SRC TR 88-108

TECHNICAL RESEARCH REPORT

The Second Synthetic Microstructures in Biological Research Conference

Co-chairmen:

Dr. M. Peckerar NRL/U.MD
Dr. P.S. Krishnaprasad U.MD.
Dr. S. A. Shamma U.MD

SYSTEMS RESEARCH CENTER

UNIVERSITY OF MARYLAND

COLLEGE PARK, MARYLAND 20742

THE SECOND SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH CONFERENCE

A JOINT IEEE/ONR/NIMH SPONSORED CONFERENCE

AIRLIE HOUSE

20 - 23 MARCH 1988

Co-chairmen:

Dr. M. Peckerar NRL/U.MD.
Dr. P. S. Krishnaprasad U.MD.
Dr. S. A. Shamma U.MD.

CONFERENCE GOALS

- BRING TOGETHER SCIENTISTS AND ENGINEERS WORKING IN THE (SEEMINGLY) DIVERSE DISCIPLINES OF MICROSTRUCTURE SCIENCE AND BIOLOGY FOR A SHARING OF TECHNIQUES AND TO ACQUAINT EACH OTHER WITH MUTUALLY BENEFICIAL DEVELOPMENTS IN THE TWO FIELDS

- ALLOW MICROSTRUCTURE SPECIALISTS TO BECOME ACQUAINTED WITH THE PROBLEMS OF THE BIOLOGIST WHICH CAN BE ADDRESSED BY MICROSTRUCTURE TECHNOLOGY

- ALLOW BIOLOGISTS TO ACQUAINT MICROSTRUCTURE SPECIALISTS WITH NOVEL METHODS OF MICROSTRUCTURE FABRICATION INSPIRED BY LIVING SYSTEMS

Section I
GENERAL INTRODUCTION
Area Summary

The aim of this session was to present an overview of research areas in Neural Information Processing and related implementations that shed some light onto the needs of the biological community to specialized microstructures, and current attempts to meet these needs. The talks addressed global issues from biological and theoretical perspectives. The first talk by Dr. Matthyse of the Mailman Research Center (Abstract not included) presented mathematical models of neural processing and addressed methods of extracting information from local measurements in the system. On a similar but more experimental vein, Dr. Richmond of NIH presented neurophysiological data of responses in the visual cortex of the monkey. This work illustrated the temporal aspects of visual coding in contrast to the usual spatial code. Dr. Gross followed by a talk that dealt with neural processing in monolayers cultured in dishes with a large array of electrodes fabricated on them. Finally, Dr. Brockett of Harvard University reviewed the state of the art in the fabrication of micromechanical actuators, a necessary prerequisit for the fabrication of dexterous robotic structures.

SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH

21 MARCH, SESSION I:
MICROSTRUCTURES AND BIOLOGICAL SYSTEMS RESEARCH

GENERAL INTRODUCTION: 8:45 a.m.-9:00 a.m.

9:00 a.m.-10:00 am

1. EXTRACTING GLOBAL INFORMATION FROM LOCAL MEASUREMENTS IN COMPLEX SYSTEMS (Steven Matthyse, Mailman Research Center, McLean Hospital, Belmont, Massachussetts)

10:00 a.m.-10:45 a.m.

2. CRACKING THE NEURAL CODE (Barry J. Richmond, Laboratory For Neuropsychology, NIMH, Bethesda Maryland)

BREAK: 10:45 a.m. 11:00 a.m.

11:00 a.m.-11:45 a.m.

3. NETWORK ANALYSIS AND MONOLAYER CULTURES (Guenther Gross, North Texas State University, Denton, Texas)

11:45 a.m.-12:15 p.m.

4. MICROMECHANICAL ACTUATORS (Roger Brockett, Gordon McKay Laboratory, Harvard University, Cambridge Massachussetts)

CRACKING THE NEURON'S CODE: CODES BASED ON TEMPORAL MODULATION OF THE SPIKE TRAIN ALLOW SINGLE VISUAL NEURONS TO TRANSMIT INFORMATION ABOUT MULTIPLE STIMULUS DIMENSIONS.

Barry J. Richmond and Lance M. Optican, Laboratory of Neuropsychology, NIMH, and Laboratory of Sensorimotor Research, NEI, Bethesda, MD 20892.

What does the response of a visual system neuron say about the stimulus? The answer depends upon how the response is interpreted. The function of visual system neurons is most frequently interpreted in terms of those features that yield the strongest responses. The response strength indicates the match of the stimulus with the receptive field, a retinotopically defined spatial region that contains subregions each sensitive to a different brightness. This approach assumes that the critical measure of the response is its strength. The use of a single response measure then leads to the assumption that the representations of different stimulus features are confounded within the response, and that individual features can be unconfounded only by looking across a large population of neurons. Essentially this is a population code.

This approach overlooks the fact that neurons show modulation of the pattern as well as the strength of their activity. Different stimulus features, e.g., color, luminance, and pattern, often simultaneously influence the responses of single neurons. When we applied principles of information theory and defined an orthogonal, two-dimensional set of black and white stimuli as an input code, and the optimal orthogonal set of temporal patterns to represent the responses as an output code, we were able to show that neurons in areas throughout the visual system vary the strength and pattern of their responses independently. The stimulus-related information is carried in three or more simultaneous and independent patterns of activity, i.e., information about different stimulus features is transformed into temporally modulated messages that are multiplexed into the spike train. Application of Shannon's information theory revealed that the amount of information carried by the temporal modulation is at least twice as great as that carried by the response strength alone. Further, information about different stimulus parameters is not confounded but is carried separately in different parts of the neuronal code.

Based on our multiplex filter hypothesis, we have formed quantitative models that accurately predict the temporally modulated responses of striate cortical complex cells and lateral geniculate parvocellular neurons to arbitrarily constructed black and white stimuli. Recently we have moved substantially closer to our goal of decoding the neuronal messages. We have identified a potential structure for a neuronal code.

Our findings imply a new interpretation of the functional roles of single neurons. In this new view, single neurons are not abstracting visual features, but, instead, each conveys a multidimensional low resolution description of the stimulus in temporally modulated messages that are multiplexed onto the spike train. This principle of temporal modulation leads to an integrated interpretation of many different phenomena from biophysics, neuropharmacology, neurophysiology, neuroanatomy, and network activity. These results have substantial implications for the design and implementation of simulations of human vision, and potentially for the design of devices that communicate directly with neural structures.

MICROMECHANICAL ACTUATORS

Roger W. Brockett

Division of Applied Sciences
Harvard University

In this talk we will describe technologies capable of producing actuators on a scale which is approxiimately one order of magnitude smaller than the standard electric motors available today. The idea is to create vibratory motion in an elastic strip together with the phase motion of a pair of brakes to rpoduce linear displacement. The forces available through this kind of continuum kinematical system are quite substantial. The driving mechanism can be magnetic, electrostatic, piezoelectric, magnetostrictive or even of the electrocapillary/electrowetting type. A difficult problem is that of achieving rapid acting and precise braking. Issue include the question fo whether or not the holding surfaces should be smooth or precisely engraved with teeth which can be engaged and disengaged. Materials which can be used include plastics, metals, and silicons. Surface treatments for enhancing frictional effects may be useful.

This work was supported in part by ARO/DARPA grant DAAG29-85-K-0096 and by NSF grant MEA-83-18972.

Section II
PROBES AND INTERFACES
Area Summary

This session concentrated on the difficult technological questions faced by all microstructures, specifically the device interfaces. The openning talk was by Dr. Heetdirks of NIH who reviewed the prosthetics program at NIH and the different types of probes and microstructures that their program is involved in over the last few years. Dr. Smith from MIT followed with a review of the different packaging approaches that they are investigating, and the criteria they have found most useful in evaluating the reliability and utility of different schemes. Dr. Regher of CALTECH presented the latest developments in recording systems of cultured neurons. A detailed report of this exciting work is include within. Finally, Dr. Edell of MIT reviewd the status of the design and fabrication of their multielectrode array system, and the results of the experimental tests carried out so far.

SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH

21 MARCH, SESSION II:
PROBES AND INTERFACES

1:30p.m.-2:15p.m.
1. MICROSTRUCTURES FOR PROSTHESES (William Heetdirks, National Institutes Of Health, Bethesda, Maryland)
2:15 p.m.-3:00 p.m.
2. PACKAGING FOR BIOSENSORS (Rosemarie Smith, Massachussetts Institute Of Technology, Cambridge, Massachussetts)
BREAK:3:00 p.m. - 3:15 p.m.
3:15p.m.-4:00 p.m.
3. ADVANCES IN LONG-TERM ELECTRICAL CONNECTION TO CULTURED NEURONS (Wade Regher, California Institute Of Technology, Pasedena, California)
4:00 p.m.-4:45
4. NEURAL INFORMATION TRANSDUCERS: DESIGN AND FABRICATION (David Edell, Massachussetts Institute Of Technology, Cambridge, Massachussetts)

MICROSTRUCTURES FOR NEURAL PROSTHESES

William J Heetderks, M.D.
Neural Prosthesis Program
Division of Fundamental
Neurosciences
NINCDS, NIH

Abstract:

Neural prosthetic systems are being developed for restoration of function in neurologically impaired individuals. These systems require methods to record and stimulate reliably and chronically from small populations of neurons and muscle cells. Microfabrication and integrated circuit technology, applied to the design of integrated electrode arrays, will help to provide reliable stimulation and recording.

Microstructures for use in a neural prosthesis must function as part of a system. Thus a recording electrode array needs the fine microstructure of the recording sites and the electrode shank, a method to amplify and multiplex the transduced signals, and a method to transmit the signals off the microstructure to the rest of the system. These elements of the microstructure must all function reliably over the lifetime of the implanted individual . They must function in an environment that is relatively hostile and they must be biocompatible. Starting with the biological signals, with emphasis on measuring the potentials associated with extracellular action potential currents, several designs for recording and stimulating microstructures will be discussed in terms of transduction, transformation and transmission of the signals. Also the requirements for encapsulation of microstructures for use in neural prostheses will be considered.

Packaging of Biosensors

Rosemary L. Smith
Massachusetts Institute of Technology

The design and packaging of microsensors for *in vivo* biomedical applications is considerably complicated by the surrounding, conductive and corrosive biomedical environment. The encapsulation of active components becomes critical to the usefulness of the microsensor. How much of the system to integrate and the relative positioning of circuits sensor and i/o leads becomes intimately dependent on the packaging capabilities and the effects of the environment on the functioning of the system. Microelectronic components are themselves very sensitive to temperature, pressure, humidity and chemical species, and usually non-selectively to all of these parameters. Response of the transducer is not easily isolated from that of the circuitry. The integrated microsensor therefore poses a challenging calibration problem. The package itself may contribute to the response due to environment induced strain, e.g. the swelling of epoxy encapsulants during hydration or from thermal expansion.

From the discussion above, it is clear that the packaging of microsensors is a system design problem. In the case of biomedical sensors, increased information gathering capability per unit volume is often the driving factor for integration. However, the difficulty in encapsulating devices often limits performance. Therefore, a packaging technology need be provided for the microsensor. Since microsensors are application specific, the package is a custom design feature, and must be designed along with the sensor from the very start. The package will determine the placement of sensor, circuitry, and i/o pads with regard to function, isolation and encapsulation.

Materials selection is another critical factor. Several solid state insulators, such as silicon nitride, have been shown to be excellent barriers to the diffusion of water and ions. However, their behavior in aqueous electrolytes has only recently been explored, and little is understood about long term behavior. The same can be said for organic thin films. In addition, there are few organic materials which lend themselves to photolithographic patterning and other planar processing techniques. If that were not problematic enough, the biosensor designer is faced with biocompatibility requirements and electrical safety.

In this presentation, a review of the critical issues involved in microsensor design and encapsulation, pertaining to packaging, is given. These issues are demonstrated by a detailed description of a specific packaging scheme, for a chemical microsensor. In this design, a combination of solid state encapsulants and integrated circuit isolation techniques with silicon micromachining is employed, to produce a wafer level, but hybrid, process. In this approach, the microsensor is divided into two principal physical parts, an electrode and electronics containing substrate, and a micromachined membrane package. The fabrication and, hence, the resultant performance of each part is independently optimizeable. The final microsensor is constructed by, first, binding the two parts together at the wafer level, followed by die separation, and lead attachment. The micromachined membrane holders are then filled with liquid, chemically selective membranes, to yield functioning sensors. A calcium ion sensor fabricated by this method is demonstrated. It is shown how this technique will enable the creation of a miniature, ion-selective electrode array.

Towards an Ideal Neural Information Transducer

David J. Edell, Associate Professor

Massachusetts Institute of Technology
Building 20A-127
Cambridge, Massachusetts 02139

This research program is principally concerned with the science, technology and medical applications of microelectronic devices designed to function within biological systems. Of major interest is neural information transducer technology. Neural information transducers are designed to establish communication between nerves and electrical systems. Potential benefits of this research include expanding our knowledge of neurophysiology and fabrication technology as well as providing a basis for clinical rehabilitation of the physically disabled.

The ideal neural information transducer would have the capacity to exchange all possible information with the neural structure of interest. This would require hundreds of bidirectional information channels per square millimeter which could arbitrarily be selected as inputs or outputs. The relatively high bandwidth of neural signals (10kHz) and low power requirements for implant technology would preclude development of such a system. However, in the most general system, only the time of occurrence of action potentials need be transduced. Significant bandwidth reduction could be achieved since action potentials occur at less than 1000/sec, and more generally average 10-100/sec. More specific systems may require even less information transfer, such as the mean rate of action potential occurrences. If integrated circuit detectors are designed and fabricated to transduce this information at the contacts themselves, then it becomes reasonable to consider the possibility of transducing all of the information associated with the neural structure rather than a subset of the information.

In order to develop the technology base required for such a system, a number of fundamental issues are currently being investigated. These issues include: refinements related to fabrication of the probe structures; modeling and manipulation of the nerve-electrode interface; assessment of the electrical properties of the microvolume of tissue surrounding the probe; understanding receptive fields; optimization of the electrode design for selectivity; and understanding the statistical nature of the physical relationship between the device and neural sources. Of paramount importance is the development of a saline compatible integrated circuit technology using thin film protection techniques.

While practical applications requiring integrated circuits on the neural interface must await the outcome of the above research, it is important to begin considering appropriate circuit design strategies. An example of a wideband, low power, low noise, current steering multiplexing system being implemented at MIT will be presented as an example of a design approach that may eventually lead to the VLSI information transducers of the future.

Support for these projects provided by
The Whitaker Foundation
NIH-NINCDS contract NO1-NS-7-2399

To be submitted for publication in the *IEEE Transactions on Electron Devices*

Progress in Long-Term Electrical Connections to Cultured Neurons Using Integrated Circuit Technology

WADE G. REGEHR, CHI-BIN CHIEN, TINA J. KRAMER,

DAVID W. CRANK, DAVID B. RUTLEDGE, and JEROME PINE

California Institute of Technology, Pasadena, CA 91125.

Abstract—**Many interesting experiments for studying the electrical activity of neurons in cell culture cannot be performed due to limits of current technique. Using conventional glass-pipette electrodes, it is extremely difficult to simultaneously stimulate and record from more than three cells, or to perform an experiment lasting more than a few hours. This paper describes three devices built with integrated-circuit technology which can overcome these limits of number and time.**

1. ***Multielectrode dishes*, comprising a planar array of extracellular electrodes, have been used for stimulation of and recording from cultured neurons, both vertebrate and invertebrate. They have proved most effective for large invertebrate neurons, where they have been used to monitor spontaneous activity for up to 13 days. Transparent electrode leads are well-suited for simultaneous voltage-sensitive dye recording or video microscopy. Cultures of rat sympathetic neurons have been stimulated with dish electrodes and simultaneously recorded from with dyes.**
2. ***Diving-board electrodes* are movable extracellular electrodes made by micromachining silicon. When placed on neurons, they form chronic two-way electrical connections. These electrodes have been used with cultured vertebrate and invertebrate neurons whose cell bodies are 20 μm in diameter or larger; they have been used to stimulate and record from cultured invertebrate neurons for up to 4 days.**
3. ***Multiwell dishes* are designed to combine the superior electrical properties of the diving boards with the multiple electrodes of the multielectrode dishes. Each well is about the size of a cell, and has an electrode in its floor. A neuron is placed in a well when it is young and grows to fill the well, which is designed to hold the cell in intimate contact with the electrode.**

This paper describes progress with these techniques, and compares them with standard methods.

I. Introduction

The nervous system is tremendously complex, and many different approaches are required as we try to understand it. One strategy is that of cell culture, where neurons are removed from the animal and placed in the artificial environment of a petri dish. Here they exhibit many of the same properties that they would in the animal, but in a greatly simplified system where access is easy and the environment can be carefully controlled. Standard glass-pipette electrodes have provided much information about the electrical behavior of cultured cells, but conventional experiments are extremely inconvenient for more than two cells, and can only rarely last longer than ten hours. Many important phenomena, such as neuronal growth and development, and synaptic plasticity, occur over longer times; also of great interest is the behavior of networks of more than two cells. Devices made using integrated-circuit technology offer the possibility of long-term two-way electrical contacts (i.e., for both stimulation and recording) to many neurons at once, making these experiments possible.

A great variety of neuronal cell types can be grown in culture [1], providing that they are maintained under exactly the right conditions. In addition to the many types of embryonic vertebrate neurons that have been cultured, it has recently been possible to culture identified neurons from various adult invertebrates, including the leech [2], and the mollusks *Helisoma* [3] and *Aplysia* [4]. Fig. 1 shows examples of an invertebrate and a vertebrate culture system: neurons from the snail *Helisoma*, and neurons from the rat superior cervical ganglion (SCG) [5]. The study of invertebrate culture systems is simplified

greatly by the large size and sturdiness of the neurons; however, vertebrate systems are more likely to approximate the complex behavior of an intact vertebrate nervous system.

Electrophysiology

A brief description of neuronal electrophysiology and of conventional electrophysiological techniques will motivate the use of integrated devices. Fig. 2 shows recordings made from a *Helisoma* B19 neuron and from a rat SCG neuron. These signals, called *action potentials*, characteristically have an amplitude of about 100 mV and a duration of 1–5 ms. A neuron will fire an action potential once its intracellular voltage is raised past a certain threshold level; in these two cases, the neurons were stimulated to fire by injecting current through an intracellular electrode (explained below). Action potentials carry signals over long distances within the same neuron; *postsynaptic potentials* (PSPs) carry information between neurons. PSPs are smaller in amplitude (usually less than 20 mV) and longer in duration than action potentials. They are caused when one cell synapses on (makes a connection to) another cell: when the presynaptic cell fires an action potential, the postsynaptic cell will have a PSP, which in some cases will be suprathreshold and cause the postsynaptic cell to fire an action potential as well. The electrophysiologist would like to record action potentials and PSPs as they occur, as well as the DC potential across the cell membrane, and also would like to stimulate the cell to fire action potentials.

