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## Sealing cultured invertebrate neurons to embedded dish electrodes facilitates long-term stimulation and recording

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Recently it has become possible to form small networks of synaptically connected identified invertebrate neurons in culture. Using conventional saline-filled glass electrodes, it is difficult to simultaneously stimulate and record from more than 2 or 3 cultured neurons and to perform experiments lasting longer than several hours. We demonstrate that it is possible to overcome these limitations by using planar arrays of electrodes embedded in the bottom of a culture dish. The arrays employ conductive leads and insulation that are transparent, making the dishes compatible with voltage-sensitive dyes and inverted microscopy. Identified neurons from leech *Hirudo medicinalis*, slug *Aplysia californica*, and snail *Helisoma trivolvis*, have been grown on these arrays. Due to their large size (soma diameter 40–200  $\mu\text{m}$ ) these neurons form seals over the dish electrodes. Individual electrodes can then be used to stimulate and to record action potentials in the associated neuron. With sealing, action potentials have been recorded simultaneously from many neurons for up to two weeks, with signal-to-noise ratios as large as 500:1. We developed and tested a simple model that describes the voltage waveforms measured with array electrodes. Potentials measured from electrodes under cell bodies were primarily derivatives of the intracellular potential, while those measured from electrodes under axon stumps were primarily proportional to local inward  $\text{Na}^+$  currents. While it is relatively easy to record action potentials, it is difficult to record postsynaptic potentials because of their small size and slow rate of rise.

### Introduction

Following pioneering work on leech neurons (Ready and Nicholls, 1979), many types of identified invertebrate neurons have been grown in culture (Kaczmarek et al., 1979; Dagan and Levitan, 1981; Wong et al., 1981; Schacher and Proshansky, 1983). They have proven to be useful preparations for studying a variety of neuronal properties including neurite outgrowth (Cohan and Kater,

1986) and synaptogenesis (Kaczmarek et al., 1981; Dagan and Levitan, 1981; Schacher and Proshansky, 1983). Studies have been limited by the inability to reliably and non-invasively stimulate and record from specific neurons in a cultured circuit over extended time periods. While the use of voltage-sensitive dyes to optically measure the change in membrane potential from large numbers of neurons simultaneously is promising (Rayburn et al., 1984; Chien et al., 1987; Parsons et al., 1989), it is limited by the unavailability of a complementary means for stimulating and by dye phototoxicity. Another approach is to grow neurons on a planar array of stimulating and recording electrodes.

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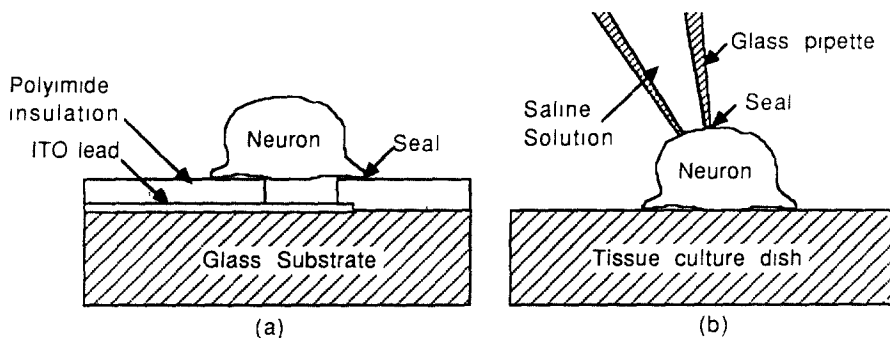


Fig 1 a schematic of a neuron growing over a dish electrode and forming a seal. b: a large diameter glass electrode that has been placed in contact with a neuron to form a seal

Planar arrays of electrodes embedded in the bottom of a culture dish have been used to record from a variety of cell culture preparations (Thomas et al., 1972; Pine, 1980; Gross et al., 1982; Israel et al., 1984; Novak and Wheeler, 1986). In general it has been possible to use these multielectrode dishes to record small extracellular signals resulting from current flow associated with an action potential. It may be difficult to interpret these signals, since the signal-to-noise ratio is often poor, and it is not always possible to get a one-to-one electrode–neuron correspondence. It is possible to stimulate neurons using these electrodes (Pine, 1980). A current pulse passed through an electrode creates a voltage gradient in the medium sufficient to depolarize nearby axons and cell bodies, causing them to fire action potentials. Such stimulation techniques suffer from the disadvantage that it is difficult to use array electrodes to detect the resulting action potentials; therefore, an independent means of stimulus verification such as voltage-sensitive dyes (Salzberg, 1982; Grinvald, 1984; Cohen and Leshner, 1986) or intracellular recording is required.

