = 0.870)$ as a function of frequency (0.1–5 kHz). Images on the right are SEM micrographs of a gold recording site and a platinized site. Scale bars = 5 μm.

5000 Hz with a lock-in amplifier and fit to a power law [Fig. 3]. The impedance of the metal microelectrode/electrolyte interface is given by $Z_e \propto f^m$, with typical m values between -0.4 and -0.9 [35]. The value of m fluctuated between -0.6 and -0.8 for platinized microelectrodes, depending on the amount of electrodeposited platinum. Microelectrode impedance influences the background Johnson noise according to [36]

$$N_{\rm rms} = \sqrt{\int_{f_1}^{f_2} [\text{real}[Z_e] + R_{\rm spread}]\, df} \qquad (1)$$

where $\text{real}[Z_e] = R_0 f^m$, and $R_{\rm spread}$ represents the resistance of the extracellular solution between the microelectrode and the platinum reference electrode. The real component of Z_e for the bare gold microelectrodes was fit using a linear regression with the parameters $R_o = 1.4 \times 10^8$ and $m = -0.69$ $(n = 20, r^2 = 0.94)$. Platinized microelectrodes were fit with parameters $R_o = 1.1 \times 10^5$ and $m = -0.36$ $(n = 9, r^2 = 0.92)$. Including an estimated value of $R_{\rm spread}$ of 58 k Ω and 19 k Ω for gold microelectrodes and platinized microelectrodes, respectively, the estimated root-mean-square (rms) Johnson noise predictions are 8.1 μV (gold) and 1.4 μV (platinized). When cells are cultured on the MEA surface, an additional resistance representing the path to ground between the membrane and microelectrode (sealing resistance, $R_{\rm seal}$) needs to be added to the resistive components in (1) (Fig. 2).

C. Surface Patterning

1) μCP: After platinization, MEAs were subjected to a one minute plasma discharge (Harrick Scientific plasma cleaner/sterilizer) and soaked in Milli-Q water for 1–2 days with frequent changes. After drying the MEA in an oven at 60 °C, μCP was used to transfer PLL patterns onto MEAs. Masters [Fig. 4(a)] were fabricated as detailed elsewhere [23]. The poly(dimethylsiloxane) (PDMS) stamps were cured on optical flats to allow fixation to a microscope stage. Fluorescein isothiocyanate (FITC) labeled PLL (Sigma #P3069) was dissolved at 1 mg/ml in borate buffer, filter sterilized through 0.22-μm-syringe filters, and applied to plasma discharge-treated PDMS stamps for 10–30 min. Stamps were blown dry with a nitrogen stream. Using a custom-made alignment tool, PLL coated stamps were aligned to and brought into contact with the MEA surface for 5–10 min under a pressure of 100 g/cm^2 [25]. Stamped arrays were rinsed in

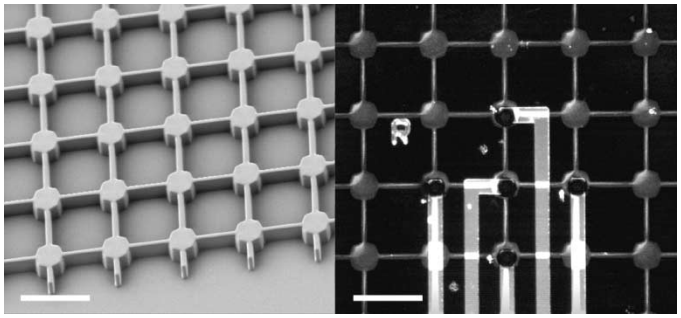

Fig. 4. (a) SEM micrograph of a PDMS stamp. (b) Pattern of FITC-labeled PLL produced by aligning the stamp's features to the recording sites. Scale bars = 50 μm.

buffer and Milli-Q water, and then dried with a dry nitrogen stream [Fig. 4(b)].

2) Lift-Off: Substrates were also patterned with PLL using a photoresist-liftoff method [3], [20]. MEAs were patterned with photoresist and exposed to an oxygen plasma for one minute to improve PLL adsorption to resist-free areas of the MEA. The arrays were then incubated in a 1.0-mg/ml solution of PLL overnight at room temperature. Substrates were then sonicated in acetone for 30 min, and a final application of a cotton-swab doused with acetone helped to remove remaining photoresist residue. In this process, a large percentage $(\sim 60\%)$ of the deposited platinum on microelectrodes is removed, increasing the microelectrode impedance, but cell network fidelity to the PLL pattern was compromised when this residue was not removed. Modifications to the photoresist protocol (manufacturer, prebake, and post-bake) are being explored to eliminate the need for mechanical removal of resist residue. Finally, arrays were rinsed in isopropanol and soaked in deionized water with several changes for 1–3 days.

