Supporting Text

Microwire Array Fabrication

The main design used in this study consists of three basic parts: (*i*) an array of S-isonel-coated tungsten microwire electrodes, (*ii*) a printed circuit board (PCB) to which the microwire electrodes are connected, and (*iii*) a high-density, miniature connector that is attached to the opposite side of the PCB (see Figs. 1 *A*-*C*). PCBs (Fig. 1*A*) are made of a 0.008-inch thick (200 μm), rigid material and contain conductive traces etched onto their surfaces. The spacing between the traces can be defined by the user. In the example shown in Fig. 1*A* the traces are 0.004 inches (100 μm) wide and spaced 0.012 inches (300 μm) apart (center to center) at the end where electrode wires are attached.

Individual microwire electrodes are attached to the board by using a custom-made "jig" that facilitates the process of establishing electrical contact between a wire and individual traces. A high-density connector is also attached to the other end of the board. The conductive traces from the electrode wires terminate in solder pads onto which the connectors are soldered. This connector forms the interface between the electrode wires and external signal amplifiers. In addition, the PCB has two long tabs. These tabs are used for mechanical support of the PCB during assembly of the electrodes and to hold the electrode assemblies during surgical implantation.

The custom-designed jig is used to hold the microwire in parallel alignment and to align them with the traces on the PCBs. First, the wires are threaded into the jig on one end, and the PCB is plugged into the other end. PCBs that use the rigid material have the connector already soldered onto the appropriate pads. The jig uses a drilled array that is consistent with the PCB thickness and trace spacing. With the wires threaded into the array and the PCB plugged in to the opposite side, a small bead of cyanoacrylate gel is laid down along the edge of the board. The wires are then placed on their traces as established by the array. This completes the mechanical connection from the electrode wires to the connector. The electrical connection to the wires is formed by using a highly

conductive colloidal silver paint applied to the end of the wire and its respective copper trace. Once the paint is dry, the electrode assembly is unplugged from the jig and the wires are cut to length. Different lengths are used for different implant procedures. A new PCB is plugged into the jig and the process continues until the jig is expended of its wire. Usually five electrode assemblies can be produced with the jig fully loaded. After the electrode assembly is removed from the jig, ground wires are attached to specific pads of the PCB and the assembly is potted in a dental acrylic compound. The electrode assembly is then ready for use. If an electrode assembly is to have more than one row of wires, multiple PCBs are used.

The high-density connectors (see Fig. 1*C*) used with the rigid PCB depicted in Fig. 1*A* are commercially available (Nano series connectors, Ohmnetics, Minneapolis). The particular connector used in this study was a dual row connector with 18 connectors per row and pin spacing of 0.025 inches. These high-density connectors, which are very rugged, light, and extremely compact, attach to the PCB by using surface-mount solder pad connections.

Surgery for Implantation of Microwire Arrays

On the day of surgery, the animals' head and extremities are shaved under light ketamine anesthesia. For induction, a combination of ketamine, propofol, and isoflurane are used. Monitoring of various physiological parameters (e.g. temperature, heart rate, oxygen saturation, end tidal carbon dioxide, blood glucose, and basic electrolytes) is continuously performed during our surgeries. Noninvasive blood pressure monitor is used to record blood pressure trends, and, in conjunction with heart rate measurements and EEG recordings, provide a quantitative way to judge the depth of anesthesia.

Preoperatively, i.v. broad spectrum antibiotics and a single dose of dexamethasone are given. Throughout surgery the animal receives lactated Ringer's solution i.v. and small amounts of pediatric formula through a feeding tube placed in the stomach.

After a midline incision, the periosteum is elevated with the scalp flap, and the temporalis muscle is partially dissected off the skull. Craniotomies are drilled, using an air-powered dental drill, until the soft dura is reached to assure proper grounding. After each craniotomy is drilled, the dura mater is then opened.

The bent tip of a tuberculin syringe needle is used to hook the dura, and a 15-blade knife is used to incise it. Extreme care is used not to injure the cortical vasculature and pia. We use the hooked end of a tuberculin needle to pierce and mechanically open the most superficial layers of the arachnoid/pia. This maneuver is carried out with extreme care, under X40 magnification.

In macaque monkeys, the arachnoid/pia is a clearly identifiable but relatively tough layer for blunt microwire arrays to penetrate. After a small patch of arachnoid/pia is removed from the implantation site, a microwire array is attached to the stereotaxic manipulator by a custom-designed holder that tightly grips on the two long tabs of the array's PCB and centered over the target. Fine-tipped microdissecting forceps are then used to enlarge this opening by pulling on opposite edges of the slit arachnoid/pia, thus effectively peeling it back off the cortex. This can be performed with minimal disruption of the cortical vasculature or the deeper layer of the pia. Should bleeding occur, gel foam soaked in thrombin is applied temporarily to promote rapid hemostasis.

