

An ultra small array of electrodes for stimulating multiple inputs into a single neuron

Spencer L. Smith^{a,b,*}, Jack W. Judy^c, Thomas S. Otis^b

^a Neuroscience and Neuroengineering Programs, 1320 Gonda Center, 695 Young Drive South, University of California, Los Angeles, CA 90095, USA

^b Department of Neurobiology, School of Medicine, University of California, Los Angeles, CA 90095, USA

^c Department of Electrical Engineering, School of Engineering and Applied Sciences, University of California, Los Angeles, CA 90095, USA

Received 7 July 2003; received in revised form 27 September 2003; accepted 3 October 2003

Abstract

We have developed an ultra small, translucent array of electrodes for use in the parasagittal cerebellar slice preparation. This positionable array is capable of stimulating multiple independent bundles of parallel fibers (PFs), which synapse onto a single Purkinje neuron.

On a silicon substrate, a low-stress silicon nitride film was used both as a structural layer and as electrical insulation. Evaporated gold pads and interconnects were sandwiched between two such layers. A bulk anisotropic silicon etch released the individual arrays. The electrodes are supported within a 2- μm -thick cantilever of translucent silicon nitride. In one design, eight 4- μm -wide square electrodes are arranged on 8- μm -centers. Another design, half the scale of the first, was also tested.

The array was mounted on a micromanipulator and can be visualized by an upright microscope. It can then be positioned in the dendritic arbor of a Purkinje neuron while not disturbing a recording pipette at the soma. Paired-pulse facilitation experiments have confirmed that the electrodes are capable of stimulating non-overlapping bundles of PFs.

This device will be useful for exploring spatiotemporal synaptic integration in single neurons. Potential applications in experiments on cerebellar LTD are also discussed.

© 2003 Elsevier B.V. All rights reserved.

1. Introduction

To understand how networks of neurons in the brain perform computations, it is necessary to precisely measure how individual neurons integrate their synaptic inputs. To explore this integration, it is instructive to record the behavior of a neuron in response to inputs which vary not only in temporal structure, but spatial structure as well (Heck et al., 2003; Magee, 2000; Williams and Stuart, 2003).

Tight seal, whole-cell recordings of neurons provide the best method available to observe the electrical activity of individual neurons. This high-resolution technique is difficult to carry out in vivo, but is quite easily employed in the brain slice preparation, which preserves the local circuitry. Another advantage of brain slices is the ability to tightly control the synaptic input to an individual neuron. Most inputs are silent in the slice but many are still functional upon

electrical stimulation. Thus, the experimenter is able to render a tightly controlled stimulus and record the electrical response of the neuron with high fidelity.

In parasagittal slices of cerebellum, there is an added benefit from the stereotyped architecture of the cerebellar cortex. Purkinje neurons, the output cells of this structure, have a unique two-dimensional dendritic arbor that lies in the plane of the brain slice. Over 100,000 parallel fibers (PFs) lie in bundles perpendicular to this plane; these axons form synapses on the Purkinje neurons. Portions of this extensive set of inputs can be electrically stimulated to activate these PF synapses.

Methods of stimulating neural inputs in brain slices have not changed significantly since the preparation was first used. Metal or electrolyte-filled glass electrodes are positioned with micromanipulators and current is passed through the tissue to be stimulated. In some cases, researchers have used multi-pronged metal electrodes (Heck, 1995; Tominaga et al., 2001) or petridish-style multielectrode arrays (Borkholder et al., 1997; Heuschkel et al., 2002; Novak and Wheeler, 1988; Oka et al., 1999; van Bergen

* Corresponding author.

E-mail address: lavere@ucla.edu (S.L. Smith).

et al., 2003) to stimulate neural fibers. However, limitations on electrode densities have restricted the study of neighboring synapses on an individual neuron.

The technique of focal photolysis of caged glutamate offers a high degree of control over the spatiotemporal characteristics of stimuli to an individual neuron (Wang and Augustine, 1995). Although it is an invaluable tool for exploring many important questions about the spatial extent of glutamate action, it bypasses the presynaptic machinery at the synapse. It would be advantageous to have a means to stimulate several independent synaptic pathways onto a single neuron.