D. Cell Culturing

Primary cultures of hippocampal neurons were prepared from prenatal Sprague Dawley rats (18 days gestation) using previously described methods [37]. Briefly, embryos were recovered by c-sections under CO_2 anesthesia. Individual neurons were isolated by treating the dissected hippocampi with trypsin and trituration in HEPES-buffered calcium-magnesium free Hank's Balanced Salt solution (pH 7.3). MEA surfaces were patterned with PLL by μCP or lift-off as described previously. A culture chamber was made by drilling a 25 mm hole in a 60 mm tissue culture dish. The chamber was placed such that the recording sites were enclosed while excluding the wire tabs near the periphery of the wafer to allow for connection to external electronics. Culture chambers were attached to the MEA surface with a thin layer of PDMS and cured in an oven at 60 °C for 15–20 min. The culture chamber was sterilized by an overnight incubation in 0.1% gentamycin in Hank's buffer and rinsed twice with Hank's buffer before plating the cells. The neurons were plated at a density of $1.00–1.50 \times 10^5$ cells per well, in minimal essential media supplemented with 10% horse serum and 0.1% pyruvate. Horse serum containing neuronal plating media was replaced with serum-free (N2.1) or neurobasal media at least two hours after the initial plating. Two different methods were used for long-term maintenance of cultures. Co-cultures were created using a coverslip containing an astrocytic monolayer inverted above the neuronal culture in N2.1 media [37]. This method is specifically designed for low plating densities of cells, however we encountered sporadic success ($<10\%$ of MEAs would contain healthy patterned cell networks at day 14 *in vitro*) and so a second culture method was pursued. In the second method, neuronal plating media was replaced with serum-free neurobasal media, supplemented with B27 (Gibco) and 0.5 mM L-glutamine (Fluka Biochemika) [38]. Cultures were maintained at 37 °C, 5% CO_2 for more than two weeks with feedings twice a week by replacing half of the media with fresh media. Cultures were examined periodically throughout the first weeks of the culture to qualitatively monitor the fidelity of growth to the PLL pattern. The astrocyte-free method yielded patterned cell networks that survived to day 14 *in vitro* $\sim 25\%$ of the time. An extensive quantitative study of the electrical activity and long-term fidelity of low density patterned neurons on MEAs has not been conducted.

Qualitatively, the lift-off technique tended to produce healthy patterned cultures that survived for >2 weeks more consistently than μCP. Atomic force microscopy studies showed a substantial difference in the thickness of the PLL layer transferred to the substrate using μCP (0.55 nm) and the lift-off method (1.2 nm) [data not shown]. This disparity could account for differences in cell-culture success for the two techniques.

E. Electrophysiology

Electrophysiology experiments were performed after day 14 *in vitro*. A research light microscope with a custom-made resistively heated stage served as the foundation of the recording apparatus. Gravity perfusion of recording media (HEPES, held at 35 °C–37 °C) provided gradual replacement of the culture medium on the MEA and was continued throughout recording. The reference electrode for extracellular recording was a silver/silver chloride wire placed in the culture chamber. Typical noise levels were 10–20 μV pp, stemming from Johnson noise, amplifier noise, and biological noise in the form of membrane currents. Extracellular spikes were typically between 50–250 μV pp.

The signal from the recording sites was sampled (10 kHz), amplified (10 000X) and band-pass filtered (0.3–5 kHz, 40 dB/decade falloff) using A & M Systems Model 1700 and 1800 amplifiers. Between one and five microelectrodes (channels) were recorded from simultaneously. Microelectrode impedance was monitored during recordings with a 0.1 μA, 1 kHz sine wave generated by the Model 1700 amplifier. Typical *in vitro* impedances of recording sites $(|Z_e| + R_{\text{seal}} + R_{\text{spread}})$ in cell culture were from 0.3–1.0 M Ω. One of the primary losses in our setup is the capacitive coupling, C_p, between microelectrode traces and the conducting culture medium [Fig. 3(a)]. We measured C_p to be ~ 2 pF/mm, with a typical setup yielding a total $C_p = 60–100$ pF. The amplified signal was sent to an oscilloscope and a personal computer for digitization (National Instruments NI-DAQ PCI-MIO 16E, 20 kHz), using a custom scripted user interface (Mathworks' MATLAB software). Phase-contrast images were taken from an inverted microscope with a digital camera. *In vitro* images were taken using dunking objectives on the microscope with a high-sensitivity CCD camera. Neuronal viability was tested immediately after recording with Rhodamine 123 (Molecular Probes), a dye that is internalized by active mitochondria.