Next, microwire arrays are implanted slowly during continuous electrophysiological monitoring. Once final depth of implantation has been reached, a small amount of gel foam is applied around the wires, followed by a layer of bone wax and a thick layer of cyanoacrylate adhesives directly onto the electrode-brain interface. This procedure reduces the subdural space and the effect of brain pulsations, allowing bonding of the remaining pia to the electrodes and surrounding skull. Titanium skull screws are then placed for grounding purposes and for stabilization of the head cap. Approximately 15-20 screws are distributed across the skull. Dental cement is used to secure the electrode arrays in position.

The skin is then allowed to naturally abut the skull cap. The temporalis muscle is left free of its attachment and tucked under the skin abutting the skull cap. No difficulty with chewing has been noted postoperatively. After discontinuing isofluorane anesthesia, we have noted that animals regain a wakeful state in ~1 h. Buprenophrine and acetaminophen are given routinely for postsurgical analgesia. Bacitracin ointment is applied liberally to the wound edges where the dental cement meets the skin. The animal is monitored 24 h a day for the first 3 days.

Hardware Multisite, Many-Neuron Ensemble Recordings

In all experiments, multiple 16-32 channel headstages were used to provide the first level of amplification of the neural signals obtained from the arrays of microwires implanted in the animal's brain. Both commercially available head stages (Plexon) and those manufactured at Duke University were used in our experiments (1).

Each MAP used in this study allows simultaneous sampling from 128 microwires and discrimination of up to four individual action potentials per wire, for a maximum of 512 recorded neurons. A series of lightweight cables were used to route neuronal signals to the MAPs. This device allows one to simultaneously record the activity of large numbers of single neurons as animals execute a behavioral task. The MAP (and MAP cluster) then provided further amplification and band pass filtering (500 Hz to 5 KHz) to these signals. After a stage of analog-to-digital conversion, the signals are routed to DSP boards, each of which contains four digital signal processors (DSP, Motorola 56002) running at 40 MHz. Each DSP handles data from eight input channels and contains 32K 24-bit words of SRAM and 8K 16-bit words of dual port SRAM memory. A timing board is responsible for distributing timing and synchronization signals to the entire MAP. It also provides a digital time output that is used to synchronize external devices or to drive a video timer for a professional VCR used to store video records of the animal's behavior. The DSP boards also provide inputs for sampling digital pulses and analog signals (i.e., EMG signals, eye coil signals, joystick movements, lever displacement, etc.) generated by the setup to monitor the animal's behavior during execution of tasks. A single host

Pentium computer (800-1.2 MHz, with 1.0 Gbytes of RAM, and 40-70 Gbytes of disk space) running C++ host software under the Windows 2000 operating system (Microsoft, Seattle) was responsible for controlling the MAP and storing data. Recorded files were transferred to a computer server containing a CD or a DVD recorder, which are used to produce permanent records of the data. The MAP also supplies options for analog back-up by using 8-16 channel tape recorders.

Multielectrode recordings were obtained when monkeys 1 and 2 were awake and performing spontaneous arm movements. All experiments carried out in monkeys 1 and 2 used a standard 128-channel MAP. For monkey 3, we used a 384-channel MAP cluster. Because monkey 3 was not adapted to the primate chair at the time these recordings were made, the recordings reported here for that animal were obtained under light ketamine anesthesia. Recordings from monkey 1 continued for 18 months, during which time the animal learned multiple behavioral tasks. Recordings in monkeys 2 and 3 were performed for 3-4 weeks, a time that was sufficient for demonstrating that the surgical and recording techniques could be reproduced.

Statistics calculated in 3D PC space produced the same results. For channels without single units the mean ± SE values of these statistics were: F (3D) = 0.9 ± 0.05, J3 (3D) = 0.50 ± 0.02, PsF (3D) = 1,253.7 ± 194, and DB (3D) = 0.60 ± 0.02. For channels with single units the mean values were: F (3D) = 6.6 ± 0.6, J3 (3D) = 2.6 ± 0.2, PsF (3D) = 25,041.3 ± 3,719, and DB (3D) = 0.40 ± 0.01.

Single-Unit Spike Sorting

All major steps used in our off-line spike sorting procedure are described in Fig. 6.

The process of isolating individual single neurons for each channel proceeded as follows: First, an automatic algorithm, called valley-seek (2), was used to provide an initial evaluation of how many distinct clusters of waveforms could be isolated from the set of valid spikes in 2D PC space. This algorithm returned several potential cluster partition

schemes to the experimenter. Using the same OFSS options described above, the range of possible clusters resulting from this first level of analysis was scrutinized. Therefore, even though the original unsupervised phase of the off-line spike sorting process was helpful and significantly reduced the time required to sort large numbers of channels, we observed that appropriate cluster partition still required a second phase in which the user defined the final clusters manually following objective criteria.