We have developed an ultra small array of electrodes (eight electrodes in a $30 \times 30 \mu\text{m}$ area) capable of stimulating multiple, spatially distinct bundles of PFs. Many of the individual PFs in these bundles make passing synaptic connections onto a single Purkinje neuron in a parasagittal slice of cerebellum. This array is translucent, allowing precise positioning in relationship to the recorded Purkinje neuron. It also has a very small footprint to avoid obstructing access to the soma for a recording pipette. This array will enable studies of how individual neurons integrate complex spatiotemporal patterns of stimuli. It will also facilitate detailed explorations of the synapse specificity and timing parameters of plasticity at this synapse.

2. Materials and methods

2.1. Design

The electrodes and bonding pads are contained on a 1 mm-wide and 6 mm-long rectangular backbone of silicon. On one end of the backbone, a $100 \mu\text{m}$ -long cantilever of translucent silicon nitride extends out and holds the array of gold–platinum electrodes. These electrodes are connected to the bonding pads at the opposite end of the device (Fig. 1).

Four variations of the same basic array design were tested (Fig. 2). The number of electrodes and the scale of the array varied among the designs. Four-micron-wide square electrodes were arranged on $8\text{-}\mu\text{m}$ -centers for a total of either four or eight electrodes. Two other designs, both with $2 \times 2 \mu\text{m}$ electrodes on $4\text{-}\mu\text{m}$ -centers, were also tested. These geometries were selected to maximize the density of electrodes in a small space. The offset grid arrangement used in the eight-electrode designs is convenient because it permits both a large number of evenly spaced pairs of electrodes as well as several different spacings.

The electrodes were designed to be on a thin silicon nitride cantilever, only large enough to contain the electrodes and the requisite wires. This minimized the footprint to allow unobstructed access to the cell soma. Silicon nitride is also a convenient material due to its excellent electrical properties as an insulator and its biocompatibility (Kristensen et al., 2001).

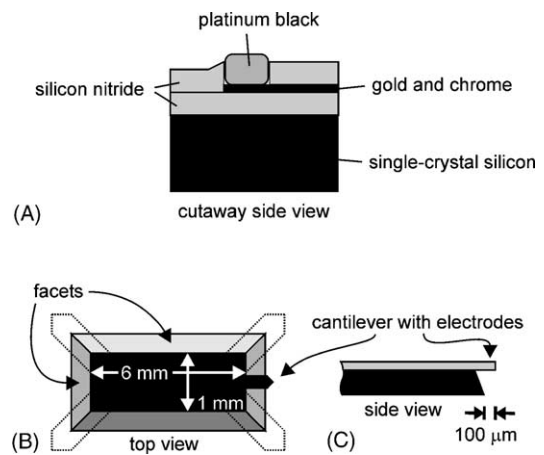


Fig. 1. (A) A cross-section view summarizing the surface-micromachining steps. The device is shown before the bulk silicon etch which will remove the silicon under the electrodes. (B) Shown with dotted outlines are the corner compensation structures. These are used for the bulk-etching step to preserve the convex corners of the device. Not to scale. (C) The nitride cantilever containing the array protrudes just enough to be seen from the backside when the device is inverted in the assembly.

2.2. Process

Conventional thin-films processing techniques were used to manufacture the microelectrode arrays. Bare, single-crystal silicon wafers were covered with $1 \mu\text{m}$ of a very low-stress ($<25 \text{ MPa}$) silicon nitride film using a plasma-enhanced chemical vapor deposition (PECVD) machine (Unaxis, St. Petersburg, FL). This was followed by an