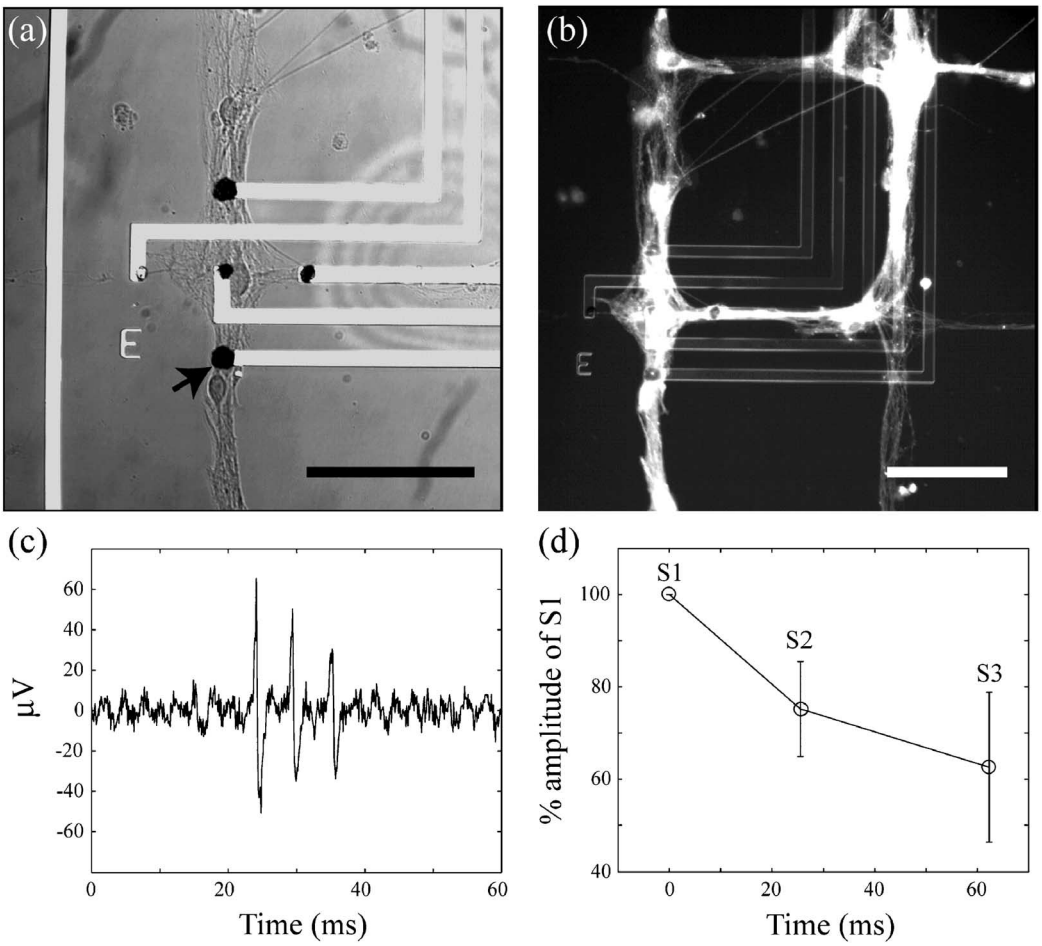

Fig. 5. (a) Phase contrast micrograph of a cell network on a MEA. Scale bar = 100 μm. (b) Fluorescence micrograph of the same network (Rhodamine 123). Scale bar = 100 μm. (c) Spontaneous activity recorded from the microelectrode indicated by the arrow in (a). (d) Average attenuation of spike amplitude from the first spike (S1) to the second (S2) and third spike (S3) during 14 spontaneous spike bursts.

Extracellular recordings necessitate the use of spike-sorting algorithms to identify single cells within spike trains. We operate under the assumption that each cell-electrode coupling event is relatively unique, and will give rise to a unique waveform. By statistically examining the waveforms of a catalog of extracellular spikes, we can then assign instances of similar spikes to a single cell-electrode coupling event. Instances of coincident activity on multiple electrodes can then be ascribed to either multiple cells with a common input firing simultaneously, or a single cell coupled to several electrodes. Spikes were detected offline using a window discriminator algorithm. We used a combination of manual and K-means clustering to group spikes into clusters representing putative single-units [39]. The multichannel spikes were extracted, and a principle components analysis was carried out on the catalog of spikes. A scatter-plot of the projection of each spike onto the first two principle components was used to identify clusters. A spike near the center of each cluster was selected, followed by a second round of K-means clustering. A final manual clustering was used to correct obvious misclassifications and remove outliers.