Here we report that identified neurons from leech *Hirudo medicinalis*, slug *Aplysia californica*, and snail *Helisoma trivolvis* can be placed on electrode arrays where they grow and form seals. The electrode then forms a geometrical relationship with the neuron similar to that formed by a patch-type glass electrode of the same diameter (Fig. 1). Three types of neurons were studied to test whether the technique was generally applicable, and to determine the importance of different

factors in obtaining reliable stimulation and recording. We found this method to be electrophysiologically similar to that employed in loose-patching (Stuhmer et al., 1983), and to recording techniques used *in vivo* (Tauc, 1962; Neher and Lux, 1969). Portions of this work have been published in abstract form (Tank et al., 1986; Regehr et al., 1988a) and related techniques have recently been reviewed (Regehr et al., 1988b).

## Materials and Methods

### *Culturing identified invertebrate neurons*

The culturing techniques for *Helisoma*, *Aplysia* and *Hirudo* were all similar. First, appropriate ganglia were removed from either juvenile or adult animals and digested in enzyme. Second, the ganglia were removed from the enzyme, washed, pinned out in a Sylgard dish and desheathed. Neurons were identified based on size, coloration, and position in the ganglia, and were removed either by suction with a pipette or withdrawn from the ganglia by stabbing with a fine-tipped glass capillary. The neurons were then transferred to a coated culture dish filled with the appropriate plating medium.

*Helisoma* B5 neurons and B19 neurons were cultured, and dishes prepared and coated, as described elsewhere (Wong et al., 1981). Gentamycin sulfate (50  $\mu\text{g}/\text{ml}$ ) was substituted as the antibiotic in conditioned medium. Neurons were plated into conditioned medium and placed near the center of the array with their axon stumps point-

ing towards the middle of the array. Following plating, the dishes were not moved for several hours to allow the cells to adhere. Neurons would often move as much as 50  $\mu\text{m}$  after they were plated.

*Hirudo* neurons were cultured as previously described (Ready and Nicholls, 1979) and as modified by Liu and Nicholls (personal communication): after removal from the ganglia, Retzius cells were incubated overnight in a culture dish filled with serum-containing medium. The cells did not adhere and lost their associated glia. This left the neuron with a clean cell surface that would adhere immediately when placed on an array electrode in medium containing no serum. A mouth pipette and a large-tipped glass electrode were used for cell placement.

Culturing techniques for *Aplysia* neurons were also similar to those reported elsewhere (Schacher and Proshansky, 1983; Lin and Levitan, 1987), with some minor differences: ganglia were pinned to a Sylgard dish containing *Aplysia*-L15 media with 1 g/100 ml methyl cellulose, and neurons were removed by impaling the soma of choice with a fine-tipped electrode using a micromanipulator. Identified neurons used were those from the left upper quadrant of the abdominal ganglia (LUQs), the giant cell R2 (Schacher et al., 1985), abdominal neurons L10–L12, and the metacerebral cell (MCC). Exact placement with *Aplysia* neurons was not critical, since due to their large size they would often cover several electrodes. Cells were placed using a 20  $\mu\text{l}$  pipette. Cells were often covered with glia which tended to impair adhesion.

#### Dish fabrication

The multielectrode arrays were fabricated using conventional integrated circuit technology. The electrode leads were made from transparent indium tin oxide (ITO) (Gross et al., 1982); the insulation was photosensitive polyimide; and the electrode tip was electroplated platinum black. The electrode pattern consisted of a hexagonal array of 61 electrodes, 12  $\mu\text{m}$  in diameter, separated by 70  $\mu\text{m}$ .