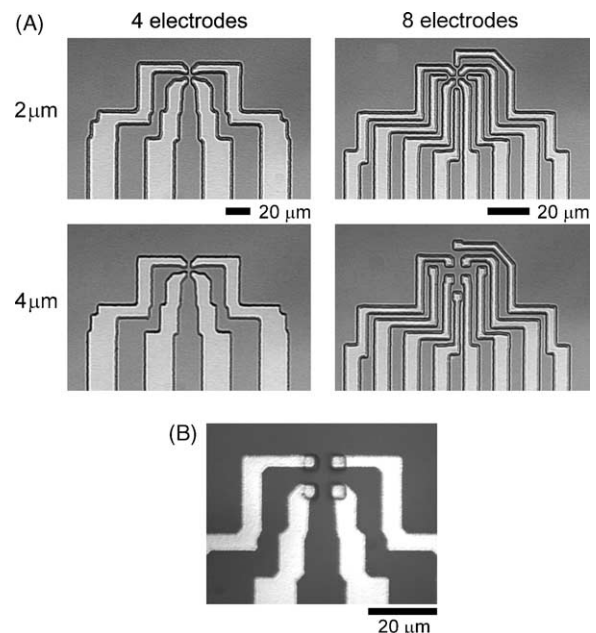


Fig. 2. (A) The four different microelectrode-array designs used. The top two designs feature $2\text{-}\mu\text{m}$ -wide square electrodes on $4 \mu\text{m}$ centers. The bottom two designs have $4\text{-}\mu\text{m}$ -wide square electrodes on $8 \mu\text{m}$ centers. (B) A close up of a microelectrode array after vias through the silicon nitride have been etched.

evaporation deposition of 5 nm of chromium, as an adhesion layer, and 200 nm of gold. After patterning the metal layer using photolithography and wet chemical etchants (TFA for gold and 1020 for chromium, both from Transene, Danvers, MA), a second 1- μm -thick film of silicon nitride was deposited. After a second photolithography step, a plasma etch was used to create vias through the silicon nitride to the electrodes and the bonding pads. This etch was also used to create the corner compensation structures (Fig. 1) after a third photolithography step. Finally, the arrays were released using a bulk anisotropic silicon etch with potassium hydroxide (KOH).

Key to the development of this microelectrode array was the refinement of a low-stress silicon nitride film. A conventional method for depositing such films, low-pressure chemical vapor deposition (LPCVD), is incompatible with low-melting temperature metals, such as gold, which is used in the present device. PECVD is an alternative, but must be optimized to yield a low-stress silicon nitride film.

It is possible to deposit silicon nitride films with either compressive or tensile stress by varying only the deposition pressure in a PECVD process. Bare silicon wafers were coated at several different pressures to find a zero-stress point (Fig. 3). All film stresses were measured with a Tencor Flexus stress measurement system (KLA-Tencor, San Jose,

CA). After optimizing pressure, the silane to ammonia ratio was adjusted to yield a film with an index of refraction of 2.00. Then a final fine-tuning of the deposition pressure was made. All the nitride films used in the present devices exhibited stresses less than ± 25 MPa. These films were highly uniform across the surface of the wafer, varying less than 3% in thickness from the center to the edge (Fig. 3). Several of the films exhibited stresses less than ± 10 MPa.

The corner compensation structures were necessary to preserve the convex corners of the device in the anisotropic KOH etch (Fig. 1). The four residual nitride cantilevers were removed prior to device assembly. Another consideration of the KOH etch is that it renders sides which slope out from the top surface of the silicon backbone. The fifth silicon nitride cantilever, on which the microelectrode array was mounted, protruded sufficiently to be visible from the backside of the device despite the sloping side.

2.3. Assembly

Since the electrodes are on the top surface of the device, it must be inverted for positioning on the top side of a brain slice. A custom PCB (Advanced Circuits, Aurora, CO) was designed to hold a 2 mm-style, eight-pin female connector (DigiKey, Thief River Falls, MN) on top and a small, surface mount chip package (Spectrum Semiconductor, San Jose, CA) on the bottom side (Fig. 4). The completed microelectrode arrays were glued to the chip packaging and an ultrasonic bonder was used to connect the device to the leads on the chip.