F. Immunocytochemistry

After electrophysiological recordings, neurons were fixed with 4% paraformaldehyde at 37 °C for 10 min and processed for immunocytochemistry using standard procedures [40]. Samples were permeabilized with 0.2% triton in HEPES Hank's buffer for 10 min at room temperature (RT) and incubated in 6% bovine serum albumin (30 min at RT) to block nonspecific staining, and incubated in primary (60 min at 37 °C) and secondary (40 min at 37 °C) antibodies in

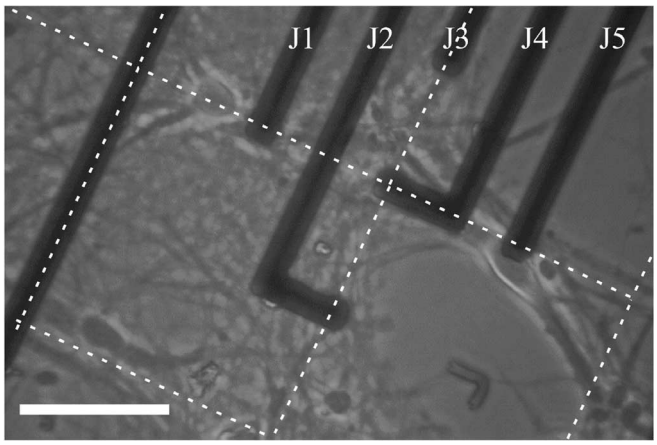

Fig. 6. Phase contrast micrograph of neurons on a MEA. The dashed grid indicates the central position of the PLL grid (25-μm-wide lines and 75-μm-wide spaces). Scale bar = 50 μm.

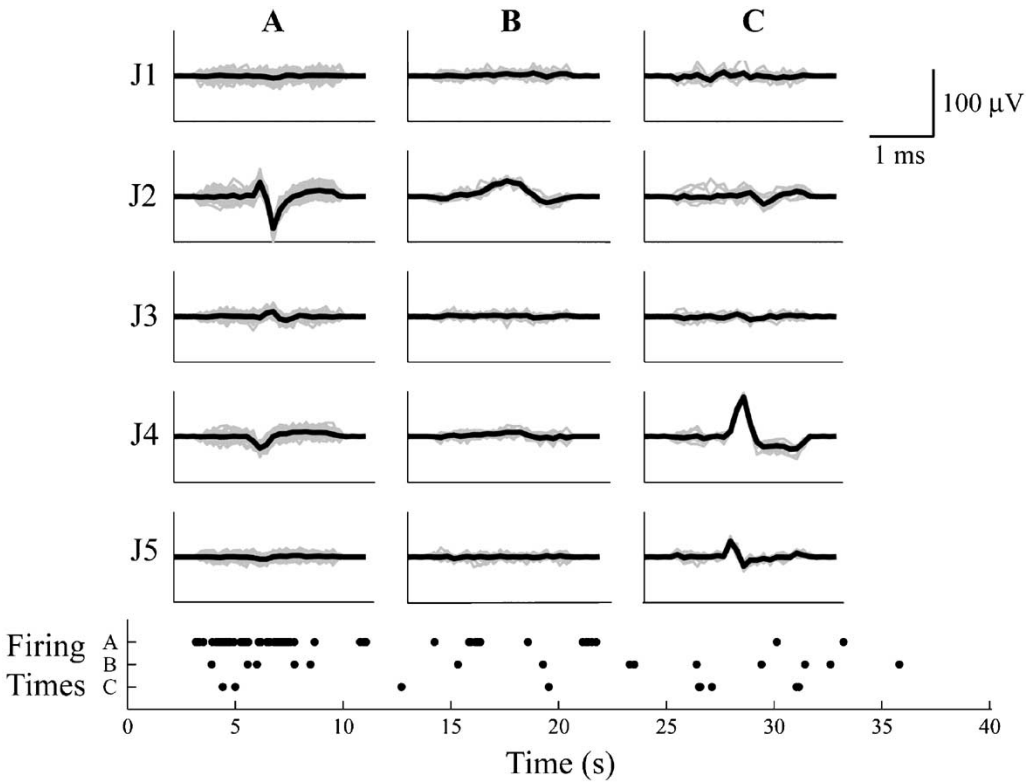

Fig. 7. Multichannel spikes grouped into three clusters: A (57 spikes), B (14 spikes), and C (9 spikes). Individual spikes (gray) are superposed and plotted in the assigned cluster on each channel with the average waveform (black). A raster plot of the firing times of each cluster is shown at the bottom.