Fabrication began with a glass substrate (0.041  $\times$  2.29  $\times$  3.55 cm), coated with a layer of ITO 100

nm thick, and with a sheet resistance of 100  $\Omega$ /square (Donnelly Corporation Midland, MI). After cleaning, an electrode and lead pattern were produced using Shipley 1350J photoresist, with a 125  $\text{mJ}/\text{cm}^2$  exposure for the central pattern and an 800  $\text{mJ}/\text{cm}^2$  exposure for the peripheral part of the wafer to remove the thick edge bead formed during photoresist spinning. The ITO was etched for 4 min in a freshly prepared solution of 50 parts  $\text{H}_2\text{O}$ , 50 parts  $\text{HCl}$ , and 1 part  $\text{HNO}_3$  (vols. of concentrated solutions) at 40°C. The photoresist was removed and a 3 nm layer of aluminum was evaporated onto the wafer, resulting in a thin layer of aluminum oxide which was an effective adhesion promoter. Next, photosensitive polyimide (MRK Selectilux HTR 3-50) was spun at 5000 rpm for 30 s, soft baked for 5 min at 85°C, patterned with the electrode mask, developed, and cured for 12 h at 200°C. The resulting polyimide was approximately 1  $\mu\text{m}$  thick. To reduce the

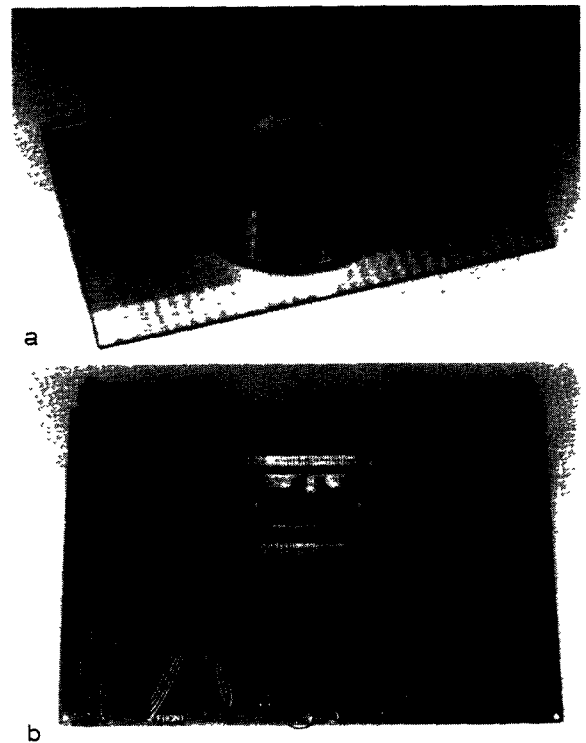


Fig 2 a completed multielectrode culture dish b: dish mounted on a circuit board

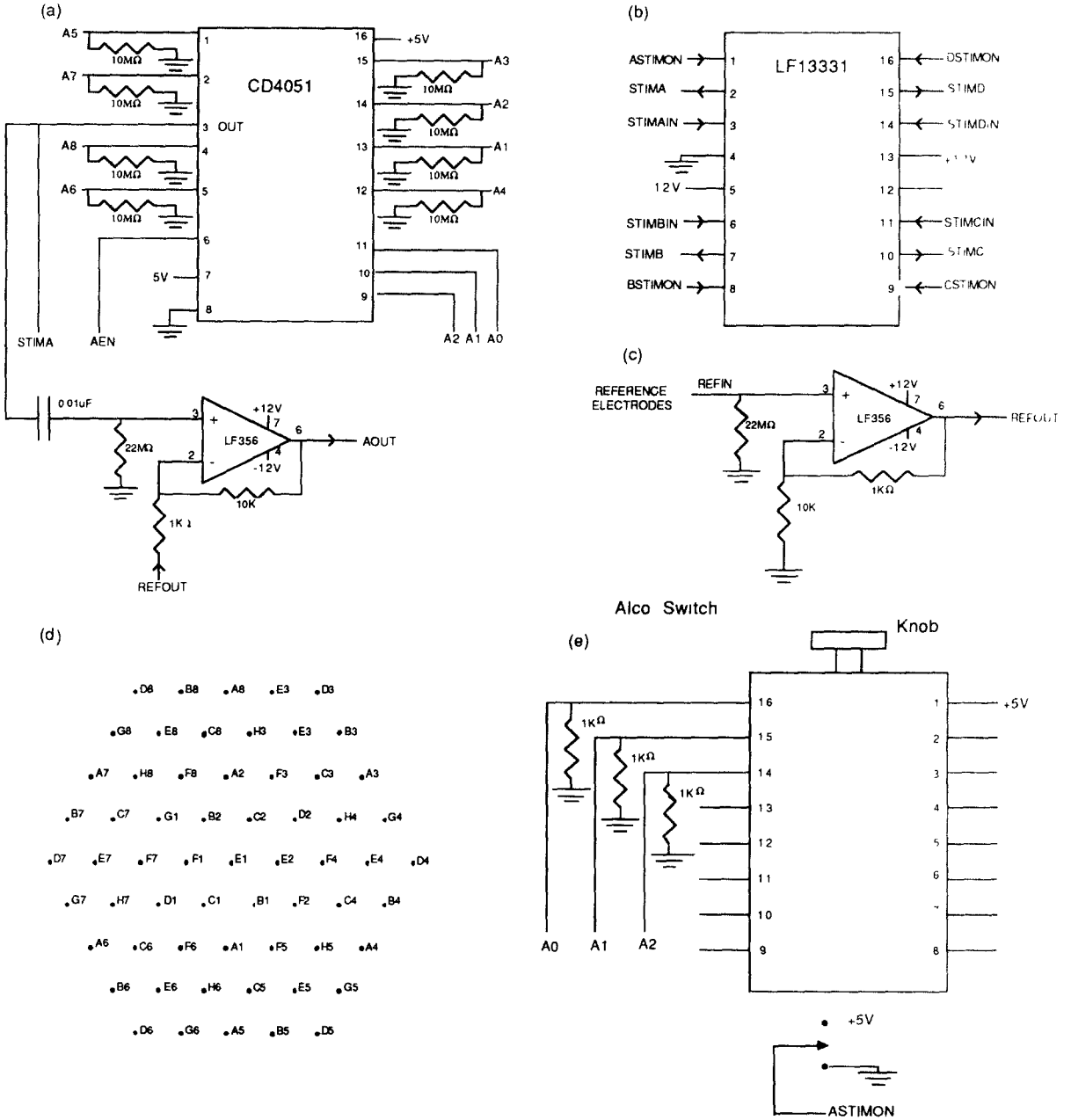


Fig. 3. Interface electronics for the multielectrode array a: channel A. b: stimulus switches. c: reference electrodes. d: dish addresses. e: control box circuitry.

electrode impedances to less than 500 kΩ at 1 kHz, the electrodes were 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<sup>2</sup> for 10 s. Then the bottom of a small tissue culture dish with a 5 mm diameter hole was glued to the top of the substrate. Fig. 2a shows a completed multielectrode dish. It took an average