Epoxy was used to insulate the assembly and secure the gold wires connecting the device to the chip packaging. On the other side of the PCB, the connector was firmly plugged

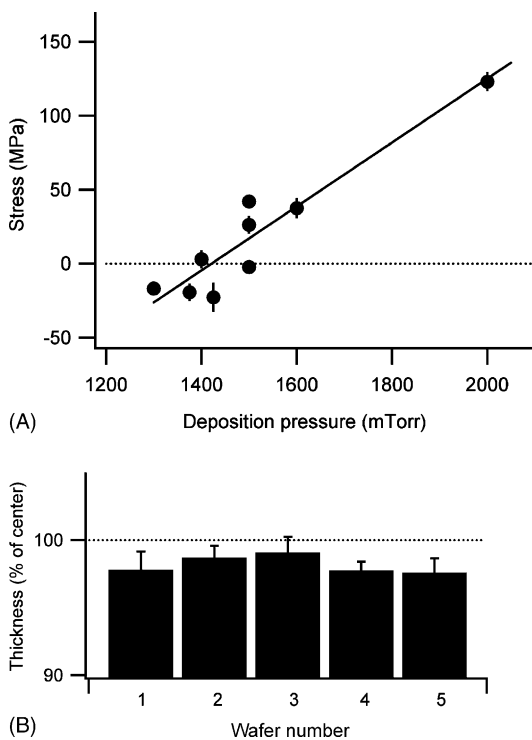


Fig. 3. (A) Stress measurements of the PECVD silicon nitride film as a function of pressure. Measurements were taken on five intersecting diameters. The error bars represent the standard deviation of these five measurements. Simple linear fit with slope of 0.21 MPa/mTorr. Zero crossing at 1450 mTorr. (B) Five films with less than ± 25 MPa stress were measured for their uniformity. The film thicknesses dropped less than 3% from the center of the wafer to the perimeter.

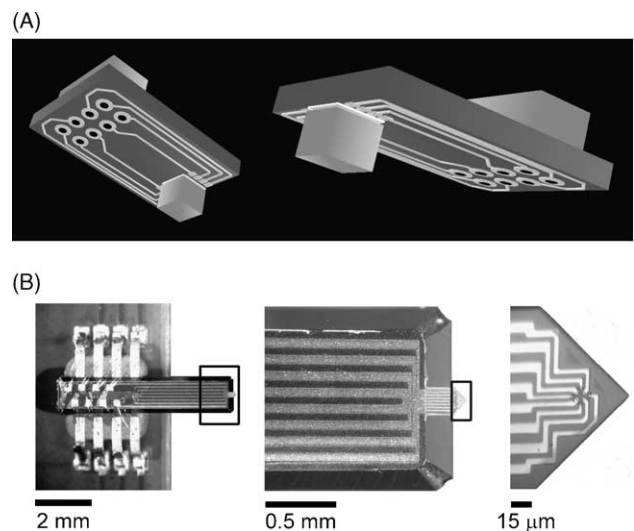


Fig. 4. (A) Custom PCB designed with through-holes for a 2 mm connector on one side and surface mount pads for the chip packaging on the other. Thicknesses are not to scale. (B) Progressive close-ups of the assembled device. In the middle panel, note how the array protrudes over the slanting side of the silicon backbone.

into a corresponding male connector and wiring harness that was rigidly attached to a standard, rod-style stimulus pipette holder (S.D. Designs, Grants Pass, OR). The mechanical engagement between the male and female connectors provided ample stability. The device was mounted horizontally, parallel to the brain slice surface, at a slight incline (between 5 and 10°). This angle insured that the electrode array contacted the slice before the opposite end of the device contacted either the slice or the bottom of the recording chamber. The difference in elevation across the electrode array was at most 5 μm . This value is less than the variation in thickness of the brain slices themselves ($\pm 20 \mu\text{m}$). The final assembly was positioned using a motorized micromanipulator (model MP-255, Sutter Instruments, Novato, CA).