Intracellular connections

For standard intracellular physiology, a neuron is penetrated by a hollow glass pipette

with a submicron tip, filled with conductive electrolyte. The same electrode can be used to inject current for stimulation and to record the intracellular potential. *Whole-cell patching* is another way to make an intracellular connection to the cell [6]. Instead of penetrating with an ultrafine pipette, a glass pipette several microns in diameter is touched to the cell and suction is applied. Under the right conditions, an extremely close contact (with a leakage resistance of gigohms) will form, and the membrane patch beneath the electrode tip may be broken to obtain an intracellular connection. Both of these intracellular techniques stimulate the neuron very easily, and record with an excellent signal-to-noise ratio. However, they have some disadvantages. They are invasive and usually damage the cell, and the electrodes must be held steady to within a fraction of a micron, requiring bulky mechanical manipulators. The connection cannot usually be maintained for longer than several hours, and the bulk of the manipulators makes it very difficult to use more than two electrodes at a time.

Extracellular connections

Extracellular electrodes, either far away from the cell or touching it, provide a less invasive (though less direct) method for stimulation and recording, since they do not pierce the cell.

When far from the cell, extracellular electrodes can record the voltage drop in the extracellular solution resulting from currents that flow during an action potential. Signals from action potentials are generally small (20-100 μV), since the extracellular solution

(typically in the range 50–500 Ω-cm) shunts the signal. Much less current flows during postsynaptic potentials, and so PSPs cannot be detected at all. A large current (5–50 μA) passed through the electrode can stimulate neurons to fire by creating voltage drops in the extracellular solution. (This stimulation technique is seldom used with conventional glass pipettes.) Using integrated-circuit technology, cells may be grown in dishes containing an integrated microelectrode array [7]–[11]. These arrays have been used to stimulate neurons [9],[10], and to record action potentials [7]–[10] for periods of up to weeks [7]; one such array is described in section II.

A glass pipette may also be placed directly against the cell to form a "loose patch" [12]. With a glass pipette 10 μm in diameter, this loose seal typically has a leakage resistance of 1–3 MΩ, large enough that the neuron can be stimulated with relatively small currents (50-500 nA), and its activity can be monitored easily. This technique is noninvasive, since it is extracellular; it gives signals much larger than those recorded with an extracellular electrode not touching the cell; and it is always clear which cell is being stimulated or recorded from. The signal-to-noise ratio is, however, still inferior to intracellular techniques.

Diving-board electrodes and multiwell dishes [16]–[18], described in sections III and IV, are integrated-device analogs of loose-patch electrodes.

Optical recording

Another noninvasive recording method is detection of neuronal activity using voltage-sensitive dyes [13],[14]. When these dyes are bound to a cell membrane, they respond to

changes in the intracellular potential with a linear change in either their fluorescence or their absorption.

The advantages of optical recording are that many cells can be recorded from at once (by using an array of photodetectors), and that PSPs can be detected, at least in some cases [15]. The technique also has limitations. It is not totally noninvasive, since photodegradation of the dye molecules produces toxic byproducts and so limits the total illumination that can be used. The signal-to-noise ratio can be poor, and a good dye must be found for each new cell type studied.

Voltage-sensitive dyes are promising for long-term recording, but must be combined with some other technique, e.g. multielectrode dishes [15], if stimulation required. Preliminary results of this combination are shown below.

II. Multielectrode Dishes

Multielectrode arrays are simple to fabricate, and can be a powerful tool for looking at the activity of networks of cultured neurons.

Large invertebrate neurons from the snail *Helisoma* grow over the electrodes and form a seal of several megohms, so that the electrodes can act as loose-patch electrodes, stimulating and recording quite reliably (*cf.* [10]).

However, in cultures of the smaller rat SCG neurons, the distance between cell bodies and electrodes is usually tens of microns. This makes it difficult to record activity: signal-to-noise ratios for action potentials are small, PSPs cannot be detected, and it is often

unclear which cell is causing a signal at a particular electrode. Using dish electrodes to stimulate these neurons by firing their processes is unreliable, probably because the processes are long and highly branched.

Fabrication

The fabrication, shown in Fig. 3, uses conventional integrated-circuit technology. The electrode leads are made of the transparent conductor indium-tin oxide (ITO) [19], the insulation is photosensitive polyimide, and the electrode tips are electroplated with platinum black [20],[21].

Fabrication begins with a glass substrate (0.016″ thick), coated with a 1000 Å ITO layer (sheet resistance 100 Ω/square), obtained from Donnelly Corporation (Midland, MI). After cleaning, the leads are patterned using *Shipley 1350J* photoresist, with a 125 mJ/cm^2 exposure for the central pattern and an 800 mJ/cm^2 exposure for the periphery of the wafer. (This extra peripheral exposure removes the thick edge bead formed when the photoresist is spun on.) The ITO is etched for 4 min in a freshly prepared solution of 50 H_2O/50 HCl/1 HNO_3 at 40°C. The photoresist is removed and a 30Å-thick layer of aluminum is evaporated on to the wafer. This layer oxidizes upon exposure to air, forming a thin layer of aluminum oxide which acts as an adhesion promoter for polyimide. Next, negative photosensitive polyimide (*MRK Selectilux HTR 3–50*) is spun on at 5000 rpm for 30 s, soft-baked for 5 min at 85°C, and patterned with the electrode mask. The Selectilux is then developed and cured for 12 h at 200°C. The electrodes are platinized in a solution of

1% chloroplatinic acid in 0.0025% HCl, plus 0.01% lead acetate, using a current density of $20\,mA/cm^2$ for 10 s, which reduces their impedance to less than 500 KΩ at 1 kHz. A plastic or glass well with a hole cut in the bottom is then glued to the substrate, completing the dish (see Fig. 4). Dishes take an average of about 1 h to fabricate, and have been routinely reused several times, for a total time in saline of 6 weeks. The leads and insulation are transparent, facilitating observation of cultures with an inverted microscope and recording of activity with voltage-sensitive dyes.

Electrode Tests Using Sealed Invertebrate Neurons

As shown in Fig. 5, cultured *Helisoma* B19 neurons have large cell bodies and often extend flat veil-like processes, which can grow over a dish electrode to form a loose-patch seal. The seal resistances are typically 1–3 MΩ; in this range, recorded signals are primarily derivatives of the action potentials as seen through the capacitance of the cell membrane. Action potentials can be detected with signal-to-noise ratios of 10–100:1; the recording in Fig. 5(b) is a typical example. Multielectrode dishes have been used to monitor spontaneous activity from *Helisoma* neurons for up to 2 weeks. The loose-patch seal makes it possible to stimulate a neuron by passing a current pulse through a dish electrode, while using the same electrode to record the resulting action potential. Fig. 5(c) shows an example; note that the dish electrode can both stimulate and record (the intracellular glass electrode is present only for this test). This capability is necessary for chronic stimulation experiments, which might last for several days, in order to insure that the stimulus is still

suprathreshold, despite the cell's motion or growth.

Electrode Tests Using Multielectrode Dishes and Voltage-Sensitive Dyes

Cultured rat SCG neurons have smaller cell bodies, do not normally form veils, and are usually some distance from the nearest electrode. Thus, they do not form loose-patch seals. Extracellular signals from their action potentials are therefore quite small, and more importantly, it is very difficult to determine which neuron is causing a particular signal without outside information. The lack of a one-to-one geometrical correspondence makes it hard to establish a one-to-one causal correspondence.

However, it is easy to stimulate these neurons using multielectrode dishes (most likely by firing their processes, which grow all over the dish). Fig. 6 shows an example of extracellular stimulation of an SCG neuron whose activity is being monitored by an intracellular electrode. The current injected through the dish electrode depolarizes a nearby process past threshold, so that it fires an action potential. This spike then propagates back to the cell body, where it is detected by the intracellular electrode.

In order to set the stimulus parameters for a particular neuron, it is necessary to know when it has been fired. As explained above, extracellular recording with the dishes will not tell when a particular cell has been fired, therefore some other technique, such as voltage-sensitive dye recording, is needed.

The combination of multielectrode dishes for stimulation and voltage-sensitive dyes for recording is attractive because it does not require intracellular penetration, and so in theory

could be employed as a noninvasive electrophysiological method for chronic experiments over days or weeks [15]. Fig. 7 shows simultaneous dish stimulation and dye recording on a two-cell SCG culture.

Unfortunately, dish stimulation of SCG neurons has proved more complicated than expected. In response to dish stimulation, we often see subthreshold responses (*e.g.*, the subthreshold response in Fig. 6(b)), instead of the full-fledged action potential that one would expect. Cultured SCG neurons have highly branched axons, and we interpret these subthreshold responses as being action potentials which have failed to propagate backwards beyond a branch point. This failure of backwards propagation may not be a problem with other vertebrate neurons which do not have such highly branched processes.

III. Diving-Board Electrodes

The diving-board electrode, shown schematically in Fig. 8, is a silicon microdevice which can be used as a loose-patch electrode [16]. Each electrode is maneuvered over a cell using a micromanipulator, then glued to the bottom of the tissue culture dish. Since the diving board is portable, it can be placed on any given cell, making a loose-patch seal between the cell and the lower surface of the electrode. This establishes a one-to-one, two-way connection between the diving-board and the cell, which can be used for both stimulating and recording. In principle diving-board electrodes can be placed on many neurons simultaneously, since the micromanipulator is free once each diving-board has been placed.

Fabrication

Integrated-circuit technology and micromachining of silicon provide the tools to fabricate small three-dimensional structures with well-defined mechanical properties [22]. The fabrication is described only briefly here, as it is given in full detail elsewhere [16]. The diving-board electrode is made by a five-mask process, shown in Fig. 9. The first mask defines the cup structure. (The cup's outer rim forms the seal with the cell, and the cup must be deep enough to keep the platinized electrode away from the surface of the cell.) The silicon is etched to a depth of 3 μm using an isotropic silicon etch: 100 HNO_3/100 CH_3COOH/15 HF [23]. The second mask defines a pedestal by using a boron diffusion for 10 h at 1170°C in a 5% O_2/95% N_2 atmosphere and a boron etch-stop process [24]–[26]. Next a 1000 Å thermal oxide layer is grown at 1000°C in steam for 10 min, and the third mask defines the contact hole in this layer. Then 100 Å of chrome, 800 Å of gold, 100 Å of chrome are successively evaporated on the wafer, and the metal pattern is defined. 1 μm of silicon oxynitride, deposited by PECVD [27],[28], forms the top insulating layer. The final mask patterns this top insulating layer, which is etched in an 85% CF_4/15% O_2 plasma [29]. A gold wire bond is made from a large gold bonding pad to each device; this wire will support the device after it has been freed from the silicon wafer. A three-hour etch in ethylenediamine, pyrocatechol, and water (EDP) [30] completely removes the undoped silicon from beneath each electrode. Each device is then separated from the wafer, and the wire bond is insulated by manually loading it into a polyimide tube and painting with negative photoresist. The final step is to electroplate the electrode

tip with platinum black.

Fig. 10 shows scanning electron micrographs of the cup structure and an overview of a finished electrode. Each device takes an average of about 45 minutes to fabricate and test; they can be reused several times. Defective electrodes are eliminated on the basis of optical observations and impedance measurements made before platinization.

Electrode Tests

The diving-board electrodes are placed while being viewed with an inverted microscope equipped for phase and epifluorescence optics. A drop of UV-setting adhesive is placed next to the cell, then the electrode is placed with its pedestal on the glue and the tip of its diving-board in contact with the cell. When a seal resistance of several megohms has been obtained, the drop of glue is exposed to a spot of ultraviolet light from the epifluorescence illuminator. Fig. 11 (a) shows a diving-board electrode in contact with a *Helisoma* B19 neuron.

Two-way connections to cultured *Helisoma* neurons have been established for up to 4 days. Experimental results [16] obtained using diving-board electrodes (R_{seal}=1–5 MΩ) were similar to those obtained with multielectrode dishes when the cell body was sealed over the dish electrode as shown in Fig. 8.

Preliminary studies have also been performed with dissociated rat SCG neurons. It has been possible to stimulate these neurons and to record action potentials with a signal-to-noise ratio of 10:1 (R_{seal}=0.4 MΩ). (Long-term stimulation and recording studies have

not yet been performed.) The success of the diving-board electrodes on SCG neurons demonstrates their versatility: the present devices should be generally useful for neurons 20 μm in diameter or larger, and could be scaled down for use with smaller neurons.

IV. Well Electrodes

Diving-board electrodes and multielectrode dishes have converse properties. Diving-boards form loose-patch seals, and so make good two-way, one-to-one connections; however, they would be hard to use with many cells, since they are time-consuming to place. Multielectrode dishes can easily be used with many cells, but do not make such reliable electrical connections. Multiwell dishes are designed to combine the good characteristics of each (Fig. 12 shows a schematic). Instead of maneuvering an electrode onto the cell to form a seal, the cell is maneuvered into a well which contains an electrode. This scheme also has the added advantage of restricting cell movement, which is desirable for long-term experiments. As a cultured cell on a flat substrate grows and forms connections, its processes pull in various directions, moving it about. This motion would certainly change stimulus thresholds when using multielectrode dishes, and would probably pull the cell out from under a diving-board electrode. A cell in an electrode-well, in contrast, is trapped in contact with the electrode once its processes have grown out through the grillwork and its cell body has enlarged.

Fabrication

Fig. 13 shows the fabrication procedure for multiwell dishes. The first three steps,

forming the lead and electrode insulation, are similar to the fabrication of the multielectrode arrays, while the last three steps form the well and grillwork. The fabrication begins with the same glass-ITO substrate used for the multielectrode arrays. The first aluminum exposure mask, later used to define the electrode tip, is made with a chlorobenzene liftoff [31]. 1350J photoresist is spun on, exposed at 100 mJ/cm^2, soaked in chlorobenzene for 10 min, baked for 5 min at 125°C, and developed. A 2000 Å-thick layer of aluminum is deposited by evaporation, then the photoresist and unwanted aluminum are lifted off in acetone, leaving the exposure mask (Fig. 13(a)). The leads are then patterned, etched, and coated with a thin aluminum-oxide adhesion layer, just as in the microelectrode dish fabrication (Fig. 13(b)). A thin layer of Selectilux is spun onto the surface at 2000 rpm for 10 s, baked for 20 min at 85°C, back-exposed through the glass at 220 mJ/cm^2, and then developed. Since Selectilux is a negative polyimide, this removes the *unilluminated* area over the aluminum exposure mask (Fig. 13(c)). The structure is hardened by baking for 10 min at 200°C . The second exposure mask, used to define the well, is patterned in the same way as the first (Fig. 13(d)). The 12 μm depth of the well is then built up of two layers of Selectilux (Fig. 13(e)). Each layer is spun on at 800 rpm for 10 s, left sitting at room temperature for 10 min to flatten the edge bead, baked for 30 min at 85°C, back-exposed at 1100 mJ/cm^2, developed, then baked for 10 min at 200°C. (This procedure can be repeated if deeper wells are required.) Back-exposing through these built-in exposure masks and building up multiple layers of polyimide is more reliable than a single thick layer patterned with a front-side exposure. Wells are consistently free of residue,

mask-contact problems are eliminated, and finer geometries are possible. The bars over the well are formed by spinning on a thin layer of Selectilux at 5000 rpm for 10 s, baking for 10 min at 85°C, front-exposing at 55 mJ/cm^2, and developing. This short exposure produces a thin, self-supporting grillwork. The finished polyimide structure is cured for 12 h at 200°C. Finally, the aluminum is etched out of the wells and electrode contacts using *Transene aluminum etchant type A* at 50°C for 40 s, and the electrode tip is platinized (Fig. 13(f)). Fig. 14 is a scanning electron micrograph of a completed well.

Growth tests

In order to study cell growth within the confines of a well, prototype multiwell dishes without leads or electrodes were fabricated using anisotropically-etched silicon. One to eight hours after neurons were plated, they were pushed into the wells using a very fine glass tool held by a micromanipulator. Fig. 15 shows a rat SCG neuron in such a well, with many processes growing out. Once cells were placed in these wells, their cell bodies remained trapped, and they seemed to grow normally, extending many processes outside the well. SCG neurons have been cultured in well prototypes for up to six weeks.

The multiwell dishes have since been modified to use the polyimide-on-glass fabrication described above. This transparent device is much more convenient for microscopy, and the fabrication process is simpler than the anisotropic silicon process. Wells with leads and electrodes have been made, and electrical tests with neurons are currently underway.

V. Discussion

The ultimate goals of this work are to obtain long-term connections for both stimulation and recording, and maintain such connections with many neurons at once. Our experience shows that a reliable two-way electrical connection requires close contact between electrode and cell body, at least close enough to form a loose-patch seal of several megohms.

Rat SCG neurons grown on multielectrode dishes do not form these close contacts, making recording from specific neurons very difficult. Combining multielectrode dishes for stimulation with voltage-sensitive dyes for recording offers the capability of detecting synaptic connections; however, stimulation does not seem to be reliable with these cells, probably due to their highly branched geometry. Other cell types, such as cultured hippocampal neurons [32], have fewer branches and may be better suited for stimulation with these dishes.

Close contacts *are* formed by large invertebrate neurons on multielectrode dishes, and by both vertebrate and invertebrate neurons with diving-board electrodes. We expect that vertebrate neurons in electrode-wells shall form close contacts. These loose-patch seals make it easy to record action potentials and to tell which neuron is firing, and also make it easy to stimulate neurons to fire action potentials. Multielectrode dishes and diving-boards have both been used to make long-term connections to invertebrate neurons, which do not move much. Vertebrate neurons are held in place by the prototype multiwell dishes, and so should make long-term connections with electrode-wells, once they are grown in

the complete multiwell dishes. Multielectrode dishes are the simplest way to make chronic connections to cultures of many invertebrate neurons, while multiwell dishes should be the method of choice for cultures of many vertebrate neurons.

There are improvements in the electrical connection which still might be made. The current seal resistances of a few megohms do not allow recording of subthreshold PSPs. One exciting possibility is that this resistance could be improved to hundreds of megohms or greater, as is possible with glass pipettes, perhaps by chemically treating the electrode surface. The membrane patch under the electrode could then be broken down with a voltage pulse, forming the integrated-device equivalent of a whole-cell patch electrode. Such a chronic *intracellular* connection would allow recording of fast PSPs. (However, metal electrodes are fundamentally limited to AC connections, and so could not record slow events, or the DC intracellular potential.)

Chronic experiments not amenable to conventional techniques have already been made possible by these three devices. In the future, they promise to make it possible to perform long-term experiments on cultures of 3–10 neurons, recording from all cells simultaneously, and stimulating any cell at will.

VI. Acknowledgments

The authors are grateful to Dr. S. B. Kater for supplying *Helisoma* neurons and for making his laboratory available for experiments, and to Dr. C. Cohan for helping with the *Helisoma* physiology. Thanks also to the indispensable B. Tanamachi, for culturing SCG

neurons. This research was supported by NSF grant #BNS-8603713 and NIH grant #5 R01 NS22450-02.

References

[1] P. G. Nelson and M. Lieberman, eds., *Excitable Cells in Tissue Culture*, Plenum Press, New York, 1981.

[2] D. F. Ready and J. F. Nicholls, "Identified neurons isolated from the leech CNS make selective connections in cell culture," *Nature*, vol. 281, pp. 67–69, Sept. 1979.

[3] R. G. Wong, R. D. Hadley, S. B. Kater and G. Hauser, "Neurite outgrowth in molluscan organ and cell cultures: The role of conditioning factor(s)", *J. Neurosci.*, vol. 1, pp. 1008–1021, 1981.