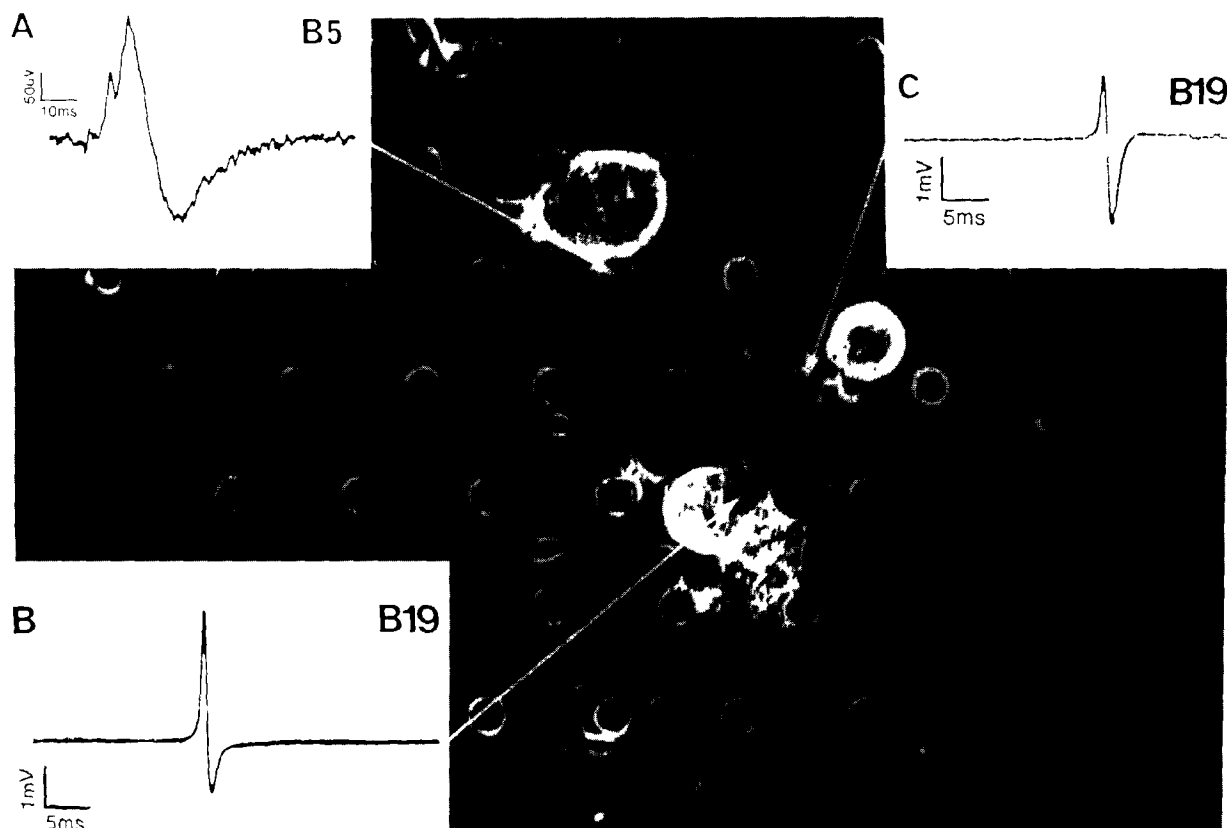


Fig 4 Spontaneous activity recorded from *Helisoma* neurons using a multi-electrode array (bandwidth 10 Hz–1 kHz). The electrodes are 70  $\mu\text{m}$  apart

of about 1 h to fabricate each dish, and they were routinely reused for several experiments.

#### Dish electronics

Fig. 2b shows a dish mounted on the interfacing printed-circuit board, which in turn mounts to the microscope stage. Sixty-three leads go to the inputs of eight 8-to-1 multiplexers, with outputs designated as channels A-H. The multiplexer output for each channel is amplified with a gain of 11, and is differential with respect to two large dish ground electrodes. A JFET switch can connect each channel to an externally generated stimulus. Two 34-wire flat cables connect the printed circuit board to interface electronics, which generates 5 control signals for each channel. Fig. 3a shows 1 of 8 identical channels, channel A. AEN enables multiplexer A; and A0, A1, A2 select the input to multiplexer A. The 8 stimuli are

turned on and off using two quad JFET switches, and ASTIMON connects STIMAIN to STIMA (Fig. 3b). The reference amplifier is shown in Fig. 3c.

Electrodes are addressed as in Fig. 3d. By sending the correct control voltages, any one electrode of channels A-H can be accessed, making it possible to record and stimulate simultaneously using 8 electrodes. A control box is used, having 8 switches that set the binary code to address the multiplexers A-H, and 8 switches to enable stimuli A-H (Fig. 3e).

## Results

### Recording

We used multi-electrode arrays to record spontaneous electrical activity from small networks of