Hank's buffered saline. After final washing, the samples were mounted under a coverslip using mounting media (1:1 HEPES Hanks/glycerol containing n-propyl gallate). Neuronal processes were labeled using a polyclonal antibody against a neuron-specific tubulin subunit, β III tubulin (BabCo/Covance; 1:10 000). Secondary antibodies were conjugated to Texas Red (Molecular Probes; 1:200). Fluorescent images were obtained using a Noran confocal scanning laser system on an inverted Olympus microscope. All data are presented as through focus projections.

G. MEA Reconditioning

After use, culture chambers were removed from the MEAs and discarded. MEAs were then cleaned with household bleach for 30 min and a subsequent water rinse for 1–3 days [9]. If this treatment proved insufficient, a 30-minute Nanostrip (a stabilized formulation of sulfuric acid and hydrogen peroxide) treatment was used.

III. Results and Analysis

A. Bursting Activity With Amplitude Attenuation

The MEAs shown in Fig. 5(a) were stamped with a PLL pattern with large feature sizes (20-μm-wide lines spaced by 200 μm), and the co-culture method was used for growing the neurons. There were 14 instances of spike bursts during a recording session taken after 15 days in culture (56 total spikes in 90 s of recording). This particular network exhibited several spike trains in which the amplitude of each spike in the train progressively attenuated [Fig. 5(c)]. The average amplitude attenuation in these spike trains was $25 \pm 10\%$ from the first spike to the second spike, and $38 \pm 16\%$ from the first spike to the third spike [Fig. 5(d)]. The time interval between the first and second spike (25 ± 25 ms) and the first and third spike (62 ± 63 ms) was averaged from the 14 cases. Further work is needed to examine this phenomenon in our cultures, but differences in ion channel distribution and inactivation within cells are the main culprits [41], [42].

B. Coincident Activity at Multiple Microelectrodes

Fig. 6 shows another cell network at day 16 *in vitro*. The PLL pattern in this experiment consisted of a lift-off patterned grid of 25-μm-wide lines with 100-μm spacing. This network was cultured using the astrocyte-free method. Cell bodies attached to and remained on the pattern, while neurites were less confined and bridged the 100-μm spaces after several days. A low level of spiking activity was observed after application of KCL to the culture medium and the clustering results are presented in Fig. 7. The background noise on microelectrodes J2, J4, and J5 was 7.2, 5.5, and 4.6 μV rms, respectively. Three types of spike sets were detected, and the cluster firing times are depicted at the bottom of Fig. 7. In certain instances, spikes were detected simultaneously (within 1–3 samples of each other) on multiple microelectrodes. For calculating time delays between coincident spikes, waveforms were interpolated to a sampling rate of 100 kHz. The amplitude of the spikes on J2 and J4 in cluster A was 66.3 ± 9.0 μV and 31.8 ± 4.6 μV pp, respectively. Spikes detected in this cluster were biphasic and monophasic (Fig. 7). The prominent peaks in the negative phases were used to calculate the time delay between microelectrodes J4 and J2 (217 ± 57 μs). The amplitude of the spikes on J4 and J5 in cluster C was 79.2 ± 7.8 μV pp and 38.4 ± 8.5 μV, respectively. These