[4] D. Dagan and I. B. Levitan, "Isolated identified *Aplysia* neurons in cell culture," *J. Neurosci.*, vol. 1, pp. 736–740, 1981.

[5] C.-P. Ko, H. Burton, M. I. Johnson, and R. P. Bunge, "Synaptic transmission between rat superior cervical ganglion neurons in dissociated cell cultures," *Brain Research*, vol. 117, pp. 461–485, 1976.

[6] A. Marty and E. Neher, "Tight-seal whole-cell recording," in *Single Channel Recording*, eds. B. Sakmann and E. Neher, Plenum Press, New York, pp. 107–122, 1983.

[7] G. W. Gross, A. N. Williams, and J. H. Lucas, "Recording of spontaneous activity with photoetched microelectrode surfaces from mouse spinal neurons in culture," *J. Neurosci. Methods*, vol. 5, pp. 13–22, 1982.

[8] C. A. Thomas, Jr., P. A. Springer, G. E. Loeb, Y. Berwald-Netter, and L. M. Okun, "A miniature microelectrode array to monitor the bioelectric activity of cultured cells," *Exp. Cell Res.*, vol. 74, pp. 61–66, 1972.

[9] J. Pine, "Recording action potentials from cultured neurons with extracellular microcircuit electrodes," *J. Neurosci. Methods*, vol. 2, pp. 19–31, 1980.

[10] D. W. Tank, C. S. Cohan, and S. B. Kater, "Cell body capping of array electrodes improves measurements of extracellular voltages in micro–cultures of invertebrate neurons," *IEEE Conference on Synthetic Microstructures*, Airlie, 1986.

[11] D. A. Israel, W. H. Barry, D. J. Edell, and R. G. Mark, "An array of microelectrodes to stimulate and record from cardiac cells in culture," *Am. J. Physiol.*, vol. 247, no. 4, pp. H669–H674, Oct. 1984.

[12] W. Stuhmer, W. M. Roberts, and W. Almers, "The loose patch clamp," in *Single Channel Recording*, eds. B. Sakmann, and E. Neher, Plenum Press, New York, pp. 123–132, 1983.

[13] A. Grinvald, "Real-time optical imaging of neuronal activity," *TINS*, vol. 7, pp. 143–150, May 1984.

[14] B. M. Salzberg, "Optical recording of electrical activity in neurons using molecular probes, in *Cellular Mechanisms of Neurobiology*, vol. 3, 1982.

[15] C.-B. Chien, W. D. Crank, and J. Pine, "Noninvasive techniques for measurement and long–term monitoring of synaptic connectivity in microcultures of sympathetic neurons," *Soc. Neurosci. 17th Annual Meeting*, abstract 393.15, 1987.

[16] W. G. Regehr, J. Pine, and D. B. Rutledge, "A chronic *in vitro* neuron-microdevice connection," *IEEE Trans. Biomed. Engr.* (in press).

[17] J. Pine, W. G. Regehr, T. J. Kramer, and D. B. Rutledge, "Neuron-Microdevice

Communication," *Soc. Neurosci. 16th Ann. Meeting*, abstract 147.1, 1986.

[18] J. Pine, J. R. Gilbert, and W. G. Regehr, "Microdevices for stimulating and recording from cultured neurons." in *Artificial Organs: The W. J. Kolff Festschrift*, ed. J. D. Andrade, VCH Publishers, Deerfield, Florida, 1986.

[19] G. W. Gross, W. Y. Wen, and J. W. Lin, "Transparent indium-tin oxide electrode patterns for extracellular, multisite recordings in neuronal cultures," *J. Neurosci. Methods*, vol. 15, no. 3, pp. 243–252, 1985.

[20] R. C. Gesteland, B. Howland, J. Y. Lettvin, and W. H. Pitts, "Comments on microelectrodes," *Proc. IRE*, vol. 47, pp. 1856–1862, Nov. 1959.

[21] D. A. Robinson, "The electrical properties of metal electrodes," *Proc. IEEE*, vol. 56, no. 6, pp. 1065-1071, June 1968.

[22] K. E. Peterson, "Silicon as a mechanical material", *Proc. IEEE*, vol. 70, no. 5, pp. 420–457, May 1982.

[23] H. Robbins and B. Schwartz, "Chemical etching of silicon, II. The system HF, HNO_3, H_2O, and $HC_2H_3O_2$," *J. Electrochem. Soc.*, vol. 107, p. 108-111, Feb. 1960.

[24] Sohio Carborundum PDS Planar Diffusion Sources: Technical Information, 1986.

[25] D. Rupprecht and J. Stach, "Oxidized boron nitride wafers as an *in-situ* boron dopant for silicon diffusions," *J. Electrochem. Soc.*, vol. 120, no. 9, pp. 1266–1271, Sept. 1973.

[26] K. Najafi, K. D. Wise, and T. Mochizuki, "A high-yield IC-compatible multichannel recording array," *IEEE Trans. Electron Devices*, vol. ED-32, no. 7, pp. 1206-1211, July

1985.

[27] A. R. Reinberg, "Plasma deposition of inorganic thin films," *Ann. Rev. Mater. Sci.*, vol. 9, pp. 341-72, 1979.

[28] W. R. Snow, "PWS 450 COYOTE: A vertical parallel plate plasma reactor for silicon nitride deposition," Pacific Western Systems Application Note, March 1981.

[29] C. M. Melliar–Smith and C. J. Mogab, "Plasma assisted etching techniques for pattern delineation," in *Thin Film Processes*, ed. J. L. Vossen and W. Kern, Academic Press, New York, pp. 497–552, 1978.

[30] A. Reisman, M. Berkenblit, S. A. Chan, F. B. Kaufman, and D. C. Green, "The controlled etching of silicon in catalyzed ethylenediamine-pyrocatechol-water solutions," *J. Electrochem. Soc.*, vol. 126, no. 8, pp. 1406–1415, Aug. 1979.

[31] M. Hatzakis, B. J. Canavello, and J. M. Shaw, "Single-step optical lift-off process," *IBM J. Res. Develop.*, vol. 24, pp. 452-460, 1980.

[32] G. A. Banker and W. M. Cowan, "Rat hippocampal neurons in dispersed cell culture," *Brain Res.*, vol. 126, pp. 397–425, 1977.

Figure 1 Phase-contrast micrographs of cultured neurons. (a) *Helisoma* B19 snail neuron after 3 days in culture. (b) Rat SCG neurons after 2 weeks.

Figure 2 Intracellular recordings of action potentials. The top traces show current pulses injected through intracellular electrodes, and the lower traces show the intracellular potential. (a) *Helisoma* B19 snail neuron. (b) Rat SCG neuron.

Figure 3 (a) Fabrication of multielectrode array. (b) Central lead pattern of array. The hexagonal array consists of 61 electrodes, 12 μm in diameter and 70 μm apart.

Figure 4 Completed multielectrode culture dish (4 cm in length).

Figure 5 Cultured invertebrate neurons sealing over dish electrodes. (a) Schematic of a neuron sealing over an electrode. (b) Spontaneous activity recorded from *Helisoma* neuron using a multielectrode array (bandwidth 10 Hz–1 kHz). (c) A dish electrode stimulates a cell (not shown) with a current pulse, and the same electrode is used to record the resulting action potential (*top trace*). For this test, an intracellular electrode was used to confirm the stimulation (*bottom trace*).

Figure 6 Extracellular stimulation of cultured rat SCG neurons using a multielectrode dish. (a) Cell 1 was penetrated with an intracellular electrode, which monitored its activity while it was stimulated using extracellular electrode B3. (b) The top traces show a series of bipolar current pulses injected through electrode B3. The lower traces show the responses of the cell, as measured with the intracellular electrode. At first there is no response, then a subthreshold response, then a full-fledged action potential.

Figure 7 Stimulation of cultured rat SCG neurons with a multielectrode dish, combined with voltage-sensitive dye recording. (a) Activity in cells 1 and 2 (the only cells in this culture) was monitored using voltage-sensitive dye fluorescence, while stimuli were applied through electrodes B4 and D4. The squares over the cells are the fields of view of the photodetectors used to record optical signals; the two pixels over cell 2 have been added together to produce the traces shown in (b) and (c). (b) Stimulating with D4. The top trace shows the current pulse used as a stimulus; the other traces are optical signals from cell 2 and cell 1, respectively. Cell 1 fired an action potential, while cell 2 shows no activity. (c) Stimulating with B4. Cell 2 fired an action potential, while cell 1 shows a putative PSP, presumably due to synaptic input from cell 2. Six consecutive trials have been averaged in order to improve the signal-to-noise ratio.

Figure 8 Schematic of a diving-board electrode in contact with a cell.

Figure 9 Fabrication procedure for the diving-board electrode.

Figure 10 A finished diving-board electrode. (a) Top view of the cup structure. (b) Bottom view of the cup. (c) Overview of the electrode.

Figure 11 (a) Diving-board electrode in chronic electrical contact with a *Helisoma* B19 neuron. The pedestal is glued to the bottom of the culture dish and the electrode tip is in contact with the cell body. (b) Diving-board electrode in contact with a rat SCG neuron.

Figure 12 Schematic of cultured neuron in an electrode well. (a) Top view of the well. (b) Cross-section through a well containing a neuron.

Figure 13 Fabrication procedure for multiwell dishes.

Figure 14 Scanning electron micrograph of a finished electrode well.

Figure 15 Differential-interference contrast micrograph of rat SCG neurons growing from prototype wells, after 2 days in culture.

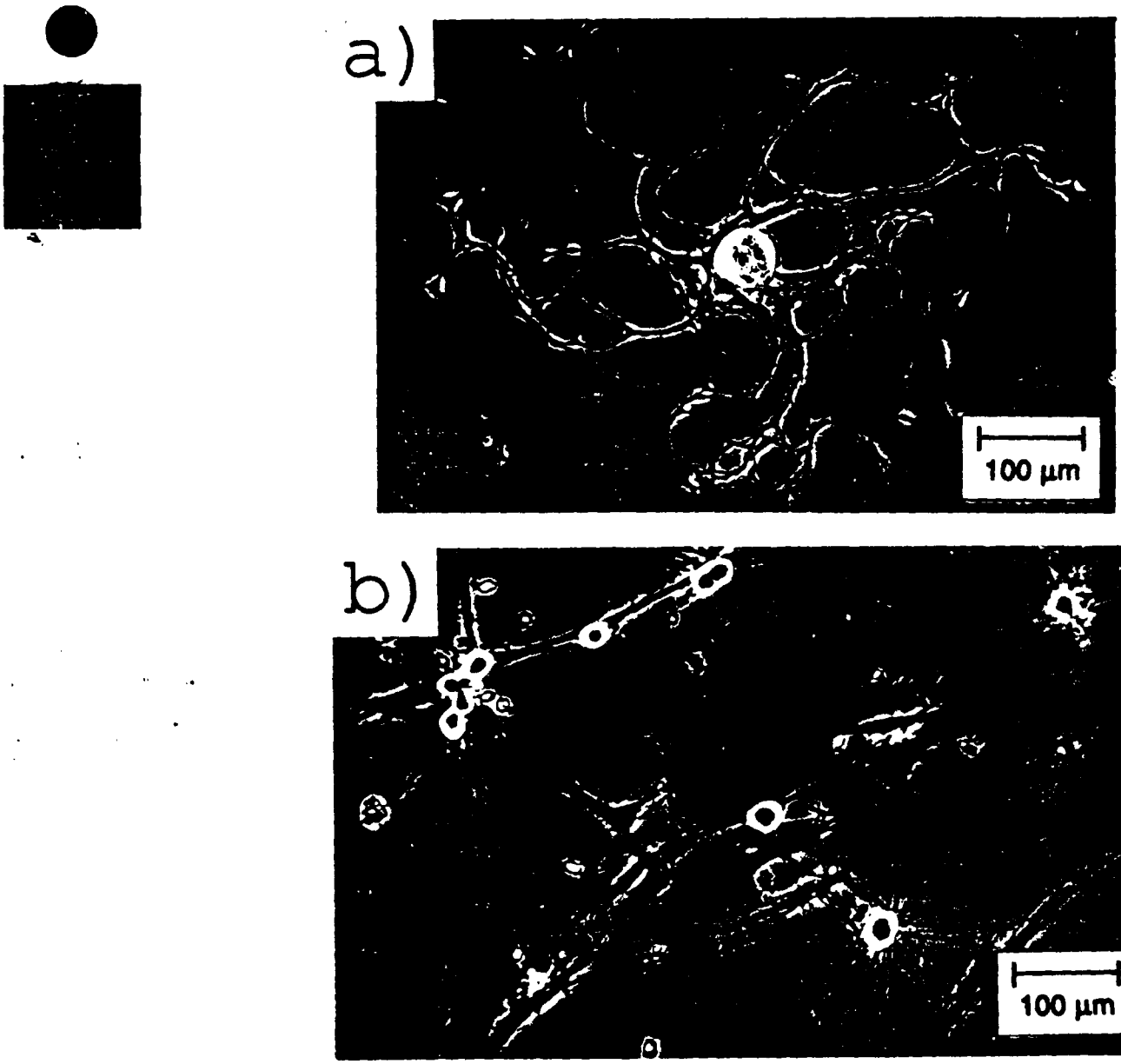


FIG. 1

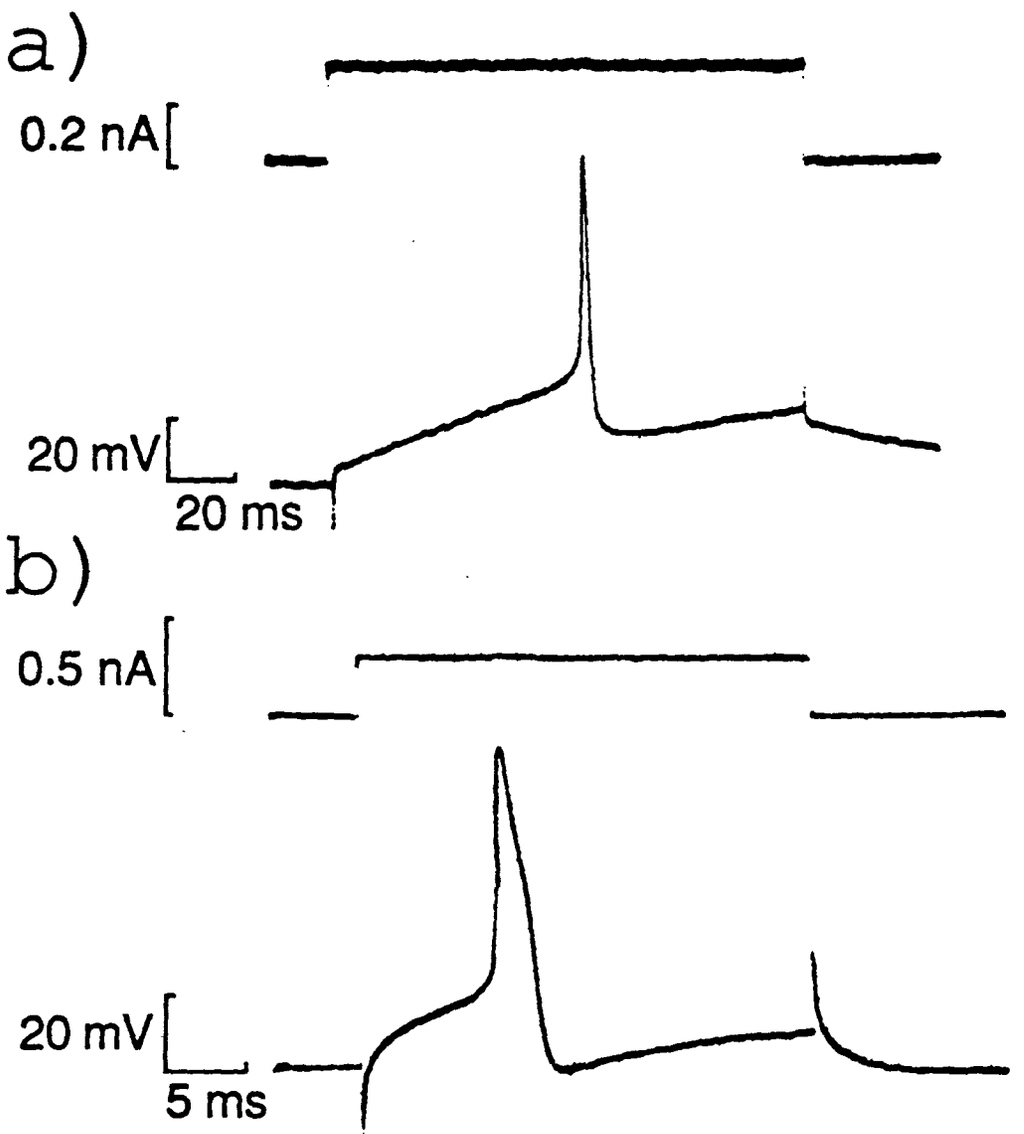
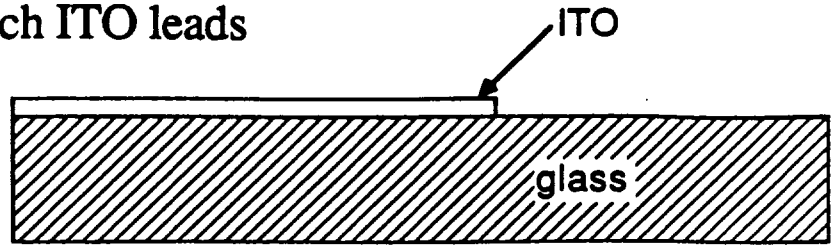


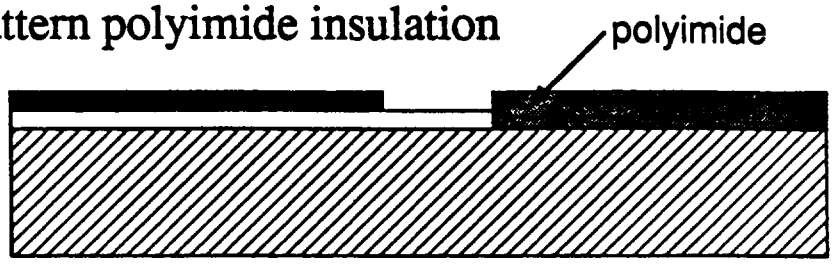
FIG. 2

a)

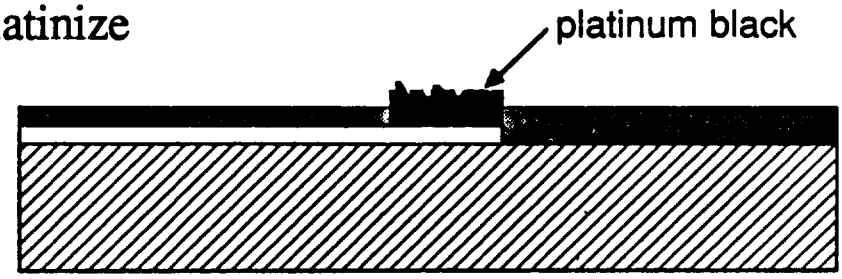
(1) Etch ITO leads



(2) Pattern polyimide insulation



(3) Platinize



b)