A solution of 1% chloroplatinic acid, 0.01% lead acetate, and 0.0025% hydrochloric acid in water was used to platinize the gold electrodes (Gesteland, 1959). A voltage of -0.5 V was placed between the ground and the electrodes with a 100 k Ω resistor in series. With this configuration, platinization was complete in a few seconds resulting in electrode resistances of $100 \pm 50 \text{ k}\Omega$ for 4- μm -wide electrodes and $500 \pm 100 \text{ k}\Omega$ for 2- μm -wide electrodes.

2.4. Testing

Parasagittal slices of cerebellum were prepared as previously described from 2 to 3-week-old Sprague-Dawley rats (Smith and Otis, 2003). An upright microscope (Leica, Wetzlar, Germany) equipped with infrared DIC optics and a CCD camera (Hamamatsu, Hamamatsu City, Japan) was used to image the tissue. Glass microelectrodes (1.2–2.5 M Ω) filled with (in mM) 122 K-methylsulfonate, 5 KCl, 1 NaCl, 10 HEPES, 1 EGTA, 2 MgCl₂, 0.4 GTP, 2 ATP were used to obtain whole-cell recordings of Purkinje neurons. The cells were held at -75 mV in voltage clamp using a BVC-700A (Dagan Instruments, Minneapolis, MN) patch clamp amplifier. Stimuli had a 100–200 μs duration and an amplitude of 10–150 μA using IsoFlex constant current stimulus isolators (AMPI, Jerusalem, Israel). Data was acquired with an Axon DIGIDATA A/D converter and Axon pCLAMP software (Axon, Union City, CA). All experiments were performed at $31 \pm 0.2^\circ\text{C}$.

3. Results

To evaluate the performance of the microelectrode array, parasagittal slices of rat cerebellum were obtained. A whole-cell recording from a Purkinje neuron was established and the array was positioned in the molecular layer on the surface of the slice above the dendritic arbor of the recorded neuron.

In order to center the array under the objective, it was convenient to use the wider field of view afforded by the eyepieces of the microscope as opposed to the CCD camera

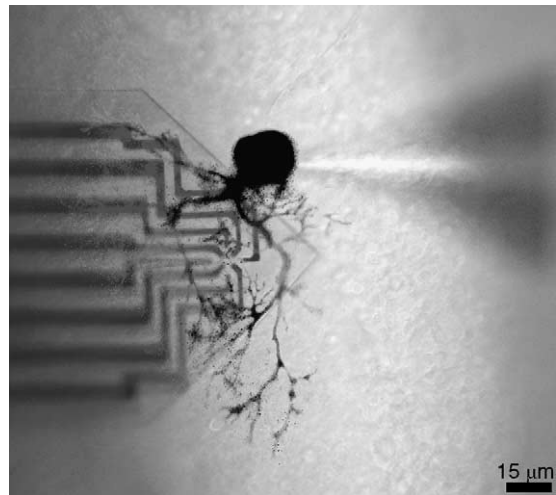


Fig. 5. The array in use. The pipette is seen approaching from the right and the array approaches from the left. The electrodes easily fit within the dendritic arbor of a Purkinje neuron of a 2-week-old rat. The silicon nitride cantilever does not obstruct access to the soma. The neuron has been filled with the dye Lucifer Yellow.

monitoring system. However, when positioning the array on a brain slice, the camera system was always used.

The translucence of the silicon nitride membrane permitted DIC imaging and facilitated positioning of the array in the dendritic arbor of the Purkinje neuron (Fig. 5). This also allowed for visual monitoring of the electrodes during stimulation. Stimulus parameters were kept in a range that avoided bubble formation on the electrode surfaces due to the hydrolysis of water.

All eight electrodes of the largest, 4- μm -wide electrode design fit well within the dendritic arbor of a single Purkinje neuron in a parasagittal slice of cerebellum from a 14-day-old rat (Fig. 5).