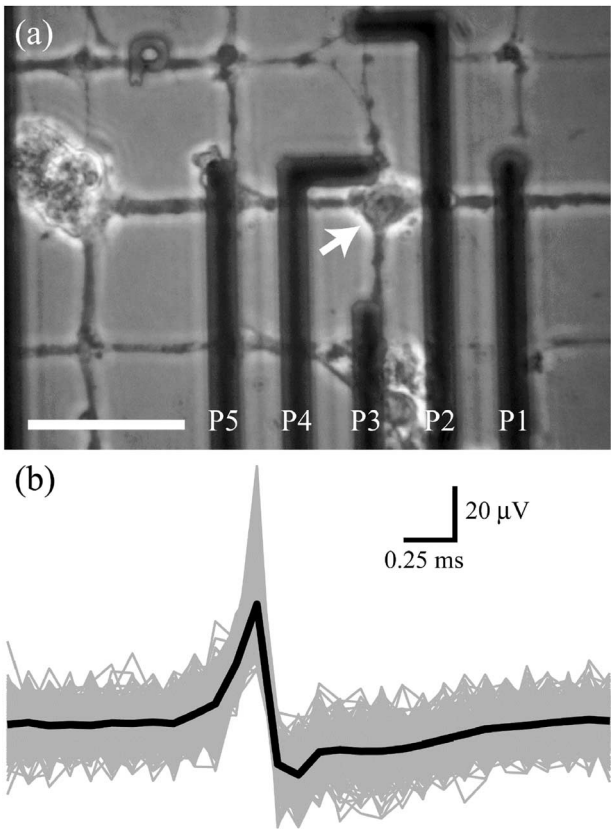

Fig. 8. (a) Phase contrast micrograph of cells on a MEA patterned with a PLL grid. The arrow indicates a putative cell body. Scale bar = 50 μm. (b) Individual spikes (gray, n = 380) and the average spike (black) detected by microelectrode P3.

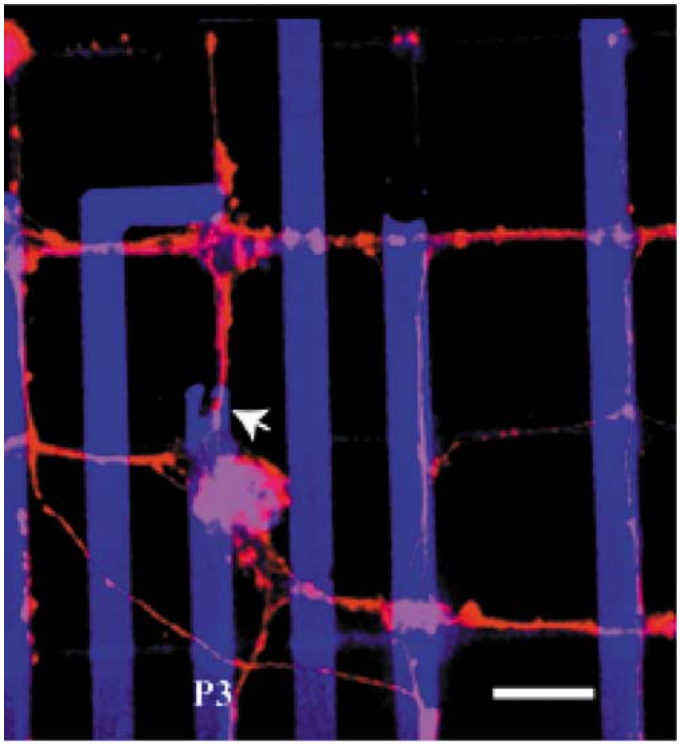

Fig. 9. Fluorescence micrograph of the cell network in Fig. 8. The white arrow indicates the bundle of neurites coupled to P3. Signals shown are the PLL and MEA traces (blue) and β-III tubulin (red). Scale bar = 25 μm.

spikes were all biphasic, and the prominent peaks in the positive phase were used to calculate the time delay from J4 to J5 (172 ± 25 μs).

C. Recording From a Guided Bundle of Neurites

Fig. 8(a) shows a 16 day-old culture in which spontaneous activity was detected at microelectrode P3 (380 spikes in 6 min). This MEA was stamped with a high-resolution PLL pattern (2-μm lines, 50-μm spaces, 15-μm nodes) and cultured in the absence of astrocytes. Upon visual inspection, the culture exhibited signs of degeneration such as neurite beading. The spike catalog and the mean spike are shown in Fig. 8(b). Microelectrodes P1 and P2 displayed no spike activity, while P4 and P5 were nonfunctional. The lack of activity on microelectrodes P1 and P2 supports our contention that the active element is within the structures directly coupled to P3, since activity from the cell body indicated in Fig. 8 would also likely be detected by P1 or P2 due to the similar distance and environment. The cluster of cells located under P3 in Fig. 8(a) were ruled out as a source of the detected activity due to previous experiments that showed such cell clusters as unhealthy and inactive. The average amplitude of the spikes on P3 was 70.0 ± 17 μV pp with a background noise of 7.0 μV rms. This culture was stained with β III tubulin to indicate the location of neuronal cellular structures (Fig. 9).