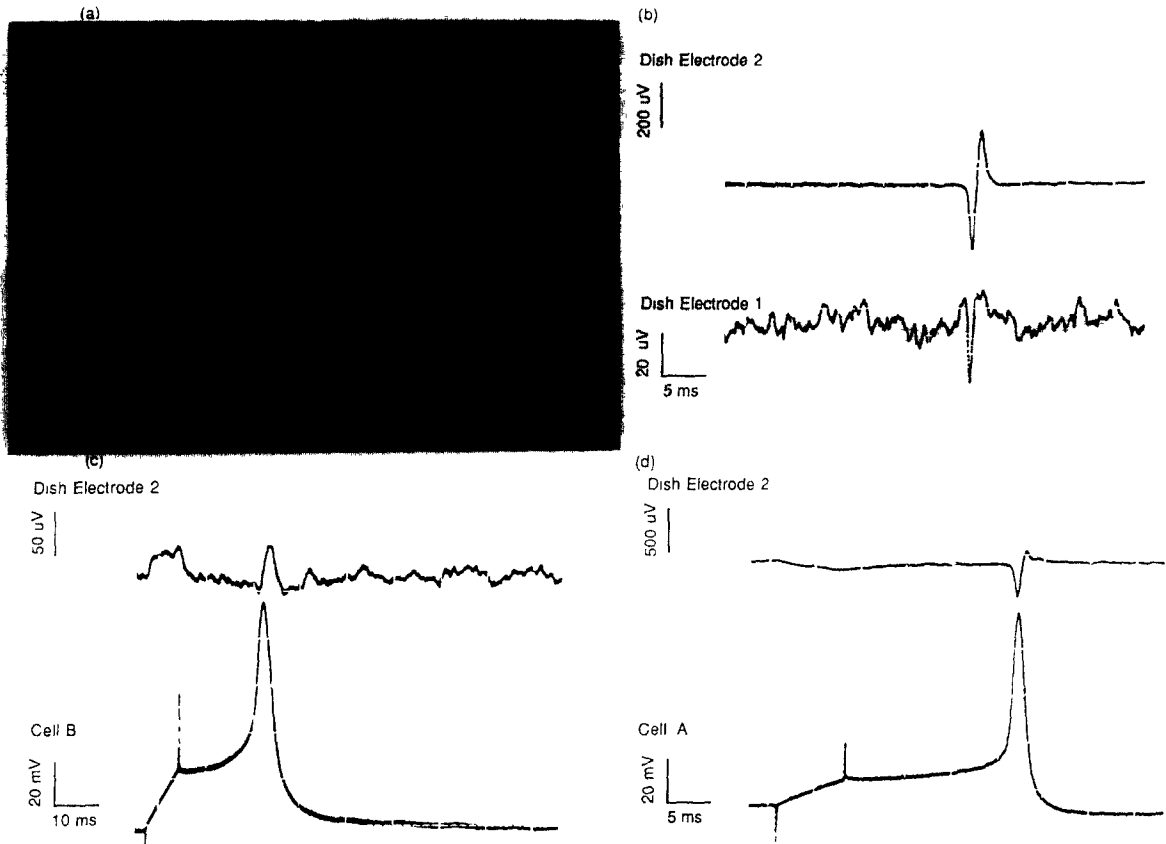


Fig 5. a. *Helisoma* neurons growing on multielectrode array Cell A is a B19 neuron and cell B is a B5 neuron. b spontaneous activity recorded by the multielectrode array c intracellular potential of cell B (bottom trace) and simultaneous array recording (top trace). d intracellular potential of cell A (bottom trace) and simultaneous array recording (top trace)

invertebrate neurons with good signal-to-noise ratios and one-to-one electrode–neuron correspondence. Outgrowth, morphology and electrophysiology of neurons grown on the arrays were indistinguishable from control neurons plated in the same medium on similarly coated glass coverslips (see Figs. 4–7).

A recording experiment using *Helisoma* neurons is shown in Fig. 4. Spontaneous action potential activity was recorded from neuron A, a *Helisoma* B5 neuron, and from *Helisoma* B19 neurons B and C. Extracellular action potential recordings exhibiting large signal-to-noise ratios were generally obtained only from array electrodes seen to have a neuron growing directly over them, thus forming a seal. Seal resistances were typically several megohms when a *Helisoma* neu-

ron was seen to completely cover an electrode. Growth such as that shown in Fig. 4, in which the cells formed lamellipodia around the cell soma, generally resulted in larger seal resistances and improved signal-to-noise ratios. Signals recorded from *Helisoma* B19 neurons were in general much larger (100  $\mu\text{V}$ –3 mV) than those recorded from *Helisoma* B5 neurons (20–300  $\mu\text{V}$ ). Often the electrical potentials measured were essential derivatives of the intracellular potential (as is the case in Fig. 4b). More complicated waveforms were also recorded. An explanation of the nature of these array recordings is given in the following section. Multielectrode dishes were used to monitor spontaneous activity for up to 13 days from *Helisoma* neurons.