Both local bipolar stimuli and monopolar stimuli with a remote ground were used. The most reliable stimuli and fastest artifacts came from local bipolar stimuli. Stimulus artifacts from a 200 μs stimulus were fast and settled $>90\%$ in $<1.5 \text{ ms}$. When responses could be elicited, differences in performance between the four and eight electrode designs were not noticeable. However, while the designs with 4- μm electrodes generated stimuli with low trial-to-trial variability (Fig. 6C), those with 2- μm electrodes required high current ($>150 \mu\text{A}$) and failed to yield stable, reproducible responses over repeated trials.

A paired-pulse protocol was used to determine whether there was any overlap among the bundles of parallel fibers stimulated by adjacent electrodes. The parallel fiber to Purkinje neuron synapse is known to facilitate strongly. When a second activation of a PF synapse follows the first by 50 ms, the Purkinje neuron response to the second stimulus is facilitated $\sim 180\%$ (Atluri and Regehr, 1996). Therefore, pairs of stimuli in which each stimulus is delivered to a separate input should not exhibit facilitation as pairs delivered to the same input do.

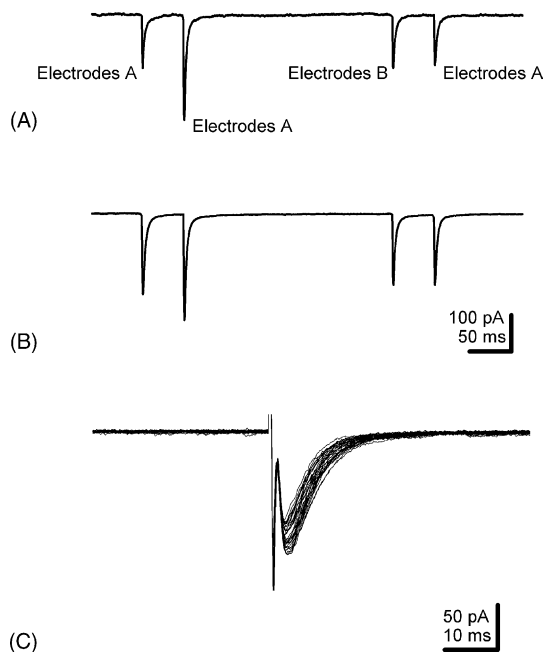


Fig. 6. (A and B) The first two EPSCs were evoked by bipolar stimuli from the same pair of electrodes (“Electrodes A”). Note the strong facilitation. The third EPSC was in response to a bipolar stimulus on a different pair of electrodes $8\text{ }\mu\text{m}$ away (“Electrodes B”). The fourth EPSC was evoked by the same pair of electrodes as the first two EPSCs. Note the absence of facilitation. Both an example trace (A), and the average of 50 sweeps (B), are shown. Stimulus artifacts have been blanked for clarity in panels (A) and (B). (C) Responses to stimuli were stable; 30 sweeps shown. Similar results were seen in two other cells.

Indeed, it was observed that the bundles of PFs stimulated by adjacent pairs of electrodes do not overlap significantly (Fig. 6A and B, $n = 7$ configurations over three cells showed independence). The electrode array could be repositioned several times in order to optimize stimulation results. Low stimulus intensities ($<50\text{ }\mu\text{A}$) were most likely to yield independent stimulation sites when pairs of electrodes were adjacent. The same stimulus amplitude was not necessarily used on each pair of electrodes. As expected, the more distant ($>8\text{ }\mu\text{m}$) pairs of electrodes were more likely to stimulate independent bundles of parallel fibers (2.7 distant independent stimulation sites per cell). However, adjacent ($8\text{ }\mu\text{m}$) pairs of electrodes could elicit independent responses as well (1.67 adjacent independent stimulation sites per cell).

After 4–6 h of use on brain slice tissue, it became necessary to clean the electrodes to assure reliable and efficient stimulation. The nitride cantilever is delicate and cannot be placed in an ultrasonic bath or coarsely handled. We found that scraping the array at an angle across the edge of a shard of glass glued to the recording chamber gave good results. This was done under observation with a microscope to assure the nitride membrane was not deflected too sharply. After cleaning, replatinization was performed if visual inspection revealed the loss of the platinum black layer from any of the electrodes.