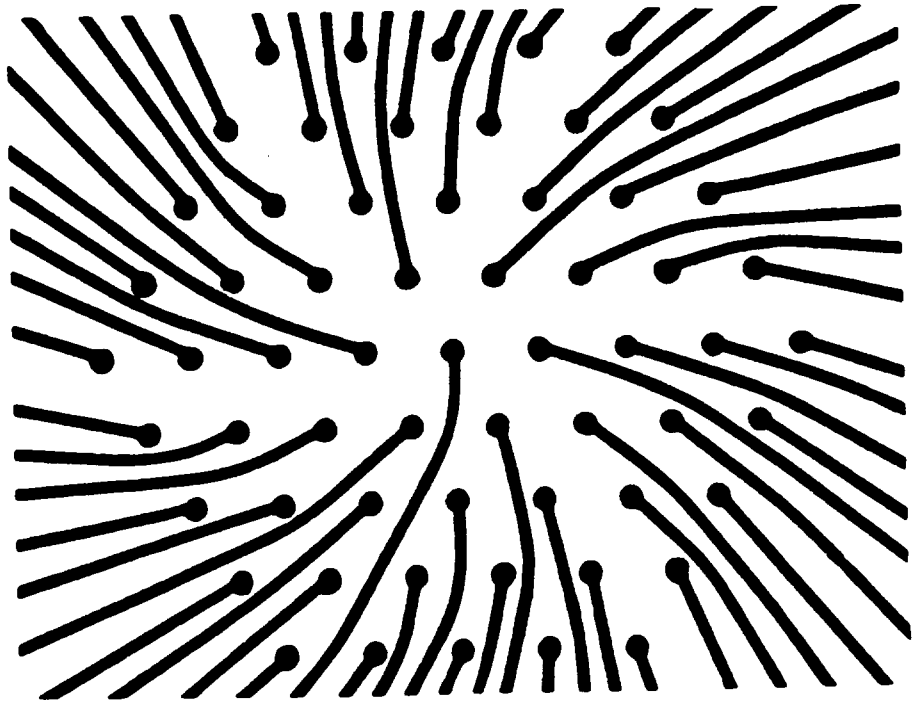


FIG. 3

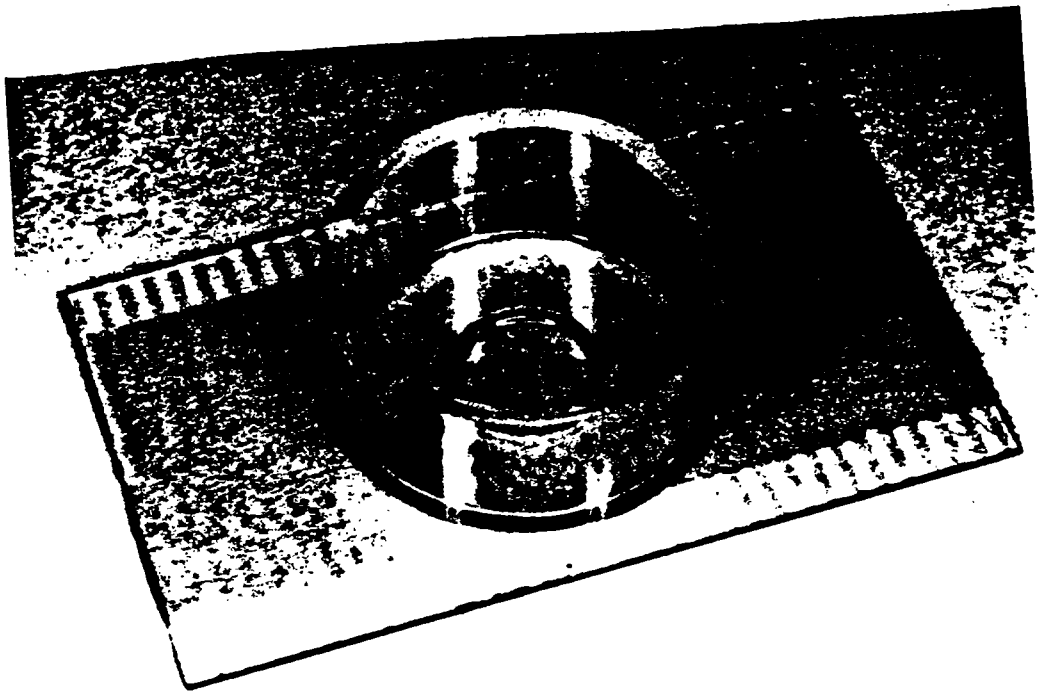


FIG.4

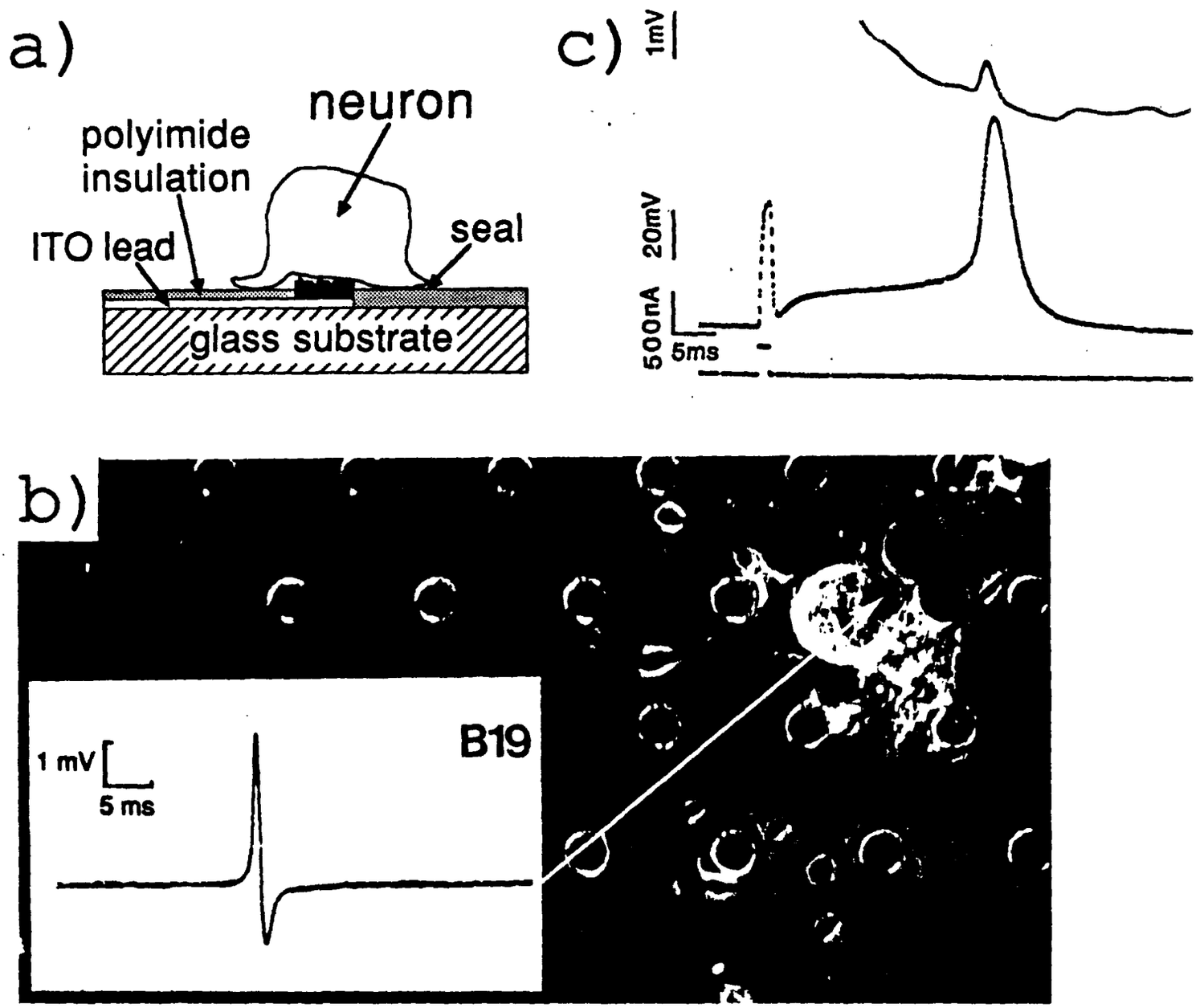
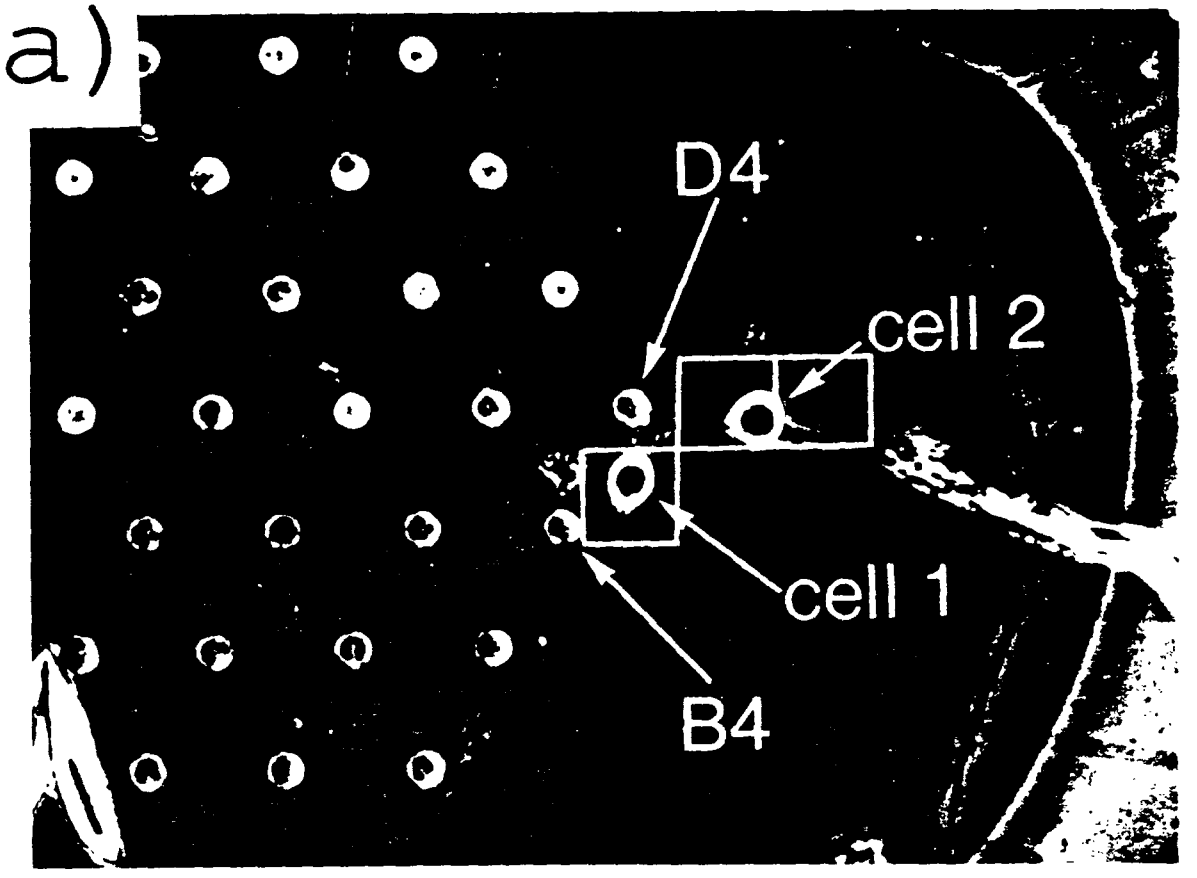

a)
neuron
polyimide
insulation
ITO lead
seal
glass substrate
c)
1mV
20mV
500nA
5ms
b)
B19
1 mV
5 ms

FIG. 5


a)
D4
cell 2
cell 1
B4

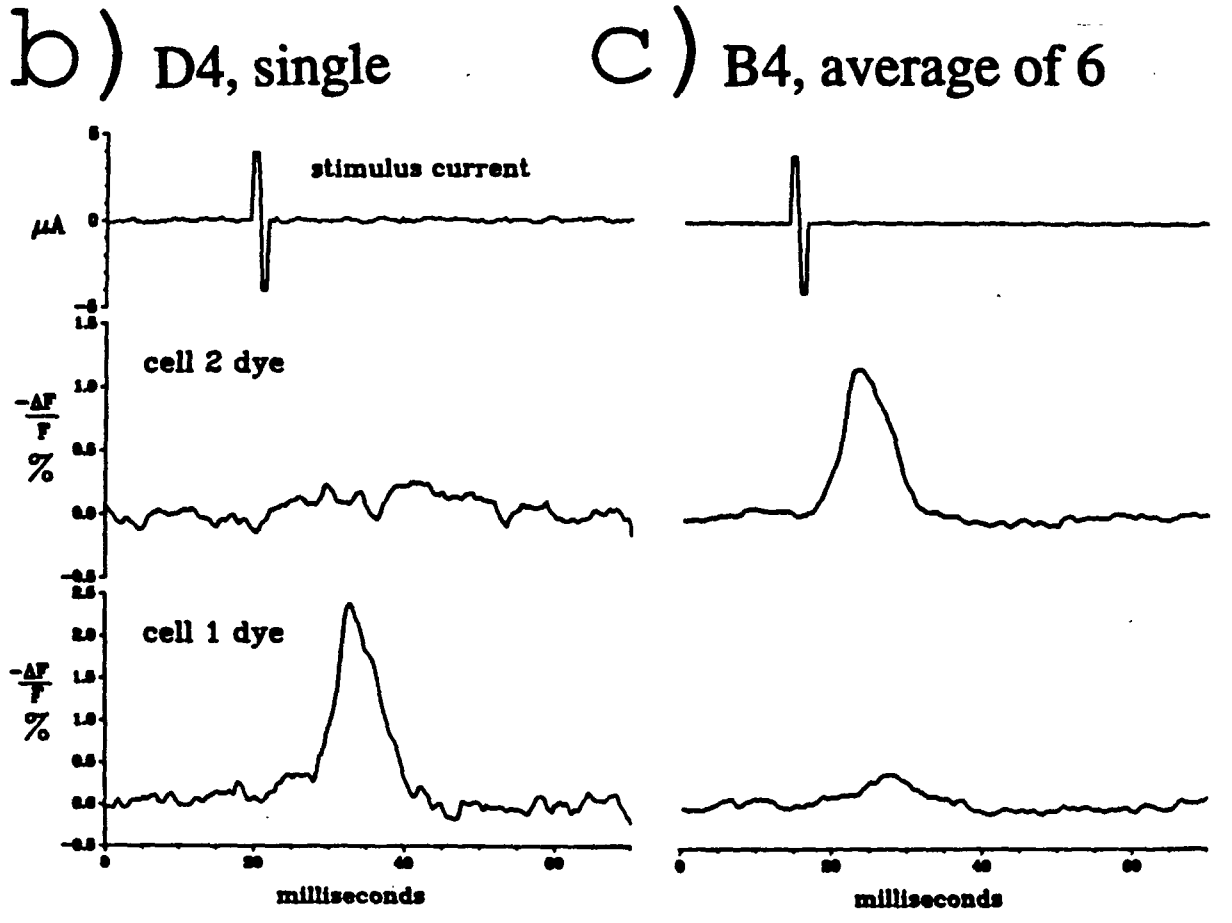

b) D4, single
c) B4, average of 6
stimulus current
μA
cell 2 dye
-ΔF/F %
cell 1 dye
-ΔF/F %
milliseconds
milliseconds

FIG 7

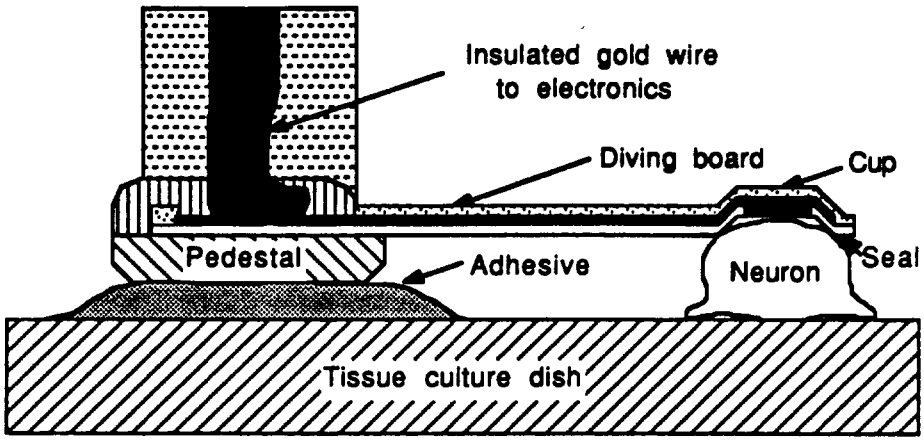
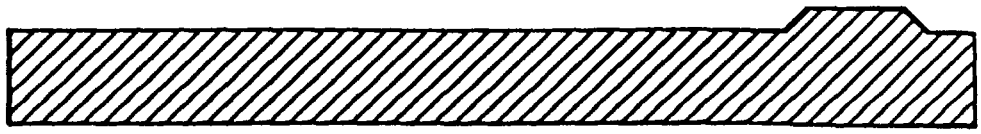
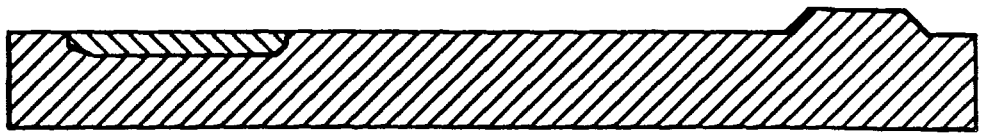

Insulated gold wire
to electronics
Diving board
Cup
Pedestal
Adhesive
Neuron
Seal
Tissue culture dish

FIG. 8

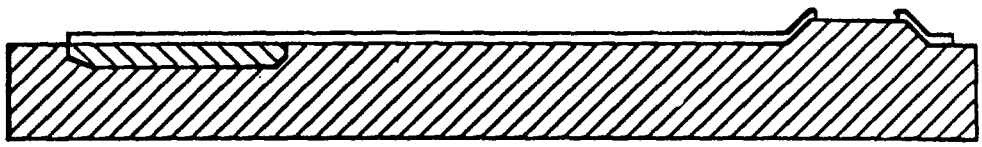
1. Mask 1.
   Define cup



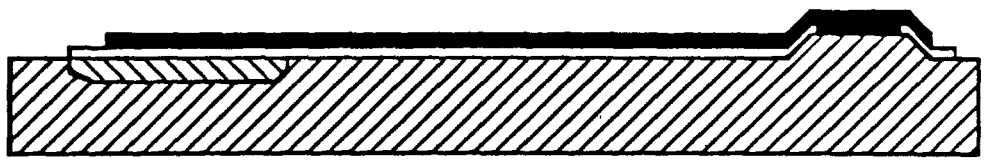
2. Mask 2.
   Boron diffusion



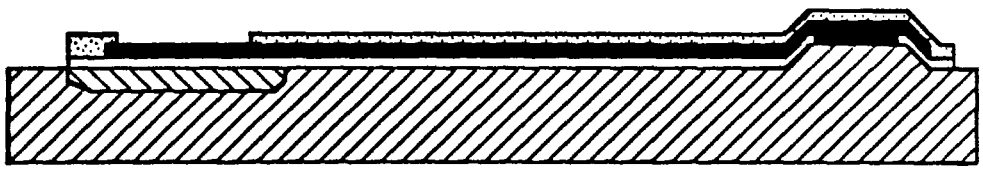
3. Mask 3.
   Silicon dioxide
   insulation