IV. Conclusion/Discussion

This paper illustrates the potential applications of chemical patterning techniques in constructing and monitoring neuronal cell networks. μCP can be used to quickly and efficiently transfer molecules to substrates, and while the lift-off technique is more time-consuming, it yielded healthier cell cultures more consistently. For long-term studies on designed cell networks, maintaining the health of the culture is one of the primary difficulties. The high-resolution PLL guidance cues used in Fig. 8 likely reduce the number of cell-cell contacts by artificially reducing cell-density and confining neurite outgrowth to small areas of the surface. This could compromise the health of the cells, as it is commonly known that these neurons prefer higher densities. Also, the co-culture and astrocyte-free culture methods need to be examined in light of the different success rates of long-term patterned networks.

The fidelity of cell networks to the PLL pattern varied with the patterning technique and the pattern geometry. Cell bodies conformed well to the patterns produced with both μCP and the lift-off method: $\sim$85% of cell bodies attach to the PLL pattern, while neurites tended to stray from the guidance cues after 4–7 days *in vitro*. Guidance of cell body attachment was successful for various PLL geometries, but the guidance of neurites varied significantly with the dimensions of the pattern. PLL grids with large duty-cycles (large ratio of PLL line-width to grid spacing) were easily traversed by neurites, while smaller duty-cycle grids achieved better guidance. For the network shown in Fig. 5(a), the duty-cycle was $\sim$10%. This confined neurites well and produced electrically active networks, but the pattern features were too large for organizing individual neurites and cell bodies. The pattern used in Fig. 6 had a duty-cycle

of 25% and demonstrated the poorest neurite guidance. The highest-resolution PLL pattern (4% duty cycle) shown in this work (Fig. 8) produced the best cell guidance at the level of individual cell bodies and neurites, and specifically guided an electrically active bundle of neurites to a microelectrode.

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Conrad D. James was born in Columbus, OH, in 1974. He received the B.S. degree in electrical engineering, summa cum laude, from the University of Notre Dame, in 1996. He received the M.S. and Ph.D. degrees in applied and engineering physics from Cornell University, Ithaca, NY, in 1999 and 2002, respectively.

Since 2002, he has been a Senior Member of Technical Staff at Sandia National Laboratories in Albuquerque, NM. His research interests include MEMS and microfluidic devices for biological and electrochemical detection applications.

Andrew J. H. Spence was born in Oxford, U.K., in 1975. He received the B.S. degree in physics from the University of California at Berkeley, Berkeley, CA, in 1997, and the M.S. and Ph.D. degrees in applied and engineering physics from Cornell University, Ithaca, NY, in 2000 and 2003, respectively. He is currently a Postdoctoral Associate at the University of California at Berkeley.

His research interests are in the areas of microfabricated devices for neural recording, neural ensemble coding, signal processing, and electronic design.

Natalie M. Dowell-Mesfin received the B.S. degree in biological sciences from the State University of New York (SUNY), Old Westbury, in 1995 and the M.S. degree at SUNY, Albany, in biomedical sciences in 2003. She is currently a doctoral candidate at the School of Public Health/ State University of New York at Albany at the Wadsworth Center.

Her interests include the investigation of the independent effects of topographical signals on neuron growth and differentiation.

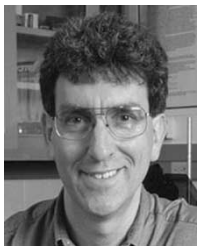
Rifat J. Hussain was born in 1959 in Pakistan. She studied chemistry and received the M.Sc. and M.Phil degrees Quaid-e-Azam University, Islamabad, Pakistan, in 1986 She received the Ph.D. degree in environmental health and toxicology with emphasis on neurophysiology from the State University of New York at Albany, Albany, NY, in 1998. From 1999 to 2000, she worked as a postdoctoral fellow at the University of Pennsylvania, Philadelphia.

Since 2000, she has worked at the Nanobiotechnology Center at Wadsworth Center, Albany, NY, as a Research Affiliate. Her research interests are in establishing *in vitro* neuronal networks on biochemically modified surfaces and characterizing them functionally with electrophysiology and morphologically with immunocytochemistry techniques.

Karen L. Smith was born in Albany, NY. She received the M.S. degree in biology from the New York State University, Albany, in 1998.

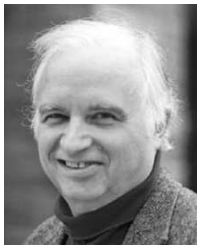
She is currently a Research Scientist at the New York State Department of Health -Wadsworth Center. For over 18 years her work and research interest has been focused on using *in vitro* electrophysiology to study epilepsy. More recently, her research has taken a turn toward the field of nanobiotechnology. The focus of this research is to study the biological reactive responses following neural prosthesis insertion.