4. Discussion

We have developed an ultra small array of electrodes for use in the brain slice preparation. This array is translucent, biocompatible, and has a very small footprint. These attributes allow it to be positioned on the brain slice surface above the dendritic arbor of a single Purkinje neuron without obstructing access to the cell soma. In this configuration, multiple independent PF inputs may be stimulated while recording from the neuron with a whole-cell patch pipette.

Four different designs were evaluated in the present study. The designs with $4\text{-}\mu\text{m}$ -wide electrodes were able to stimulate independent bundles of parallel fibers even when adjacent pairs of electrodes were used. With these experiments, there was no way to monitor exactly which parallel fibers were activated, only that they were not overlapping. It may be possible to monitor active parallel fibers by using voltage- or calcium-sensitive dyes loaded in the fibers (Brown et al., 2003). Without such data, it can be useful to use the geometry of the electrode array to estimate the separation between the two separate groups of parallel fibers. Adjacent pairs of $4\text{-}\mu\text{m}$ -wide electrodes laid at the vertices of an $8\text{-}\mu\text{m}$ -pitch grid. Therefore, it would be reasonable to assume that the two independent groups of stimulated fibers were contained in a $6\text{--}12\text{ }\mu\text{m}$ radius.

Two of the designs had $2\text{-}\mu\text{m}$ -wide electrodes which proved to be inefficient at stimulating neural tissue. Although parallel fiber stimulation was possible with the $2\text{-}\mu\text{m}$ -wide electrodes, adjacent pairs of electrodes stimulated overlapping bundles of parallel fibers. This is most likely due to electrode geometry, array geometry and high electrode resistances.

In studying how a neuron integrates its synaptic input, conventional methods make it difficult to activate more than one local presynaptic afferent. Since most neurons, and especially Purkinje neurons, receive input from many different presynaptic fibers, this array will be useful to explore how spatiotemporally complex inputs are integrated in individual neurons.

The architecture of dendritic spines, bulbous outgrowths with thin bottlenecks attached to the primary dendrite, suggests that these structures function as independent units of neuronal connectivity. However, evidence of neurotransmitter spillover at synapses argues that the situation is not so simple (Diamond, 2002). Since dendritic spines on Purkinje neurons are $0.5\text{--}3\text{ }\mu\text{m}$ apart (Palay and Chan-Palay, 1974), an array of stimulus electrodes such as has been described could find an important application in such studies.

Investigations of cerebellar long-term depression (LTD) may also benefit from the use of this device. Cerebellar LTD is a depression of PF input that is induced by pairing PF input and either climbing fiber (CF) complex spikes or short depolarizations. The spatial extent of this phenomenon is unclear (Reynolds and Hartell, 2000; Wang et al., 2000). An experiment looking at the synapse specificity of LTD is straightforward using the presently described device. By having access

to multiple independent PF bundles, the change in strength of unpaired control inputs could be carefully observed. By noting the relative position of the control pathway to a paired, depressed pathway, one may evaluate the spatial extent of depression at a single group of parallel fiber synapses.

Additionally, the timing parameters of cerebellar LTD can be efficiently probed using this device. Different independent PF inputs may be activated at different time points relative to a climbing fiber-elicited complex spike or short depolarization. The change in strength of these inputs and their relative temporal relation to the complex spike or short depolarization may yield insight into the temporal properties of LTD induction in the cerebellum.

Although this device could in principal be used to record as well as stimulate neurons, it has not been designed to do so. A reconfiguration of the device for recording would benefit from several modifications. Headstage amplifiers could be included either inline, just after the 2 mm connector, or onboard, as part of the circuitry in the silicon chip. Thicker silicon nitride insulation would help to reduce electrode capacitance. Finally, electrode and array geometry may be altered depending on the cellular architecture of the targeted brain region.