4. Mask 4.
   Metallization



5. Mask 5.
   Silicon oxynitride
   insulation



6. Wire-bonding and
   final etch

7. Insulate wire-bond
   and platinize

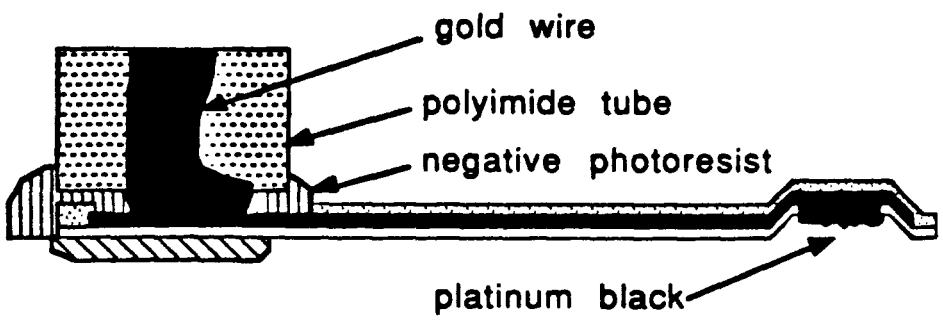


FIG. 9

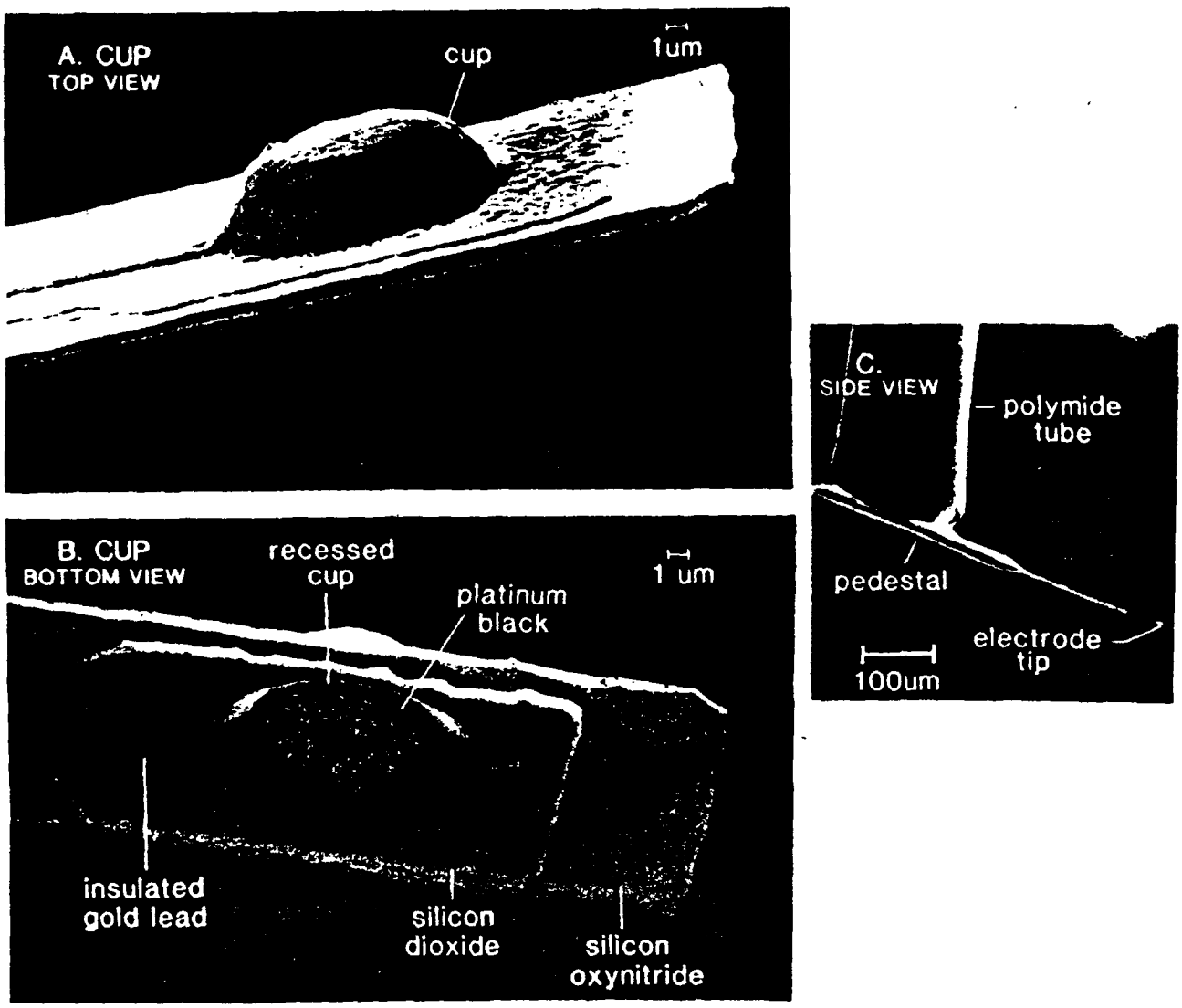

A. CUP
TOP VIEW
cup
1um
B. CUP
BOTTOM VIEW
recessed
cup
platinum
black
1 um
insulated
gold lead
silicon
dioxide
silicon
oxynitride
C.
SIDE VIEW
polymide
tube
pedestal
electrode
tip
100um

FIG. 10

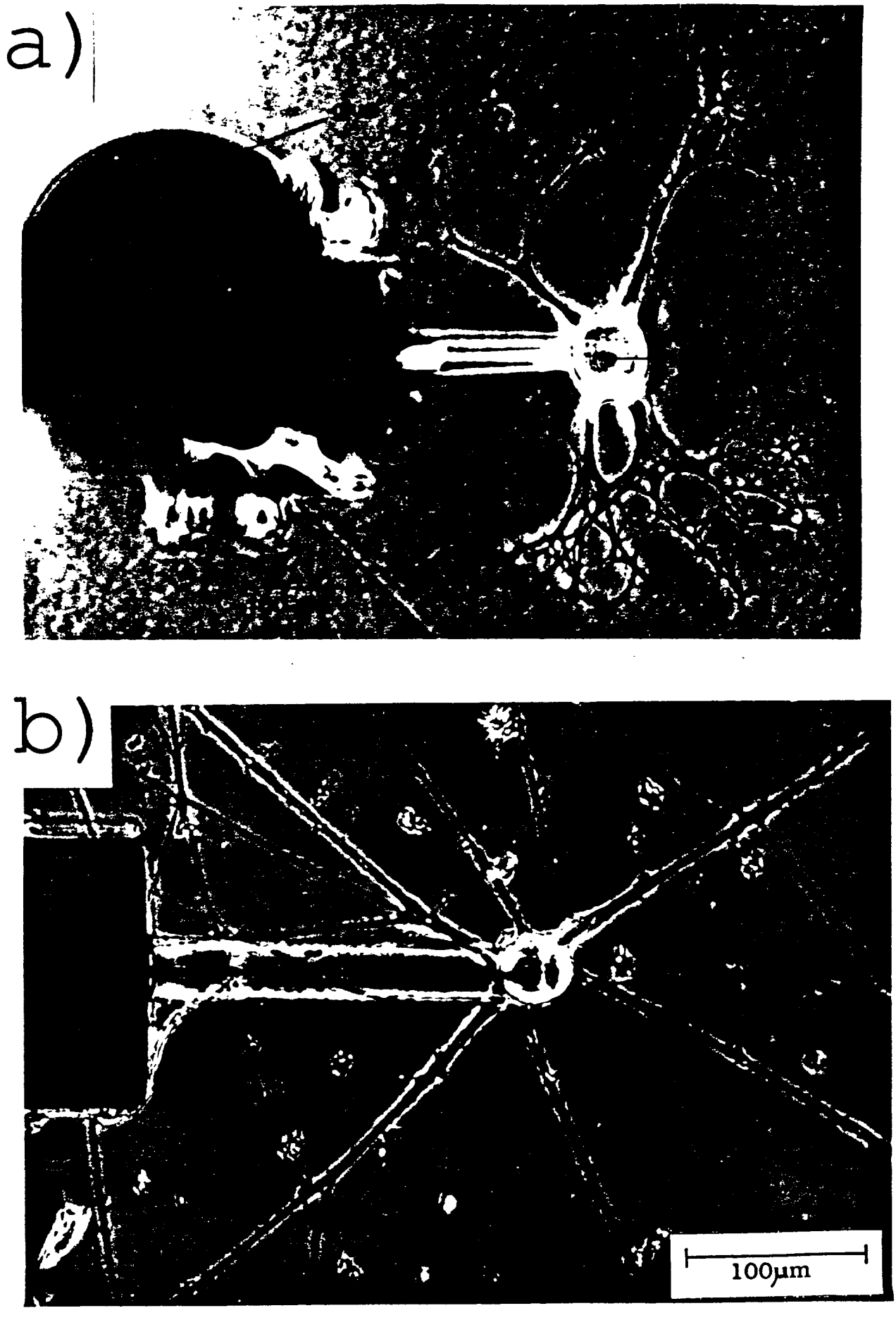


FIG 11

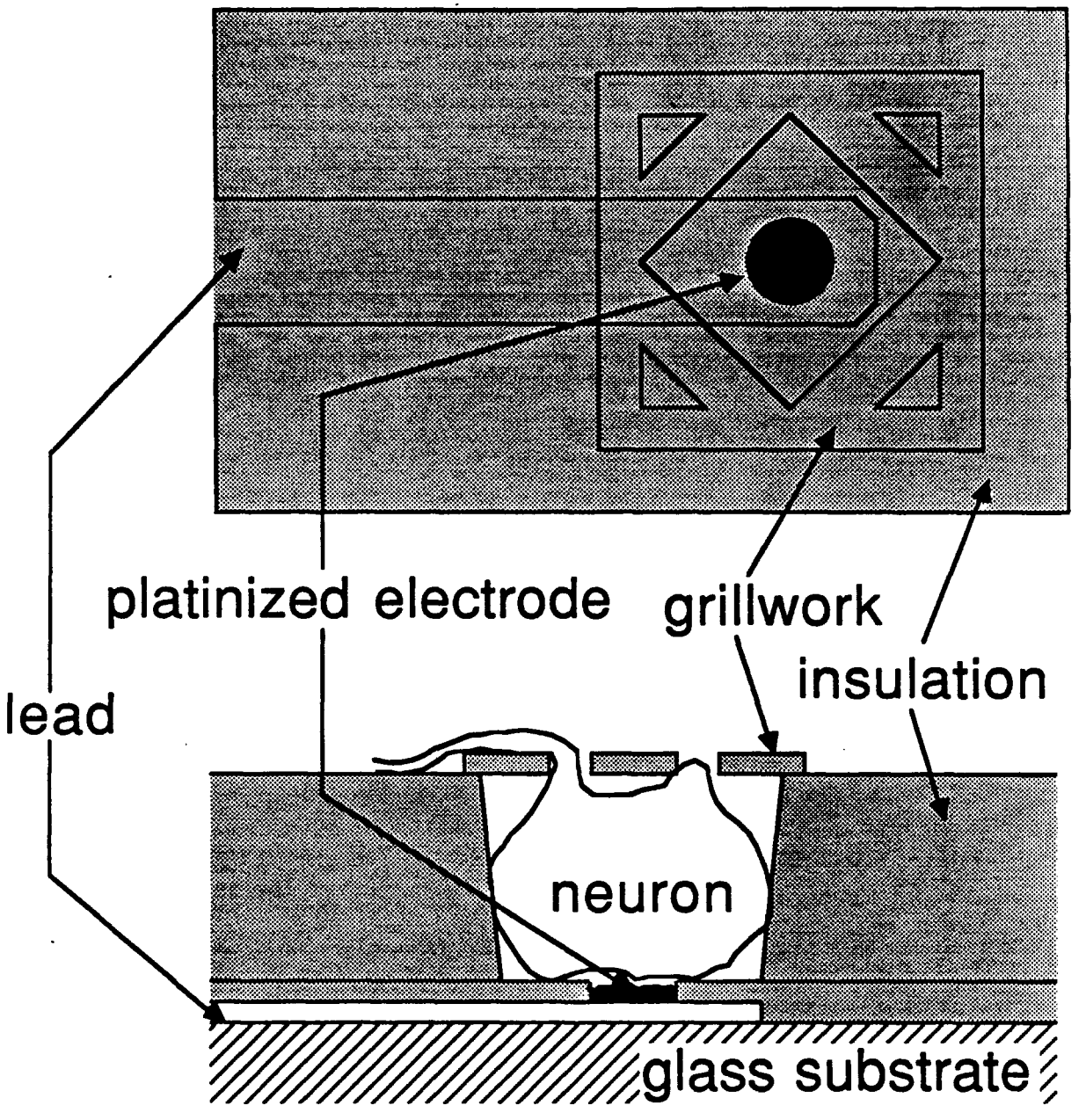
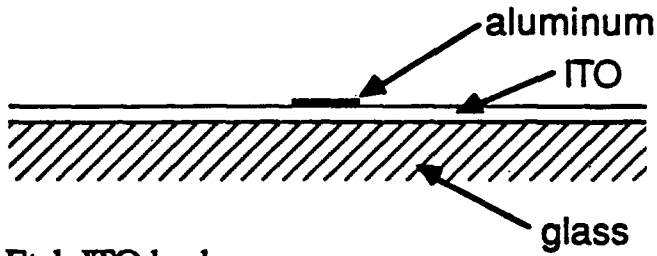


FIG. 12 (on Mac disk)

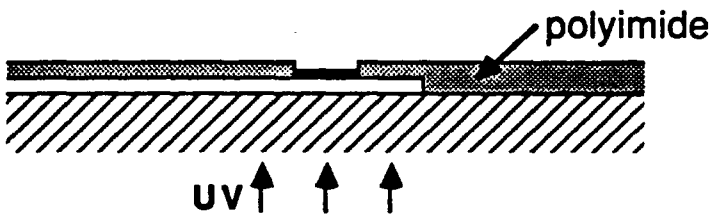
Tina's current well fabrication
6/7/88
Saved as well fab

(a) Pattern aluminum mask for contact holes

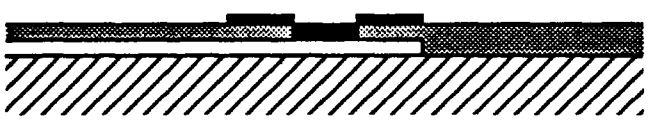


(b) Etch ITO leads

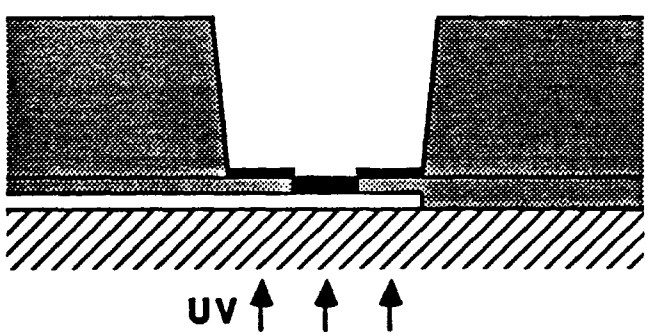
(c) Make contact holes using backillumination



(d) Pattern aluminum mask for wells



(e) Make wells using backillumination



(f) Etch out aluminum, add grillwork

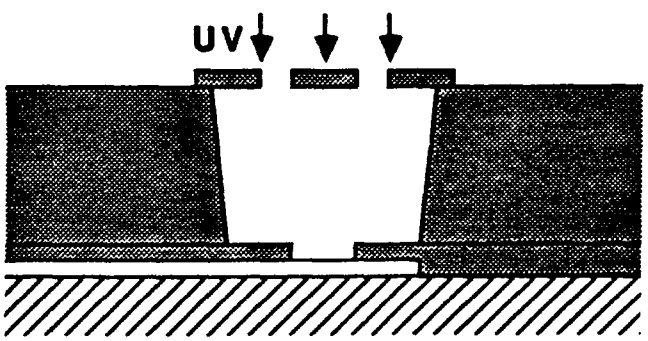


FIG. 13 (on Mac disk)