Multiunit activity was characterized by clusters defined by small action potentials (≤ 20 μV), which were characterized by very variable waveform shapes and the absence of a clear refractory period in the ISI histogram.

All four statistics used to quantify the overall separation between identified clusters in a given recording channel were calculated for both 2D and 3D PC spaces. As described by Wheeler (3), the J3 is a nonparametric statistics calculated as follows: given the feature vectors $\underline{f}_{k_i}$, the mean feature vector $\underline{m}_i$ of each cluster of N_i spikes, the overall mean $\underline{m}$ and the Euclidean distance $\| \cdot \|_E$ the following metrics for cluster separability were computed:

$$J_1 = \sum_i \sum_{k_i} \left\| \underline{f}_{k_i} - \underline{m}_i \right\|_E^2 ,$$

$$J_2 = \sum_i N_i \left\| \underline{m}_i - \underline{m} \right\|_E^2 ,$$

$$J_3 = \frac{J_2}{J_1} ,$$

J_1 reflects the average distance of points from their cluster mean and is minimized for compact clusters, whereas J_2 reflects the average distance between clusters and is maximized for well-separated cluster means. Hence, the ratio J_3 is maximized for well-separated compact clusters.

The PsF statistic was obtained by simply adjusting the value of J3 to the number of classes (i.e., clusters depicting single units and background noise) and valid spikes in the channel. The formula used is:

$$PsF = \left[(n - G)/(G - 1)\right] \times J3,$$

where n is the number of waveforms and G the number of clusters.

The DB validity index, another nonparametric measure, is based on a compromise between the spread within a cluster and the distance between clusters, *i.e.*, it tries to identify compact and well-separated clusters by calculating the ratio of the sum of within-cluster scatter to between-cluster separation. The DB index gives a small value for a good clustering and is defined as

$$DB = \frac{1}{n}\sum_{k=1}^{n} \max_{k \neq l} \left\{ \frac{S_n(Q_k) + S_n(Q_l)}{d(Q_k, Q_l)} \right\},$$

where n is the number of clusters, S_n the average of within-cluster distances (*i.e.,* the distance of all the elements of the cluster to their cluster center), d the distance between cluster centers, and Q_k, Q_l are the clusters.

Each of these statistics was calculated for channels with single units that satisfied both of the following conditions: (*i*) at least two clusters were identified in the channel, and (*ii*) at least one of those clusters was considered to contain action potentials from a single unit. For ~80% of the channels that yielded single units in this study, one of the following situations held: (*i*) in most cases, these statistics reflected the separation between a single-unit cluster and a multiunit cluster; or (*ii*) in other cases, the voltage threshold was set at a level higher than that of the multiunit activity, and the statistics reflect the separation between two single-unit clusters. In the remaining 20% of channels with single units, no cluster statistics could be obtained because the voltage threshold was set at a level much

higher than the multiunit record, and only one cluster, defining the only single unit isolated from that channel, was present in the PC space.

Statistics calculated in 3D PC space produced the same results observed in 2D PC space. For channels without single units the mean ± SE values of these statistics were: F (3D) = 0.9 ± 0.05, J3 (3D) = 0.50 ± 0.02, PsF (3D) = 1,253.7 ± 194, and DB (3D) = 0.60 ± 0.02. For channels with single units the mean values were: F (3D) = 6.6 ± 0.6, J3 (3D) = 2.6 ± 0.2, PsF (3D) = 25,041.3 ± 3,719 and DB (3D) = 0.40 ± 0.01.

To further assess the possibility of using the four statistical measurements described above to decide whether a given recording channel contained single units, we used discriminant analysis on the same data depicted in Fig. 8 *B*-*E* and *B*'-*E*'. This analysis revealed that a linear discriminant function that combined four statistics [F (2D), J3 (2D), PsF (2D), and DB (2D)] achieved a very significant discrimination between channels with or without single neurons [$F(4,251) = 40.8$, $P < 1 \times 10^{-5}$, Wilks' Lambda = 0.606]. This analysis also revealed that the most significant contribution to the model was provided by DB (2D) (F-remove = 33.9, $P < 1 \times 10^{-7}$), followed by F (2D) (F-remove = 17.1, $P < 4.7 \times 10^{-5}$) and then J3 (2D) (F-remove = 10.7, $P < 1.2 \times 10^{-3}$). PsF (2D) had no significant contribution (F-remove = 0.068, P <0.79) likely because it covaried strongly with J3. These results prompted us to use the first three statistics in a new analysis. This time, however, only data from half of the channels in each group (spikes vs. no spikes) was used to derive a linear model with discriminant analysis. These channels defined the "training" data set. Once the new model had been obtained, we asked how many channels in a "testing" data set (i.e. data that had not been used to derive the discriminant function) could be correctly classified as having vs. not having single-unit records. This analysis revealed that, on average, 73% of the testing channels could be correctly classified by a discriminant function that combined the values of F (2D), J3 (2D), and DB (2D).