In addition to the present device, some of the design strategies used in this study may find use in other microelectrode applications. Silicon nitride already enjoys widespread use in microelectrode arrays, but is often supplemented by additional materials such as polyimide to compensate for high pinhole densities and high internal stress in the silicon nitride films. Minimizing the internal stress in these films may alleviate the need for additional insulating layers and simplify manufacturing. Additionally, the results reported here suggest that a practical lower limit for planar stimulus electrode size may be around 2 μm . This can serve as a reference point for designs that minimize electrode size in order to increase spatial density in microelectrode arrays.

Acknowledgements

We would like to thank A. Hung, P. Irazoqui-Pastor, B. Matthews, and P. Motta for their valuable assistance and suggestions. This work was supported by the Whitehall Foundation (TSO) and the UCLA Neuroengineering Training Program, an NSF-funded IGERT 9972802 (SLS).

References

- Atluri PP, Regehr WG. Determinants of the time course of facilitation at the granule cell to Purkinje cell synapse. *J Neurosci* 1996;16:5661–71.
- Borkholder DA, Bao J, Maluf NI, Perl ER, Kovacs GT. Microelectrode arrays for stimulation of neural slice preparations. *J Neurosci Methods* 1997;77:61–6.
- Brown SP, Brenowitz SD, Regehr WG. Brief presynaptic bursts evoke synapse-specific retrograde inhibition mediated by endogenous cannabinoids. *Nat Neurosci* 2003;6:1048–57.
- Diamond JS. A broad view of glutamate spillover. *Nat Neurosci* 2002;5:291–2.
- Gesteland R. Comments on microelectrodes. *Proc IRE* 1959;47:1856–62.
- Heck D. Investigating dynamic aspects of brain function in slice preparations: spatiotemporal stimulus patterns generated with an easy-to-build multi-electrode array. *J Neurosci Methods* 1995;58:81–7.
- Heck D, Borst A, Antkowiak B. Passive spatial and temporal integration of excitatory synaptic inputs in cerebellar Purkinje cells of young rats. *Neurosci Lett* 2003;341:79–83.
- Heuschkel MO, Fejtł M, Raggenbass M, Bertrand D, Renaud P. A three-dimensional multi-electrode array for multi-site stimulation and recording in acute brain slices. *J Neurosci Methods* 2002;114:135–48.
- Kristensen BW, Noraberg J, Thiebaud P, Koudelka-Hep M, Zimmer J. Biocompatibility of silicon-based arrays of electrodes coupled to organotypic hippocampal brain slice cultures. *Brain Res* 2001;896:1–17.
- Magee JC. Dendritic integration of excitatory synaptic input. *Nat Rev Neurosci* 2000;1:181–90.
- Novak JL, Wheeler BC. Multisite hippocampal slice recording and stimulation using a 32 element microelectrode array. *J Neurosci Methods* 1988;23:149–59.
- Oka H, Shimono K, Ogawa R, Sugihara H, Taketani M. A new planar multielectrode array for extracellular recording: application to hippocampal acute slice. *J Neurosci Methods* 1999;93:61–7.
- Reynolds T, Hartell NA. An evaluation of the synapse specificity of long-term depression induced in rat cerebellar slices. *J Physiol* 2000;527(Pt 3):563–77.
- Tominaga T, Tominaga Y, Ichikawa M. Simultaneous multi-site recordings of neural activity with an inline multi-electrode array and optical measurement in rat hippocampal slices. *Pflugers Arch* 2001;443:317–22.
- van Bergen A, Papanikolaou T, Schuker A, Moller A, Schlosshauer B. Long-term stimulation of mouse hippocampal slice culture on microelectrode array. *Brain Res Brain Res Protoc* 2003;11:123–33.
- Wang SS, Augustine GJ. Confocal imaging and local photolysis of caged compounds: dual probes of synaptic function. *Neuron* 1995;15:755–60.
- Wang SS, Khirouq L, Augustine GJ. Quantification of spread of cerebellar long-term depression with chemical two-photon uncaging of glutamate. *Proc Natl Acad Sci USA* 2000;97:8635–40.
- Williams SR, Stuart GJ. Role of dendritic synapse location in the control of action potential output. *Trends Neurosci* 2003;26:147–54.