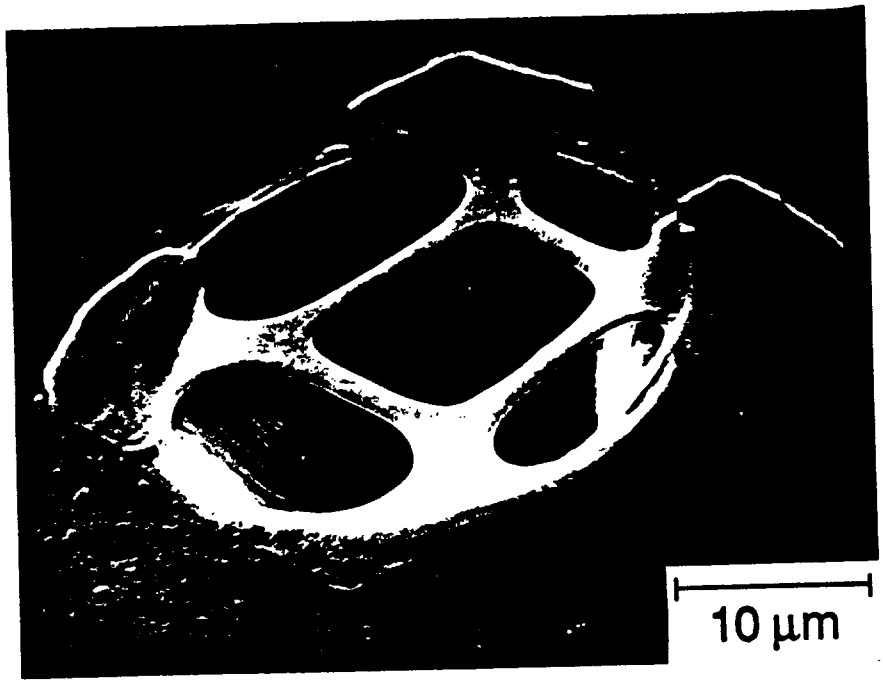


FIG. 14

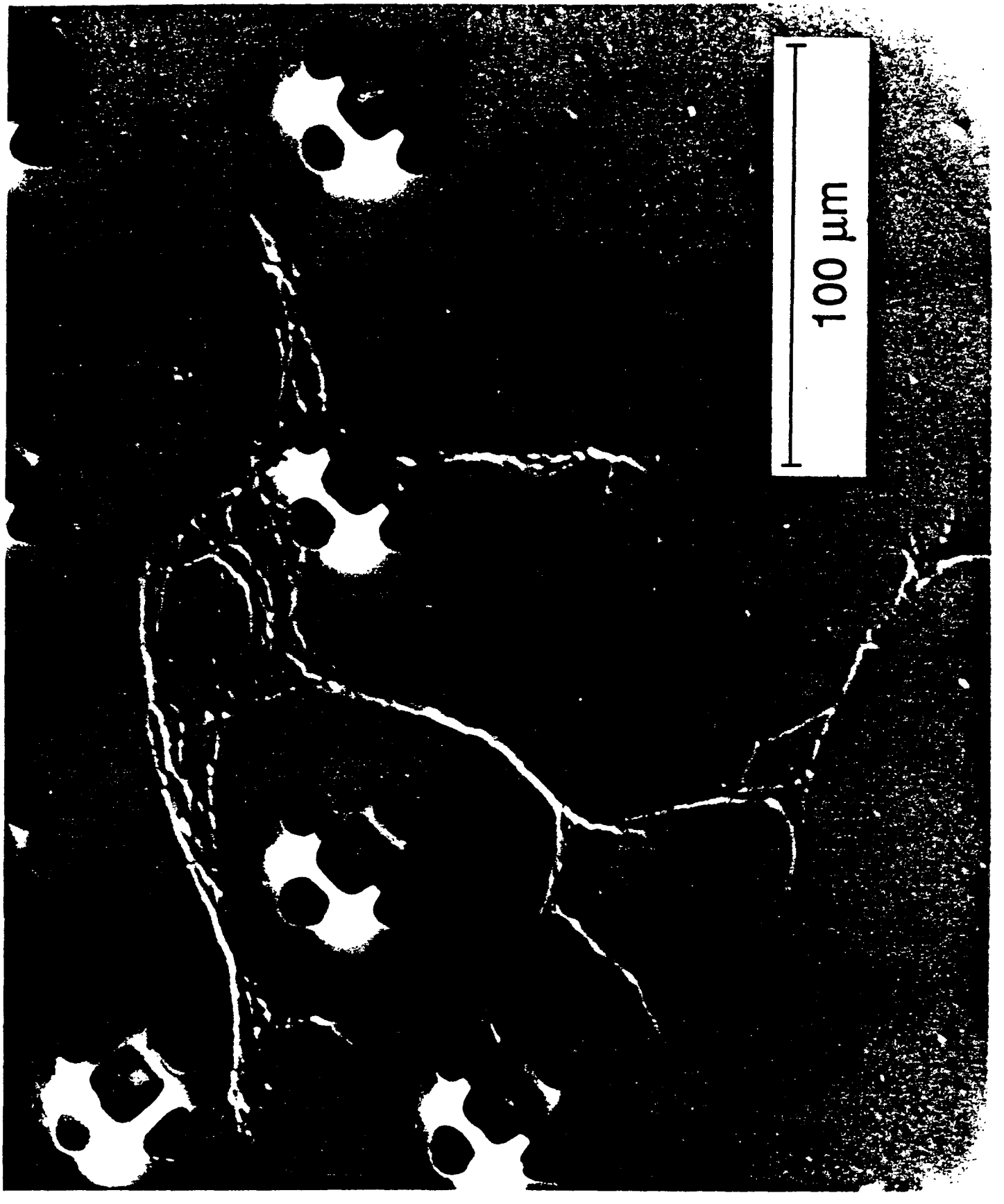


FIG. 15

Section III
PROBES AND INTERFACES
Area Summary

This session addresed methods of studying the nervous tissue and ways in which specialized microstructures can facilitate sensing, viewing, and manipulating them. The first talk reviewed new developments in X-ray microscopy. The second talk by Dr. Gebrial of AT&T presented interesting novel methods of fabricating micromechanical devices using silicon technology. Dr. Ligler then outlined the latest approaches in the fabrication of so-called receptor-based biosensors, and the developments in the key components of these systems. Finally, Dr. Krishnaprasad of the Univ. of Md. reviewed the theoretical and experimental studies his group have recently undertaken in the development of tactile sensor arrays, processing algorithms, and interface circuitry.

SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH

22 MARCH, SESSION III
MICROSCOPY AND SENSOR ARRAYS

9:00 a.m.. - 9:45 a.m.
1. MICROSCOPIC ANALYSIS OF LIVING SYSTEMS (Janos Kirz, SUNY Stony Brook, Stony Brook, New York)
9:45 a.m - 10:30 a.m.
2. MICROMECHANICAL COMPONENTS AND STRUCTURES ETCHED FROM SILICON (K. Gabriel, ATT-Bell Labs, Holmdel, New Jersey)
BREAK: 10:30 a.m. - 1045 a.m.
3. FABRICATION OF KEY COMPONENTS FOR A RECEPTOR-BASED BIOSENSOR (Fran Ligler, Naval Research Laboratory, Washington, D.C.)
10:45 a.m.- 11:30 a.m.
4. NOVEL DATA-REDUCTION TECHNIQUES FOR SENSOR ARRAYS (P.S.Krishnaprasad, University Of Maryland, College Park, Maryland)

New developments in x-ray microscopy

Janos Kirz
Physics Department, SUNY, Stony Brook, NY 11794

Synchrotron radiation from undulators make it possible to perform experiments with coherent soft x-rays. These sources can be used for holography, as well as high resolution microscopy of biological specimens. Synthetic microstructures (Fresnel zone plates) are used as the optics.

Recent experiments at the National Synchrotron Light Source demonstrate better than 100 nm resolution, and the ability to image wet biological material without fixation, stain, freezing or sectioning. This work, a collaboration between NSLS, the Center for X-ray Optics at Lawrence Berkeley Laboratory, IBM, and Stony Brook, is about to enter a new phase with the commissioning of the Soft X-ray Undulator. We anticipate better resolution, faster data acquisition, and more information on the third dimension (depth). The ultimate limitation on the imaging of sensitive biological material will be set by radiation damage.

MICRO MECHANICAL COMPONENTS AND STRUCTURES ETCHED FROM SILICON

K.J. Gabriel
M. Mehregany
W.S.N. Trimmer

Micro mechanical systems have many advantages for applications in robotics: rapid motions, higher accuracy, gentleness, reduced mass and reduced space requirements. Few systems have been developed because of the difficulties involved in making small mechanical components, actuators and sensors. New fabrication techniques, such as anisotropic and reactive ion etching of silicon, are making the manufacturing of small, precision parts practical and potentially inexpensive. These micro mechanical systems are applicable in many diverse areas: medical apparatus and services, instruments for scientific research, industrial equipment and consumer products.

We will describe some of the micro mechanical components we have fabricated using modified deposition and etching techniques originally developed in silicon electronic fabrication. Using bulk and surface micromachining techniques, free-standing, multiple and interconnected micro mechanical structures were fabricated such that they are constrained but not attached to the substrate. Turbines with gear and blade rotors as small as 125 μm in diameter and 4.5 μm thick were fabricated on 20 μm-diameter shafts. A clearance as tight as 1.2 μm was achieved between the gear and shaft. Gear-trains with two or three sequentially aligned gears were succesfully meshed. A sub-millimeter pair of tongs with a 400 μm range of motion at the jaws was also fabricated.

FABRICATION OF A RECEPTOR-BASED BIOSENSOR

T.L. Fare, A. Singh, K.D. Seib
J.W. Smuda, P. Ahl, F.S. Ligler
Naval Research Laboratory
Bio/Molecular Engineering Branch
Washington, DC 20375-5000

Address for Correspondence: T.L. Fare, Naval Research Laboratory, Bio/Molecular Engineering Branch, Code 6190, Washington, DC 20375-5000 (202) 767-4302

ABSTRACT

A three-pronged approach has been taken for the development of this receptor-based biosensor: First, asymmetric bilayer membranes were developed for protein incorporation (one monolayer adapted to the particular receptor of interest, the other monolayer polymerized to enhance membrane stability). Second, alamethecin and calcium channel complexes were introduced into the stabilized membrane and tested for ion channel function. And third, a porous support for the receptor-containing membrane was fabricated which is compatible with silicon technology. First-generation devices incorporating these components have been constructed.

KEY WORDS: Biosensor, receptor, polymerized lipid, porous electrode

ABBREVIATIONS: a.c., alternating current; BJT, bipolar junction transitor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; d.c., direct current; $DC_{8,9}PC$, 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine; LB, Langmuir-Blodgett; MaPC, 1,2-bis(12-methacryloyloxy-dodecanoyl)-sn-glycero-3-phosphocholine; pS, picoSiemens

INTRODUCTION

A receptor-based biosensor uses biomolecules with specific molecule-recognition capability to detect chemicals. For the receptor-based biosensor to be discussed here, proteins composed of binding sites and ion channels are embedded in a lipid bilayer. The protein-lipid membrane is deposited onto a transducing electrode (1,2). The lipid bilayer has two purposes: first, it serves as the medium to support the proteins, and, second, it acts as an insulating membrane on the transducing electrode. When a chemical of interest binds to the receptor site, the ion channel opens to permit an ionic current to flow through the membrane. The presence of the chemical of interest may thus be detected by the ion current.

Receptors with ligand-gated channels have several advantages as detection elements. Receptors (such as the acetylcholine receptors, olfactory receptors, and GABA receptors) recognize families of chemicals, thus one receptor can be used to monitor a number of physiologically active compounds. Unlike antibody-based sensors, a toxic chemical does not have to be specifically identified before a sensor is constructed. Any chemical which induces the opening of the ion channel is identified as being toxic. The measurement of the physiological function can also be used to detect combinations of chemicals, each of which may not be harmful, but which act in tandem to become toxic. Since the detection mechanism is based on a physiological response rather than solely on ligand binding, the incidence of false positives is greatly reduced. The potential advantages of such systems include exquisite sensitivity, tailored specificity, and speed of response (seconds).

In the work described here, a three-pronged approach has been taken to the development of an amperometric receptor-based biosensor. First, methodology for stabilizing the lipid bilayer has been developed. These asymmetric membranes include a polymerized lipid monolayer designed to enhance membrane stability and a nonpolymerized monolayer in which the lipids are chosen so as to optimize function of the receptor protein. Preliminary work on these asymmetric membranes was reported by Dalziel et al. (3).

Second, two proteins which form voltage-induced channels were tested in the asymmetric membranes. Alamethicin (4-7) was selected because it is well characterized and commercially available, is stable for long periods at room temperature, and can be added directly to the aqueous phase without additional detergents or lipids. Most importantly, alamethicin does not function in membranes which are thicker than bilayers (4,8), so its function implies that a bilayer and not a multilayer has been constructed. The calcium channel, by comparison, is a much larger, mammalian protein (9-11), semipurified from bovine brain the week of the experiment. The calcium channels used here were selective for barium (12) and appear to be similar to the calcium channels isolated by Takahashi and Catterall (11, J. Smuda, data not shown).

Third, an electrode has been fabricated which is: (a) porous so that there is an aqueous space below the receptor-containing membrane and (b) hydrophilic so that the lipid membrane adheres well to its surface. A more complex version of this porous electrode with the potential for increased sensitivity is also described.

METHODS

Materials. Asolectin, dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine, and egg phosphatidylcholine were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol was obtained from Calbiochem (La Jolla, CA). Soy lipid was obtained from the American Lecithin Co. (Atlanta, GA). 1,2-bis(12-methacryloyloxy-dodecanoyl)-sn-glycero-3-phosphocholine (MaPC) was synthesized by the method of Regen et al. (13). 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine ($DC_{8,9}PC$) was synthesized as described by Singh and Schnur (14). Photopolymerizable lipids were checked by thin layer chromatography before the start of an experiment to ensure that all the lipid was in the monomer form. Alamethicin was obtained from Sigma (St. Louis, MO). Calcium channels were isolated from bovine basal ganglia by differential centrifugation and ion exchange chromatography and incorporated into asolectin/cholesterol (4:1, w/w) liposomes in the presence of 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS)(Sigma) by the method of Borsotto et al. (9).

Experiments involving calcium channels were performed using 10 mM BaCl, 20 mM HEPES pH 7.5 buffer. All other experiments were performed using 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM $MnCl_2$, pH 7.4 unless otherwise stated. All water used was double distilled. Hexane, chloroform, and methanol were chromatographic grade from Fisher Scientific (Pittsburgh PA).

Analysis of bilayers and ion channel function. Microelectrode current measurements through lipid bilayers were performed using the patch clamp apparatus as described by Coronado and Latorre (8), using equipment previously specified (15). Briefly, lipid films were spread over buffer in a home-made Langmuir trough. Monolayers of asolectin were spread from a 5 mg/ml hexane stock solution by carefully adding the lipid to the air-water interface (16). Asolectin/cholesterol (4:1 w/w) films were formed by self-assembly of monolayers from liposomes at the air/water interface (17) and used only in experiments involving calcium channels. All polymerizable lipids were spread from chloroform. Polymerization was initiated with a mercury penlamp (Ultra-Violet Products Inc., San Gabriel, CA) placed above the monolayer film. Asymmetric membranes were made by first passing the patch pipette up through the asolectin monolayer and then passing the same pipette down through the polymerized lipid film.

Alamethicin stock solutions of 10 ug/ml in $MeOH/H_20$ (1:3) were prepared to prevent alamethicin aggregation. Alamethicin (1 ug/ml buffer) was added to the NaCl buffer in the subphase and partitioned into preformed bilayer membranes by diffusion (7). For the incorporation of calcium channels, vesicles containing the channels were

added to the BaCl buffer (5 ug lipid/ml buffer) in the trough following the method of Schindler (17,18). The asolectin/cholesterol film containing protein formed at the air-water interface. The patch electrode was passed upward through this film and subsequently down into the second monolayer (asolectin/cholesterol or $DC_{8,9}PC$) to form the bilayer.

Fabrication of the porous electrodes. Porous electrodes were constructed from an etched silicon substrate with either a platinum wire (backside contact) or gold coating (frontside contact) for electrical connection. The silicon was then anodically bonded to pyrex glass slides and modified with the addition of the porous, hydrophilic surface. To construct the device, the basic structure of porous substrate/silicon/glass was coated with layers of silicon dioxide and poly-silicon. Microlithography was used to define a strip down the center of the device. The poly-silicon and the oxide were etched away from the front side and a gold contact was deposited on the end of the strip (the small square apparent at the right end of the small device). The final porous surface measured approximately 1mm by 5 mm.

Deposition and analysis of films on porous electrodes. Lipid films were deposited onto the porous electrodes by two different techniques. Either an LB dipping technique was used to controllably deposit monolayers and multilayers of lipids onto the surface (16) or lipid layers were adsorbed to the surface in a vesicle fusion process (17-20).

In the LB dipping method, lipid monolayers were spread on the surface of a Joyce-Loebl Langmuir Trough 4 (Vickers Instruments, Gateshead, UK) equipped with a servo-feedback mechanism to maintain constant surface pressure independent of surface area. The trough is modified to accommodate two ultraviolet radiation lamps (Rayonet Photochemical Reaction Lamp, Cat. no. R.P.R. 2537A, The SO.N.E. Ultraviolet Company, Hamden, Connecticut). The $DC_{8,9}PC$ monolayer is spread from a chloroform or dichloromethane/hexane (70/30, v/v) solution on a distilled water or buffered subphase. After waiting twenty minutes for the solvent to evaporate, the teflon bars of the trough are moved slowly together so that a pressure-surface area curve may be drawn. The bars are drawn together at the rate of about 2-3 mm/min to a surface pressure of 30 mN/m (dyn/cm) which takes about one hour. The film is now allowed to relax for four hours, during which time the surface pressure may drop 5 to 10 mN/m. At this stage, the film is polymerized by exposure to the ultraviolet radiation source for twenty minutes. The teflon bars are held fixed during the exposure so that the area of the film is held constant. The surface pressure of the film increases by 15 to 20 mN/m during the polymerization to a final surface pressure of about 40 mN/m. A final surface pressure of 35 to 37 mN/m is reached after about an hour relaxation.

The trough was equipped with a speed-controllable dipping arm to pass substrates through the standing monolayer. A clean, hydrophilic substrate was passed up through the air-water interface to deposit the first monolayer; subsequent passes of the substrate through the film may result in the deposition of multiple layers. The surface pressure was maintained at 35 mN/m by a servo-feedback. The amount of lipid deposited onto the surface was measured from the reduction in surface area. The transfer ratio was calculated by comparing the change in the surface area of the film to the area of the substrate.

The second technique for depositing lipid layers was to adsorb lipid films onto the substrate. Unilamellar, soy lipid vesicles (0.3-3 mg/ml) were added to 150 mM NaCl 20 mM phosphate buffer pH 7.4 containing the porous electrode and a platinum wire electrode. The impedance, which is a function of both the conductance and capacitance of the electrode-electrolyte solution, was measured at 1 kHz. The change in the total impedance of the system was measured as a function of time as the vesicles fused into a film on the porous surface. For control experiments, the porous electrode was replaced with a second platinum wire electrode.

RESULTS

Fabrication and analysis of asymmetric bilayers. Individual monolayer films were formed in small, side-by-side Langmuir troughs. The glass patch electrode was passed upward through the first trough and down into the second trough, so that a bilayer was formed and remained in buffer during the analysis. Three different bilayers were fabricated on the patch electrode: asolectin/asolectin, asolectin/MaPC, and asolectin/$DC_{8,9}PC$. With the asymmetric membranes, there was a 10-20% success rate in forming bilayers with 1-10 gigaohms resistance. This resistance was considered to reflect a good seal between the membrane and the glass electrode and produced data with low noise levels.

The three membranes were analyzed for strength and integrity in the absence of protein channels. Voltage increasing to 1000 mV was applied across the membranes over a period of 30 sec. The asolectin/asolectin membranes broke at 386 $\pm$ 196 mV (25 tests). The asolectin/MaPC membranes broke at 851 $\pm$ 119 mV (3 tests). The asolectin/$DC_{8,9}PC$ membranes were extremely stable, maintaining their integrity at 1000 mV in all 3 tests.

Analysis of alamethicin in the bilayer membranes. To ensure that channel-forming proteins could form a pore through the polymerized monolayer and still function, alamethicin was added to the aqueous phase in the trough below the bilayer membranes. Figure 1 shows the procedure used for forming the asymmetric bilayers on the patch electrode. Alamecithin channels in the asolectin bilayer and in the asymmetric, polymerized membranes. The nature of the actual channels formed in each case was quite different. In the asolectin bilayer, the channels were open 15 msec or longer and showed the characteristic step increases in amplitude previously described (8).

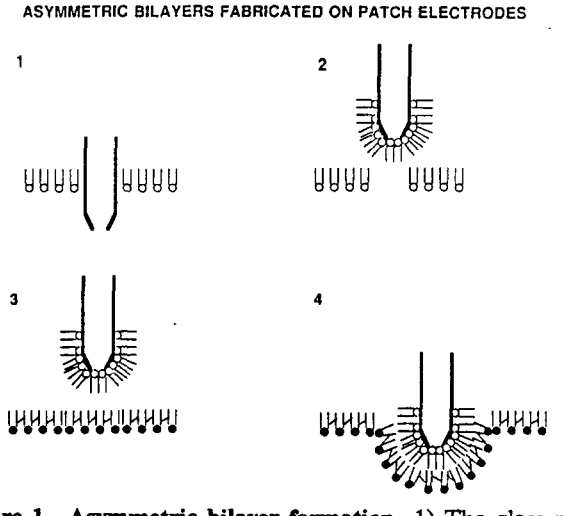


Figure 1. Asymmetric bilayer formation 1) The glass patch electrode is pulled up through an asolectin monolayer (with or without calcium channels) at the air-water interface; 2) A monomeric lipid monolayer forms across the end of the patch electrode; 3) The electrode is then dipped down through a polymerized lipid monolayer spread on a separate trough; 4) The asymmetric bilayer is formed across the orifice at the end of the patch electrode.