Quantitative Evaluation of Single-Unit Stability

Wavetracker (Plexon) uses graphical routines to evaluate the stability of both waveform shape and single neuron clustering in PC space. In one of these, for each recorded channel, the 3D PC clusters obtained in each data segment can be rendered by ellipsoids calculated by taking into account the cluster mean and 3 SDs (see Fig. 7 *A*'- *F*'). Using another graphical routine (4), a solid can be drawn by uniting the borders of stacked clusters, corresponding to 2D projections of PC clusters isolated in consecutive 30-min data segments. When the resulting 3D solid resembles a clear cylinder, the isolation of the correspondent single unit remained extremely stable throughout the recording session. This conclusion was further supported by the fact that, in these cases, no variation was observed in any of the other quantitative measurements (waveform shape, ISIs, and cluster separation statistics) chosen to evaluate recording stability.

Fig. 7 displays representative examples that demonstrate that clustering in PC space and single unit isolation remained stable throughout recording sessions that lasted for 1–2 h. In the cases illustrated in Fig. 7 *A–F*, a 2-h recording session was initially divided in four consecutive 30-min segments for subsequent analysis. Clustering in 3D PC space was then carried out for each 30-min data segment. The graphs in Fig. 7 *A–F* show waveforms isolated in each of these channels in four consecutive 30-min data segments. Analysis of these plots revealed that all discriminated sets of waveforms, each of which corresponding to an independent single unit, remained stable throughout the 2-h period. This was confirmed by the fact that neither the ISI histograms calculated for these single units nor the cluster separation statistics calculated for these channels varied significantly in time.

In Fig. 7 *A*'–*F*', ellipsoid renditions and projections in 3D PC space of the clusters identified for each channel during the first 30-min data segment are depicted. Similar plots were created for each 30-min data segment. For each channel, the identified PC clusters in 2D were then stacked on top of each other so that solids could be drawn by connecting the stacked clusters obtained from each of the 30-min data segments. Fig. 7

A''–*F*'' shows that this 3D interpolation resulted in relatively straight cylinders for each of the channels illustrated. Straight cylinders indicate that no significant variation was observed in the 2D PC clustering during the 2-h recording session, suggesting that single-unit isolation remained very stable throughout. Other examples of single-unit short-term stability are displayed in Figs. 7 *G–L*. Overall, very stable short-term recordings (1–2 h) were obtained in virtually all of the channels surveyed in the present study. A similar analysis was applied to evaluate long-term single unit stability.

The same approach detailed in Fig. 7 was used to evaluate long-term stability of single-unit isolation in our recordings, *i.e*., stability over days. Although this was not the primary goal of the present study, this analysis revealed that single units appeared to be recorded for several consecutive days. Fig. 8 illustrates representative examples of waveforms and PC space-cylinders derived from channels that were recorded for a period spanning 8 days (five recording sessions, four obtained in consecutive days, and one final session obtained 8 days after the first recording). To build Figs. 7 and 8, 1-h recording sessions were obtained per day. Then, the original file was divided in two 30-min data segments to generate two samples of waveforms and 2D PC clustering per recording day. 2D PC clusters were then stacked and drawn as solids. In the channels depicted in Fig. 8 *A–F*, which represent ˜55% of the channels surveyed, reasonably straight cylinders suggest that the same set of single units was recorded for 8 days. When the same analysis was performed over a 2-day period, ~80% of the surveyed single units produced very straight cylinders.

In Fig. 8*G*, the blue line depicts the animal's hand trajectory in X and Y coordinates, whereas the red line illustrates the on-line predicted trajectory based on the recorded neural ensemble data. As shown in Fig. 8, a reasonably precise prediction of the animal's hand trajectory was achieved by using a simple linear model. Thus, even 10 months after the implantation, multichannel single-unit recordings remained very stable, and the relatively good condition of those neurons is reflected in the fact that a significant fraction of the motor cortical neurons recorded simultaneously showed clear modulations of their firing rate before the initiation of arm movements.

Final Comment

The statistical methods for quantifying cluster separation described in the present article should provide a certain level of confidence that the neurons remain the same from day to day. Moreover, use of antidromic activation methods can provide virtual proof of recording stability across days. Because antidromic latencies for a given neuron vary by <~0.1 ms, a cell's antidromic latency and "collision window" serve as a "fingerprint." Furthermore, this approach provides additional information about a cell: cells antidromically activated from the pyramidal tract, for example, are layer 5 pyramidal cells, and, because a cell's antidromic latency correlates with axon and cell size (5), it is possible to estimate each cell's size and apply algorithms that attempt to correct for size-based sampling bias (6).

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