In 2000, Ms. Smith received a young investigators award from the American Epilepsy Society. She is currently the president of the Hudson-Berkshire Society of Neuroscience.



Harold G. Craighead (M'01) was born in Elizabethtown, PA, in 1952. He received B.S. degree in physics, with High Honors, from the University of Maryland, College Park, in 1974. He received the Ph.D. degree in physics from Cornell University, Ithaca, NY, in 1980.

He joined the faculty of Cornell University as a Professor in the School of Applied and Engineering Physics in 1989. From 1989 until 1995, he was Director of the National Nanofabrication Facility at Cornell University. He was Director of the School of Applied and Engineering Physics from 1998 to 2000 and Director of the Nanobiotechnology Center from 2000 to 2001. He served as Interim Dean of the College of Engineering from 2001 to 2002. In July 2002, he returned to the Nanobiotechnology Center as Co-Director for Research. His research interests include the use of nanofabrication for biological applications. He is also involved in projects on new molecular species for microfabrication, cell growth and molecular manipulation.



Michael S. Isaacson (M'03) received the B.S. degree in physics from the University of Illinois, Urbana Champaign, in 1965, and the M.S. and Ph.D. degrees from the University of Chicago, Chicago, in 1966 and 1971, respectively.

He joined the faculty at Cornell University, Ithaca, NY, in 1979 after serving as a staff scientist at the Brookhaven National Laboratory and a faculty member in the Department of Physics and the Enrico Fermi Institute at the University of Chicago.

Dr. Isaacson was a Hertz Foundation fellow and an Alfred P. Sloan faculty fellow; he was awarded the Burton Medal by the (Electron) Microscopy Society of America, received an Alexander von Humboldt Foundation Senior Scientist Award, and in 1993 was named a fellow of the American Association for the Advancement of Science. He was elected physical sciences director of the (Electron) Miocroscopy Society of America from 1986 to 1988, and president of the Microscopy Society of America in 1993. He has been an associate editor of *Ultramicroscopy* since 1975, and has served on the editorial boards of the *Review of Scientific Instruments, Journal de Microscopie et Spectroscopy Electronique, Journal of Electron Microscopy Research and Techniques, Nanostructured Materials*, and *Scanning.* At Cornell he is a member of the executive committees of the National Nanofabrication Facility, the Materials Science Center, the Cornell National Supercomputer Facility, and the Cornell High Energy Synchrotron Source (CHESS). His research interests include microscopic electron optics, near-field optics, and nanofabrication and biology.

William Shain received the B.A. degree in biology from Amherst College, Amherst, NH, in 1966, and the Ph.D. degree in developmental biology in 1972 from Temple University, Philadelphia, PA.

After completing postdoctoral training as a public health fellow in neuroscience at the National Institutes of Health, Bethesda, MD, he moved to the Division of Cellular Neurobiology at the Armed Forces Radiobiology Research Institute, Bethesda, MD. He currently serves as a Research Scientist at the Wadsworth Center in the New York State Department of Health, Albany, and as an Associate Professor in the Departments of Biomedical Sciences and Environmental Health and Toxicology at the University of Albany. His current interests include the use of nanofabrication techniques to study the role of astrocytes and microglia during normal brain function and in response to disease and injury.

James N. Turner was born in Binghamton, NY, in 1944. He received the B.S. degree in engineering in 1968 and the Ph.D. in biophysics in 1973 from the State University of New York (SUNY), Buffalo.

Dr. Turner has been employed as a National Institutes of Health (NIH) Postdoctoral Fellow at the Roswell Park Cancer Institute, Isaac Gordon Center for Gastrointestinal Diseases, Rochester, NY, and the Wadsworth Center, Albany, NY. The latter is New York State's Public Health Laboratory where he directs the Three-Dimensional Light Microscopy Facility and the NanoBioTechnology Program. He is a Professor of Biomedical Sciences, SUNY, Albany, and Biomedical Engineering at Rensselaer Polytechnic Institute. He is a founding faculty of NSF's Science and Technology Center in Nanobiotechnology (NBTC), and is one of the NBTC's Platform Leaders and a member of its Executive Committee. He has co-authored over 100 technical publications, and serves as Special Sections Editor of *Microscopy and Microanalysis* the *Journal of the Microscopy Society of America*. His research interests include the development and application of nanobiotechnology, and the interface between biology and nanotechnology.