A higher voltage was required to form alamethicin channels in the asymmetric membranes. Correspondingly, a lower conductance across the membrane was observed. Once formed, the channels remained open for much shorter times. Step increases in amplitude were visible for alamethicin channels in the asolectin/$DC_{8,9}PC$ membrane, but were difficult to discern with the asolectin/MaPC membrane. Channel open times appeared slightly longer in asolectin/$DC_{8,9}PC$ than in asolectin/MaPC.

Analysis of calcium channels in asolectin/$DC_{8,9}PC$ bilayer. A second channel tested in asymmetric membranes was the calcium channel from bovine brain. In this system, the channels were first incorporated into a monomeric lipid film. The patch electrode was passed up through that monolayer and then dipped through the second monolayer to form the bilayer membrane (Figure 1). Calcium channels integrated into both the asolectin /asolectin and asolectin /$DC_{8,9}PC$ bilayers were functional. Since this channel did not exhibit the step increases in amplitude characteristic of alamethicin channels (18), the amplitude and corresponding conductance of the channels could be resolved with a fairly simple computational analysis. Figure 2 shows the conductance distribution measured for the calcium channels. The mean conductance was 27 $\pm$ 8 pS for channels in the asolectin bilayer and 31 $\pm$ 6 pS in the asymmetric bilayer.

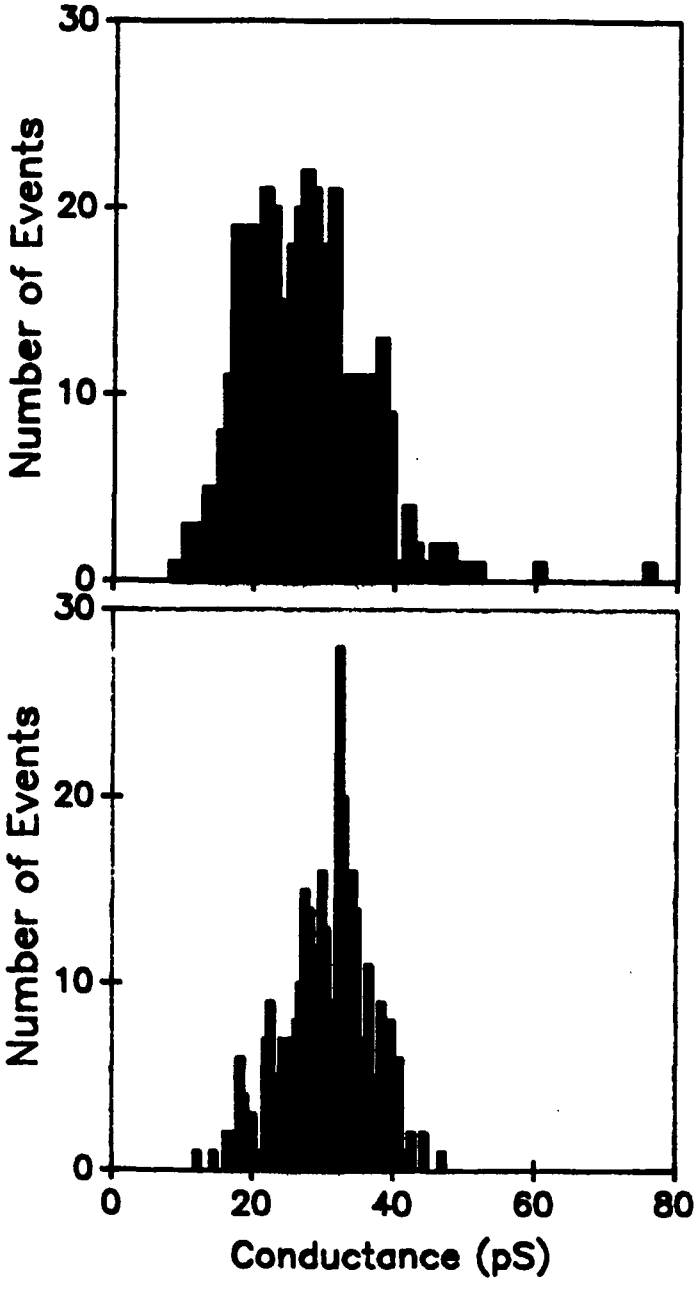


Figure 2. Conductance of calcium channels Patch electrodes were passed through an asolectin/cholesterol film containing calcium channels and then through a monolayer of asolectin/cholesterol (upper panel) or $DC_{8,9}PC$ (lower panel). Conductance was measured for 408 channels and 328 channels, respectively.

Fabrication and testing of the porous electrode. The major consideration in the development of the hardware for the biosensor was the interface between the biological and electronic components. The receptor-containing membrane must be immobilized on a compatible surface and surrounded with an aqueous buffer. In addition, electrodes must be placed on both sides of the membrane. These problems were solved by the fabrication of a hydrophilic, porous electrode. The surface of the electrode was hydrophilic so that the lipid head groups of the bilayer membrane adhered to the surface. More importantly, the surface contained holes to hold buffer beneath the membrane. Electron micrographs of these holes indicated that they could be made 1-2 microns in diameter (Figure 3) and about 2 microns in depth (determined by electron microscopy of cross-sections).

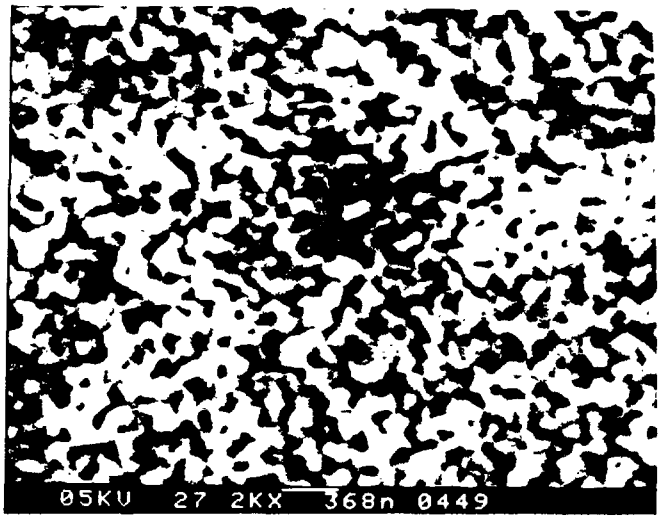


Figure 3. Electron microscopy of porous substrate Pores averaging 0.1 micron in diameter were measured in the substrate prepared by electrochemical etch and analyzed using an ISI DS-130T Scanning Electron Microscope (San Fancisco, CA) at 1680 x magnification.

LB deposition of polymerized monolayers. Initial testing of the device focused on the deposition of lipid monolayers and multilayers on the porous surface. Monolayers of dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine, egg phosphatidylcholine, monomeric $DC_{8,9}PC$, and polymerized $DC_{8,9}PC$ were deposited by passing the device through an LB film. Deposition on the porous substrate was assessed by the transfer ratio of the lipid film calculated from the reduction in area at constant pressure.

Monolayers of polymerized $DC_{8,9}PC$ were prepared as described in the Methods section. Reproducible surface pressure-area diagrams were obtained and the transfer ratio after a single pass through the LB film was consistent with a monolayer film covering the electrode. The first withdrawal of the substrate through the LB film was sufficient to change the surface of the porous electrode from hydrophilic to hydrophobic.

While one layer of lipid could be deposited on the device, attempts at depositing multiple layers of monomeric lipid films failed. The films were transferred onto the substrate, but the multilayers would peel off upon immersion or removal of the substrate from the trough during dipping. Multiple monolayers of $DC_{8,9}PC$ could be deposited as long as the film was polymerized prior to transfer. Furthermore, up to 13 layers of polymerized $DC_{8,9}PC$ could be sequentially deposited on the porous surface (Dr. Feredoon Behroozi, personal communication); however, most of the layers deposited only on withdrawal, the so-called Z deposition (16). The polymerized lipid films did not adhere to glass surfaces dipped simultaneously with the porous electrodes, but did adhere to the porous surface.

Lipid vesicle adsorption. To measure formation of a lipid film on the porous surface, a.c. current between the porous electrode and the platinum reference electrode was monitored for a fixed a.c. voltage. The a.c. current decreased and the corresponding impedance increased. The impedance usually saturated after about one hour, indicating that a lipid film formed on the porous, hydrophilic support. When buffer without vesicles was added to the porous electrode, there was no change in impedance with time; similarly, when vesicles were added to a buffer with only the platinum electrodes, no change occurred. These simple controls show that lipid adhesion to the platinum was not responsible for the increase in impedance.

DISCUSSION

INTEGRATION OF THE BIOSENSOR COMPONENTS

This paper documents three critical components of a receptor-based biosensor: a stabilized membrane in which ion channels can function, a porous surface compatible with the channel-containing membrane and electronic components, and a small device which can be used as a test structure to measure ion channel activity for a variety of receptors.

Prior to the development of the asymmetric membranes, experiments with symmetric polymerized bilayers were conducted. Some proteins, such a bacteriorhodopsin, tolerated the exposure to the ultraviolet light used to polymerize the $DC_{8,9}PC$ and continued to function in the symmetric polymerized membrane (21,22). Others, such as the acetylcholine receptor, ceased to function (3). Attempts to characterize bilayers of $DC_{8,9}PC$ or MaPC using the patch clamp apparatus failed since neither membrane, whether transferred in the monomeric or polymeric form, adhered to the bare glass electrode.

The asymmetric membranes formed from a monomeric lipid monolayer and a polymerized lipid monolayer were rugged and easily manipulated. The membranes were of high quality as judged from the low noise levels in the conductance data. Such membranes are adaptable in that the composition of the monomeric monolayer can be modified to optimize the function of receptors integrated into the membrane.

Both alamecithin and calcium channels functioned in the asymmetric membranes. The function of alamecithin, previously reported to be sensitive to the composition of the surrounding lipid layer (7), was affected by the polymerized monolayer. In the asymmetric membranes, the alamethicin channels exhibited decreased conductance and shorter open times compared to channels in asolectin bilayers. For the calcium channel, however, conductance did not change significantly in the asymmetric bilayers. Conductance values (27 ± 8 pS, 31 ± 6 pS) were remarkably consistent with the 25 pS reported for calcium channels in intact cells (10).

Adsorption of the lipid on a variety of substrates has been investigated. It was found that phospholipid monolayers would readily adhere to any silica-based surface. The difficulty is in depositing the second (tail-to-tail) bilayers. Some attempts at studying lipid layer deposition by lipid vesicle adsorption were made. Formation of a lipid layer on the porous substrate was indicated by an increase in impedance values to a steady state during immersion of the porous electrode in a solution of lipid vesicles. The increase in the impedance may be interpreted as the inclusion of a capacitor (the lipid film) in series with the electrode and solution impedance. The lack of impedance change in control experiments using (a) the porous electrode without vesicles or (b) two platinum electrodes with vesicles but without the porous substrate indicate that the changes were not due to effects of the electrolyte or the platinum. Impedance measurements in conjunction with the LB deposition technique will be employed to analyze coverage of the electrode by bilayers and to monitor incorporation of proteins into the films. Fluorescence and electron microscopy measurements are planned to assess the occurrence of pinholes in the lipid layers after deposition on the porous substrate. Conductance measurements will be performed after channels have been integrated into bilayers on the electrodes.

Recent work at this laboratory has turned to ways of incorporating proteins in the $DC_{8,9}PC$ lipid bilayers after polymerization. One means to do this is to "dope" the $DC_{8,9}PC$ layers with short chain "spacer" lipids. The spacer lipids are thought to create fluid regions in the polymerized films into which the proteins may be inserted. Preliminary results have shown that bacteriorhodopsin can be inserted into $DC_{8,9}PC$/dinonanyl phosphatidylcholine vesicles by means of a detergent dialysis. The object is to deposit a mixed lipid bilayer onto the electrode and then incorporate proteins by a method analogous to a detergent dialysis technique.

BIOSENSOR DESIGN AND DEVELOPMENT

A silicon based electrode has been developed and characterized for lipid deposition. A porous surface on the substrate would permit the deposition of the membrane over an aqueous chamber. The diameter of the holes is ideally designed to be 1-2 microns in order to minimize the strain on the membranes suspended across the hole while providing sufficient area to accommodate a large receptor protein. The surface can be made hydrophilic so that the lipid layer head groups readily adhere. Furthermore, it is structurally more stable than the hydrogels used in the past (23,24). Lipid monolayers are readily transferred onto the substrate using LB deposition techniques. Deposition of lipid monolayers and multilayers onto the electrode was demonstrated using lipid transfer measurements and optical evaluations of hydrophobicity. The difficulty of depositing bilayers of phospholipids onto substrates by dipping the substrate vertical to the water surface has been addressed (20); attempts at vertical followed by horizontal dipping are presently being investigated.

The usual configuration of the amperometric support electrode consists of a silver-silver chloride wire (or thin film) upon which a thin layer of a hydrophilic, porous polymer (23) has been deposited. A lipid bilayer is deposited over the porous polymer either by the Langmuir-Blodgett (LB) dipping technique or by brushing a mixture of lipids and solvent onto a polymer-coated electrode (25). These techniques suffer from several deficiencies: First, the polymer does not adhere tightly to the silver-silver chloride. Second, the lipid membrane deposited on the polymer does not always adhere well enough to the polymer to give a high impedance seal, which contributes noise to the measurement. Third, the background noise is inherently difficult to remove because of the large area of these electrodes. Some efforts have been made to reduce the area of these devices, but they are limited by the ability to control the deposition of the polymer. Thus, the primary drawback to the amperometric biosensors fabricated to date is the inability to control the background current contribution to the noise.

Based on the characterization of the porous electrode, a biosensor has been designed which incorporates a method for increasing the signal from the receptors without either increasing surface area of the device or increasing the number of receptors per unit area. Bipolar junction transistors (BJT) will be used as the building blocks for higher order signal processing circuitry. Figure 6 shows the scheme for using the BJT and receptor-containing membranes in a differential amplifier. Circuitry measuring the difference in signals that occur at the respective base regions 1 and 2 (See Figure 4.) will greatly decrease the noise. By blanking off base 1, the receptor signal at base 2 will be amplified without amplifying the noise at the inputs. More complex circuitry may be added to include temperature compensation.

For this circuitry, a planar BJT is fabricated in the npn configuration, as shown in Figure 4; a pnp transistor may also be used. Various technologies now available are used to fabricate such a device and the circuitry to be developed from it (26,27). The next step is to passivate the surface by depositing a silicon oxide. A hydrofluoric acid-resistant layer of poly-silicon is deposited on top of the insulating layer. This poly-silicon layer is doped and grounded to act as a shield for noise. A photo-resist is used to define an

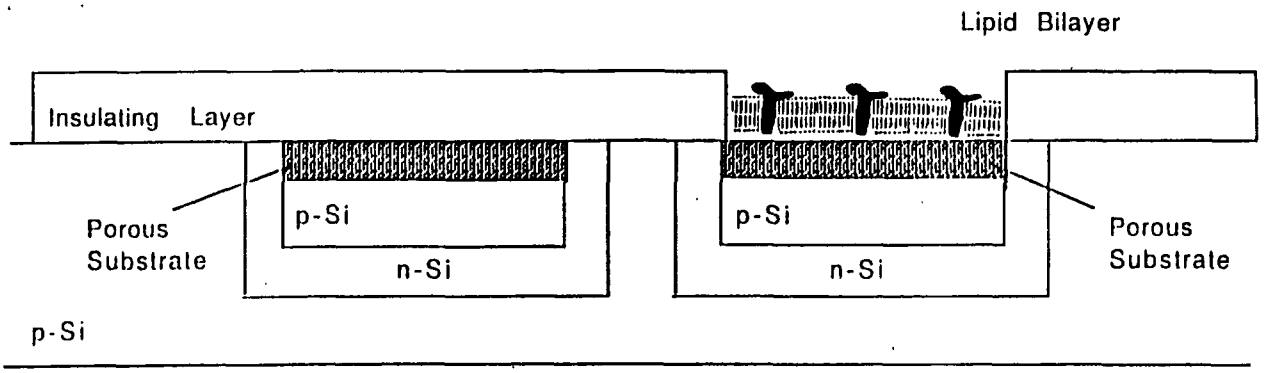


Figure 4. Schematic of next generation biosensor The next version of the porous electrode will include BJTs and provide an insulated reference bilayer (base 1, left) and a receptor-containing bilayer (base 2, right) for differential signal analysis.

area over the base. A porous surface is deposited or formed on the substrate surface. The samples are now ready for deposition of the lipid bilayers and receptor proteins. Bilayers will be deposited by traditional LB dipping techniques or by the vesicle fusion technique.

The device is placed into a reference bath with controlled pH, temperature, and/or ionic strength. The current between a reference electrode and the substrate is monitored either in the a.c. or d.c. mode. When a channel is opened in the lipid bilayer, a pulse of current is generated and recorded at the output of the device. The biosensor is operated in a similar mode as with a patch clamp electrode (28), with excitations to be applied by voltage stimulation or ligand gating. Difference measurements can be used to amplify the signal from the BJT circuits. Furthermore, the smaller geometry of pattern-defined transistors may yield reduced noise and a high impedance lipid seal.

CONCLUSION

The biosensor described here takes advantage of both the sensitivity, specificity, and physiological relevance of receptor function and the convenience and versatility of silicon-based microfabrication technology. Asymmetric, polymerized lipid membranes have been fabricated in which ion channels can function. A stable, hydrophilic, porous substrate has been integrated into electrodes to support the membrane. More sophisticated versions of the biosensor will include highly sensitive arrays of these porous electrodes.

ACKNOWLEDGEMENTS

This work was supported by an Accelerated Research Initiative of the Naval Research Laboratory and the Office of Naval Research. Discussions with Paul Yager of the Bioengineering Department of the University of Washington and with Adam Dalziel are gratefully acknowledged and appreciated.

REFERENCES

1. M.T. Jarvis: Biosensors: Today's Technology, Tomorrow's Products SEAI Technical Publications, Madison, GA, vol 155 pp, 1986.
2. A.P.F. Turner, Karube I., Wilson GS: Biosensors: Fundamentals and Applications Oxford University Press, New York, NY, pp 770, 1987.
3. A.W. Dalziel, Georger J., Price R., Singh A., Yager P.: Progress report on the fabrication of an acetylcholine receptor-based biosensor. in Goheen SC: Membrane Proteins: Proceedings Membrane Protein Symposium Bio-Rad Publishing Co., New York, NY, pp 643-673, 1987.
4. R. Latorre, Alvarez O.: Voltage-dependent channels in planar lipid bilayer Membranes. Physiol. Rev. vol 61: pp 77-149, 1981.
5. M.D. Cahalan, Hall J.E.: Alamethicin channels incorporated into frog node of Ranvier. Calcium-induced inactivation and membrane surface charges. J. Gen. Physiol. vol 79: pp 411-436, 1982.
6. R.O. Fox Jr, Richards F.M.: A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1.5-A resolution. Nature vol 300: pp 325-330, 1982.
7. I. Vodyanoy, Hall J.E., Balasubramanian T.M.: Alamethicin-induced current-voltage curve asymmetry in lipid bilayers. Biophys. J. vol 42: pp 71-82, 1983.
8. R. Coronado, Latorre R.: Phospholipid bilayers made from monolayers on patch-clamp pipettes. Biophys. J. vol 43: pp 231-236, 1983.
9. M. Borsotto, Barhanin J., Fosset M., Lazdvnski M.: The 1,4 dihydropyridine receptor associated with the skeletal muscle voltage-dependent Ca^{++} channel. J. Biol. Chem. vol 260: pp 142555-14263, 1985.
10. M.C. Nowycky, Fox A.P., Tsien R.W.: Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature vol 316: pp 440-443, 1985.
11. M. Takahashi, Catterall W.A.: Identification of an alpha subunit of dihydropyridine-sensitive brain calcium channels. Science vol 236: pp 88-91, 1987.
12. B. Hille: Ionic Channels of Excitable membranes Sinauer Associates, Inc., Sunderland, MA, pp 426, 1984.
13. S.L. Regen, Singh A., Oehme G., Singh M.: Polymerized phosphatidylcholine vesicles. Synthesis and characterization. J. Amer. Chem. Soc. vol 104: pp 791-795, 1982.
14. A. Singh, Schnur J.M.: A general method for the synthesis of diacetylenic acids. Synthe. Commun., vol 16: pp 847-852, 1986.
15. P. Yager: Patch clamping of bacteriorhodopsin and its reconstitution in a polymerizable lecithin. Biophys. J. vol 47: pp 899-906, 1985.
16. G.L. Gaines Jr: Insoluble Monolayers at Liquid-Gas Interfaces Interscience Publishers, New York, pp 326-346, 1966.
17. H. Schindler: Exchange and interaction between lipid layers at the surface of a liposome solution. Biochim. Biophys. Acta vol 555: pp 316-336, 1979.
18. B.A. Suarez-Isla, Wan K., Lindstrom J., Montal M.: Single-channel recordings from purified acetylcholine receptors reconstituted in bilayers formed at the tip of patch pipets. Biochemistry vol 22: pp 2319-2323, 1983.
19. S.L. Regen, Kirszensztejn P., Singh A.: Polymer-supported membranes. A new approach for modifying polymer surfaces. Macromolecules vol 16: pp 338-339, 1983.
20. H.M. McConnell, Watts T.H., Weis R.M., Brian A.A.: Supported planar membranes in studies of cell-cell recognition in the immune system, Biochim. Biophys. Acta vol 864: pp 95-106, 1986.
21. P. Yager: Functional reconstitution of a membrane protein in diacetylenic polymerizable lecithin. Biosensors vol 2: pp 363-373, 1986.
22. R. Pabst, Ringsdorf H., Koch H., and Dose K.: Light-driven proton transport of bacteriorhodopsin incorporated into long term stable liposomes of a polymerizable sulfolipid. FEBS Letters vol 154, pp 5-9, 1983.

23. A. Arya, Krull U.J., Thompson M., Wong H.E.: Langmuir-Blodgett deposition of lipid films on hydrogel as a basis for biosensor development. Anal. Chim. Acta vol 173: pp 331-336, 1985.
24. M. Thompson, Krull U.J., Worsford P.J.: The structure and electrochemical properties of a polymer supported lipid biosensor. Anal. Chim. Acta vol 117: pp 133-155, 1980.
25. K.I. Hongyo, Joseph J., Huber R.J., Janata J.: Experimental observation of chemically modulated admittance of supported phospholipid membranes, Langmuir vol 3: pp 827-830, 1987.
26. B.G. Streetman: Solid State Electronic Devices Prentice Hall, Inc., Englewood Cliffs, NJ, pp 463, 1972.
27. J. Millman: Microelectronics: Digital and Analog Circuits and Systems McGraw-Hill Book Co., New York, NY, pp 881, 1979.
28. P. Yager: Biosensors from membrane proteins reconstituted in polymerized lipid membranes. US Statutory Invention Registration No. H201, Jan. 6, 1987.

Section IV
ORGANICALLY DERIVED MICROSTRUCTURES
Area Summary

Microstructures are used as probes of biological systems. Organic chemical systems in turn serve as models in the creation of new microstructurs. Examples of such technology are given by Schnur et al. of NRL who show how lipids can organize spontaneously to form tubular microstructures (not included). Framer et al. illusrated this technology with the fabrication of lipsome encapsulated hemoglobin systems. Dr. Clark of the university of Glasgow outlined their research results in the development of cell cultures that are guided in their growth patterns by finely paterned silicon and other material surfaces. Finally, Dr. Erickson of the university of North Carolina reviewed the design and synthesis of Beta-protiens (not included).

SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH

22 MARCH, SESSION IV:
ORGANICALLY DERIVED MICROSTRUCTURES

1:30 p.m.-2:15 p.m.
1. BIOMOLECULAR MATERIALS, SELF-ORGANIZATION AND ALL THAT...(Joel Schnur, Naval Research Laboratory, Washington, D.C.)
2:15 p.m. - 3:00 p.m.
2. CELL NETWORKS AND ELECTRODE SYSTEMS FORMED BY MICROELECTRONIC TECHNIQUES (Peter Clark, et al., University of Glasgow, Glasgow, Scotland)
BREAK: 3:00 p.m. - 3:15 p.m.
3:15 p.m. - 4:00 p.m.
3. LIPOSOME ENCAPSULATED HEMOGLOBIN: EFFICIENCY, STABILIZATION, STORAGE (Alan Rudolph, et al., Naval Research Laboratory, Washington, D.C.)
4:00 p.m. - 4:45 p.m.
4. DESIGN AND SYNTHESIS OF BETA-PROTEINS (Bruce Erickson, University Of North Carolina, Chapel Hill, North Carolina)

Cell Networks and Electrode systems formed by micro-electronic techniques

P. Clark, J.A.T. Dow, P. Connolly, A.S.G. Curtis and C.D.W. Wilkinson
Department of Cell Biology
Department of Electronics and Electrical Engineering
The University, Glasgow G12 8QQ
Scotland, U.K.

Biological cells in vivo are guided inter alia by typographic cues; we have shown that cultured cells are strongly guided on surfaces patterned into steps, ridges and grooves by the techniques of micro-electronic fabrication.[1] Our present aim is to combine guidance of cells with the use of extra-cellular electrodes[2]. This promises to allow the extended observation of cells with continuous monitoring of their electrical activity while the cells are arranged in a well defined network.

A brief introduction to the results on the probability of cells crossing steps, and the degree of alignment produced by grooves and ridges of different pitches will be given. Arrays of hillocks, representing the epithelial surfaces over which many types of cells migrate during development, have been found to be very effective in aligning BHK cells. Extra cellular electrodes used to record the beating of single chick heart cells were formed by etching slots into silicon nitride lying above a small metal electrode; the signal to noise ratios of different types of electrode patterns will be compared. Extra-cellular recordings of neuronal activity have been obtained from excised segmental ganglia of leech (*Hirudo medicalis*).

Our progess on combining guidance and external electrical measurement on both cultured heart cells and nerve cells will be reported.

1) P. Clark, J.A.T. Dow, P. Connolly, A.S.G. Curtis and C.D.W. Wilkinson, 'Topographical control of cell behaviour I Simple step cues', Development **99**, 439-48, 1987
2) P. Clark, J.A.T. Dow, P. Connolly, A.S.G. Curtis and C.D.W. Wilkinson, 'Novel methods for the guidance and monitoring of single cells and simple networks in culture. J. of Cell Science **Suppl.8**, 55-79 (1987)

ABSTRACT FOR PROGRAM

LIPOSOME ENCAPSULATED HEMOGLOBIN: EFFICACY, STABILIZATION, AND STORAGE

Alan S. Rudolph, Martha C. Farmer, Lewis P. Stratton, and Frances S. Ligler

Bio/Molecular Engineering Branch, Naval Research Laboratory

Washington, DC 20375-5000

For the past five years, the Naval Research Laboratory has been developing liposome encapsulated hemoglobin (LEH) for use as a blood substitute in trauma medicine and combat casualty care. The present form of LEH has the advantages of being blood-type free, virus-free, easily adaptable to scale-up production, and more easily metabolized than other blood substitutes. The physicochemical properties of LEH and the scale-up procedures to produce liter quantities of this material have recently been reported. Small animal studies show the LEH to be an effective oxygen carrier with an *in vivo* half-life of 16-20 hours. Additives have been developed for preventing oxidative damage to the hemoglobin and for stabilizing the LEH during storage.

Section IV
NEW FRONTIERS IN MICROSTRUCTURE TECHNOLOGY
Area Summary

The talks in this session focused on the latest technological developments that will facilitate the fabrication of smaller and more powerful sensor systems. Dr. Pease of Stanford started the session with an outline of the new lithographic techniques for fine-line structures. He was followed by Dr. Clark of Harvard who reviewed a range of noval fabrication techniques for sensor arrays. The session concluded with a critical assessment of neural probe array technology by Dr. Marty Peckerar of NRL.

SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH

23 MARCH, SESSION V
NEW FRONTIERS IN MICROSTRUCTURE TECHNOLOGY

9:00 a.m.-9:45 a.m.
1. NOVEL LITHOGRAPHIC TECHNIQUES FOR FINE-LINE STRUCTURES (Fabian Pease, Stanford University, Palo Alto, California)
9:45 a.m. - 10:30 a.m.
2. NOVEL FABRICATION TECHNIQUES FOR SENSOR ARRAYS (J. Clark, Harvard University, Cambridge Massachussetts)

BREAK:10:30 a.m.-10:45 a.m.
10:45 a.m. - 11:30 a.m.
3. FABRICATION SCHEMES FOR PROBES AND ARRAYS: A CRITICAL COMPARISON (Martin Peckerar, Naval Research Laboratory, Washinton, D.C)

Novel Circuit Techniques For Sensor Arrays

ABSTRACT

James J. Clark
Division of Applied Sciences
Harvard University
Cambridge, MA 02138

In this lecture we introduce a technique for integrating solid state sensing elements with sensor processing circuits on a single CMOS integrated circuit. The sensor processing circuits implement convolutional and thresholding operations, and are reminiscent of neurons found in the lower levels of mammalian vision systems.

The design of the sensor processing elements is based on the principles of current mode circuitry. In current mode systems information is represented by currents flowing through wires or active devices. Convolution is performed by summing scaled replicas of currents at summing nodes, using Kirchoff's current law. The scale replicas of the currents are obtained through the use of current mirrors, which act as current amplifiers. Circuits can be constructed which invert the direction of current; hence negative scalings are attainable. This allows the implementation of center/surround spatial filtering operations, such as are performed in animal retinae.

We discuss some of the major issues in the development of integrated sensor processing networks. The foremost of these is the wiring problem. In implementing spatial convolutions it is neccessary that sensor processing elements be able to communicate electrically with elements in its immediate neighborhood. This need for communication can result in a congested mass of wires. In standard IC fabrication technology there is a limit to the number of separate wiring layers which can be overlapped. Hence there will be a limit to the density of wiring that can be obtained. This will limit either the density of our sensor processing networks or the size of the neighborhood that can be used in a convolutional operation. A solution to the wiring problem is to perform time multiplexing of many signals onto a single wire. However, this results in increased circuit complexity and makes the implementation of temporal filters very difficult.

The wiring problem is affected by the sensor distribution. Often one desires a non-rectangular, or even a non-uniform distribution of sensors and sensor processing elements. For example, a foveal vision system requires a foveal image sensor which has a high sensor element density near the center of the array and a low sensor element density near the periphery of the array. This nonuniformity of the sensor array will cause difficulties in designing circuitry for accessing or scanning of the sensor and in communicating the outputs of the sensors to the sensor processing elements.

We provide an example of the application of the current mode sensor processing networks to a real world sensor, a 64 by 64 element magnetic sensor array. This array has been implemented in 3 micron CMOS, through the MOSIS facility and is being used in a novel compliant tactile sensor system. The magnetic sensor array consists of 4096 split

drain MOSFETs that produce a differential current pair whose difference is proportional to the applied magnetic field. As currently fabricated, this array contains only sensing elements, in a rectangular distribution, and has no sensor processing integrated with it. The outputs of the sensing elements are time multiplexed in a raster scan fashion onto an output bus. We present in this lecture the design of a current mode sensor processing network, which can be integrated onto the magnetic sensor chip, which computes the spatial gradient of the magnetic field, and which also performs edge, or discontinuity, detection on the magnetic field pattern.

Techniques similar to the ones described here for magnetic field sensor arrays can be extended to other sensory modalities, such as light or chemical activity.

This research was supported in part by the Office of Naval Research under grant N0014-84-K0504 and the Joint Services Electronics Program.

FABRICATION SCHEMES FOR PROBES AND ARRAYS: A CRITICAL COMPARISON

M.C. Peckerar and C.T. Yao

Naval Research Laboratory
Washington, D.C. 20375-5000

A variety of miocroelectrode probes and arrays have been fabricated over the past 20 years which employ more-or-less standard integrated circuit processing for their manufacture. These include microelectrode arrays on metal or on silicon substrates. The silicon substrates have frequently included dopant-etch stops. Furthermore, the silicon arrays have also incorporated on-board circuitry. All these various technology have been compared. It has been concluded that there are no fundamental technological barriers to the successful fabrication of probes and arrays by any of these techniques. In the case of the implanted electrode, though, etching of the thick substrate base makes for a very low-yield process. Factors affecting critical dimension control (such as lithographic resolution and etch control) are outlined and techniques for providing minimum-dimension probes are disclosed.

SYNTHETIC MICROSTRUCTURES IN BIOLOGICAL RESEARCH

POSTERS

THE DEPOSITION AND CHARACTERIZATION OF STRUCTURED INDIUM AND THIN FILM LAYERS FOR BIOLOGICAL IMMUNOASSAY RESEARCH (Harry Charles, et. al., Johns Hopkins Applied Physics Lab, Laurel, Maryland)

DEPENDENCE OF ETCHING ROUGHNESS ON MOLARITY, TEMPERATURE AND SURFACE PREPARATION FOR SILICON IN AQUEOUS KOH (Edward Palik, Naval Research Laboratory)

DIFFERENTIAL RECORDING OF ACTION POTENTIALS WITH MICROELECTRODE ARRAYS (Shihab Shamma, University Of Maryland, College Park Maryland)

MICROELECTRONIC PROGRAMABLE ANALOG SYNAPSES (Fritz Kub, Naval Research Laboratory, Washington, D.C.)

The Deposition and Characterization of Structured Indium and Tin Film Layers for Biological Immunoassay Research

Harry K. Charles, Jr., Ph. D.
Norman A. Blum, Ph. D.
Steven J. Wajer

The Johns Hopkins University
APPLIED PHYSICS LABORATORY
Johns Hopkins Road
Laurel, Maryland 20707
(301)-953-5000 X8050

Abstract

Thin indium film layers when coated with appropriate antigen (antibody) layers will respond by a change in visual appearance (a darkening when viewed in transmitted white light) to solutions containing a matching antibody (antigen). The structure and characteristics of the thin indium films (10 to 25 nm) and the recently discovered tin films[1] which produce optimum visual responses in the immunoassay process (antibody - antigen bonding) are not well understood at this time. The primary focus of the current research is to produce controlled thickness indium and tin (and their associated alloys) film layers with known microstructure for immunoassay testing. Emphasis has been placed on the association of fundamental thin film properties with the strength and quality of the immunoassay visual response. All immunoassay evaluations have been conducted with the antigen IgG (human immunoglobulin), a protein found in blood. The best immunoassay results (greatest contrast between antigen-antibody double protein layer[2] and the background) for indium films slowly deposited on substrates at room temperature (25°C) occurred at film thickness between 13.5 and 17.5 nm when the nucleating grains appeared to be uniform in size with an average grain diameter of 0.15 μm. When similar morphology was obtained at other deposition temperatures and rates (while maintaining similar optical transmission) the immunoassay response was again peaked. Thin tin films displayed similar physical characteristics.

[1] Invention Disclosure: H. K. Charles, Jr. et al. "Immunoassay Technique Using Thin Tin Films" JHU/APL File No. 5301-327.

[2] C. L. Burek, et al. "The Indium Slide Immunoassay: A Tool for the Rapid Simplified Detection of Antigen," Clinical Immunology Elsevier Science Publishers, Amsterdam, 1987, p. 235-238.

Dependence of Etching Roughness on Molarity, Temperature and Surface Preparation for Si in Aqueous KOH

E.D. Palik and O.J. Glembocki
Naval Research Laboratory, Washington, D.C. 20375
I. Heard, Jr. and P.S. Burno
Lincoln University, Lincoln University, PA 19352
L. Tenerz
Uppsala University, Box 534, S-751 21 Uppsala, Sweden

The quality of a Si (100) surface etched in aqueous KOH varies appreciably with molarity, temperature and surface preparation. While such roughness may be negligible for micromachined structures with dimensions on the many tens-of-micrometers scale, it could become important as the structure dimensions are reduced to the few-micrometers scale.

We have made measurements of etch rates and etch roughness for (100) surfaces of n- and p-Si in the resistivity range 1-5 ohm cm as a function of molarity, temperature, and surface preparation regarding HF rinses and H_2O washes. We used Si wafers masked with blocks of SiO_2 with exposed Si streets and avenues. After etching, a mechanical stylus was used to determine the groove depth and the peak-to-peak roughness at the bottom of the grooves.

We noticed that at room temperature, the etch roughness δR mimiced the etch rate R as a function of molarity, being largest at ~5M (R=400 Å/min, δR=0.4 μm for unstirred solution) and decreasing to lower and higher molarity. For intermediate molarities the roughness lead to a drop in the apparent etch rate as measured from the short-term to the long-term (minutes to hours). This decrease in etch rate was presumably due to the exposure of slow-etching (111) planes by the roughening process; we would expect the (100) planes to continue etching at a fixed rate. This etching-roughening process produces a texture on the surface similar to orange-peel.

In general, the data were somewhat sensitive to the procedure for stripping the native oxide with a dilute HF rinse followed by an H_2O wash, and to how the solution was mixed - no stirring, or stirring with a magnetic stirrer or ultrasonically. We also observed that as the temperature was raised toward 100°C, the etch roughness decreased for several molarities between 1.7 and 10.3, while the etch rates increased rapidly. Also, we noticed at 5M (where roughness is largest at room temperature), that the roughness increased with time of etching up to 160 min.

Thus, we are confronted with a matrix of variables which can be adjusted to minimize etch roughness. The investigation of this matrix is a tedious job, and we have not been able to determine the roughness mechanism yet. However, we will discuss possibilities such as non-uniform and non-stoichiometric native oxide, and psuedo masking produced by the silicate etch products and by the sticking of hydrogen bubbles to the surface.