In addition to recording from somata sealed to

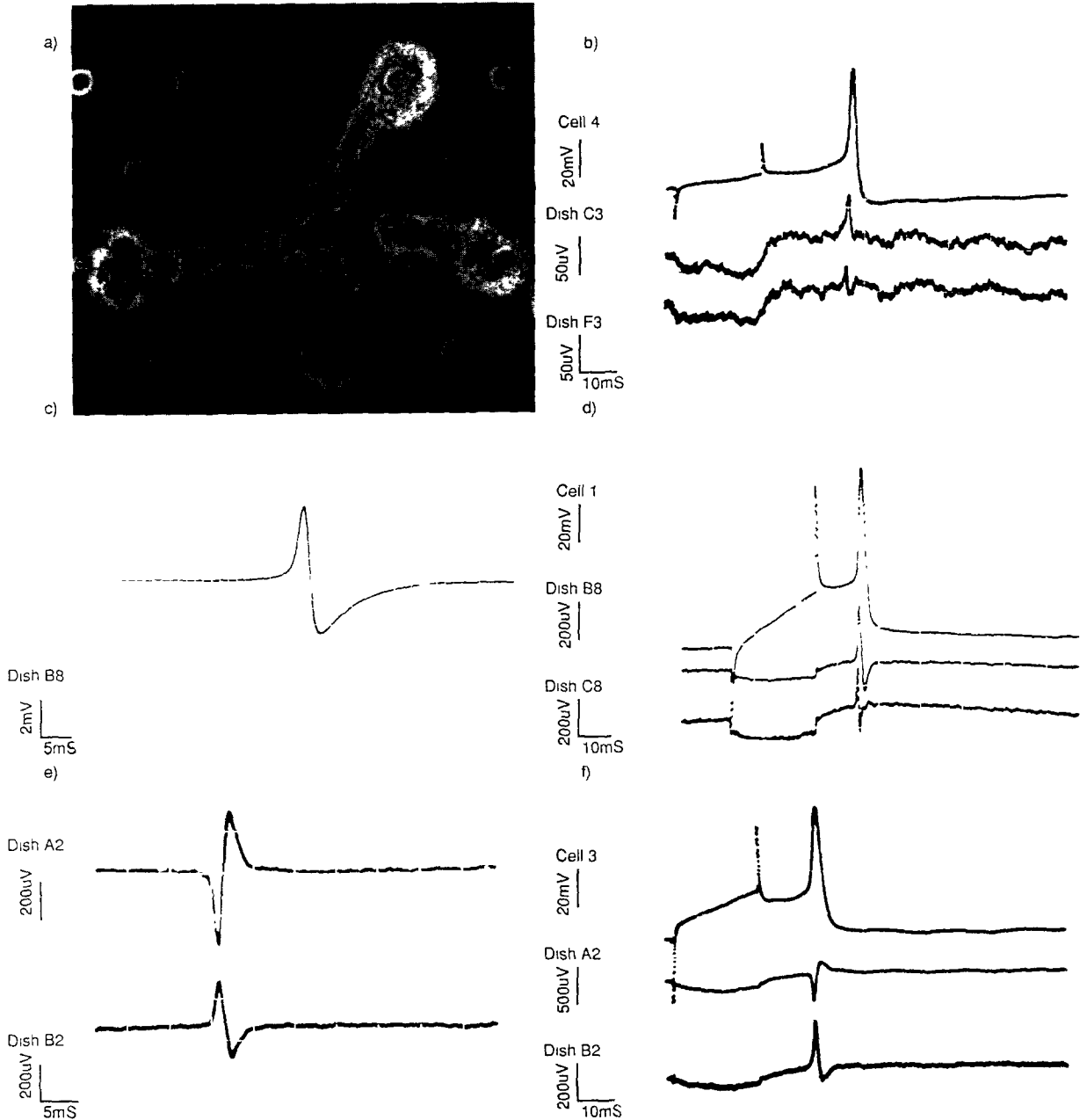


Fig. 6 a) four Retzius cells grown on multi-electrode array coated with air-dried concanavalin A (24 h after plating) b) intracellular potential of cell 4 (top trace) and waveform recorded by dish electrodes C3 and F3 (bottom traces) c) spontaneous activity recorded from electrode B8 d) intracellular potential of cell 1 (top trace) and waveform recorded by dish electrodes B8 and C8 (bottom traces) e) spontaneous activity recorded from electrodes A2 and B2. f) intracellular potential of cell 3 (top trace) and waveform recorded by dish electrodes A2 and B2 (bottom traces).

dish electrodes, it was also possible to record from neurites as illustrated in the experiment shown in Fig. 5. Cell A is a *Helisoma* B19 neuron with an

axon stump under cell B, which is a B5 neuron. Spontaneous activity was recorded by dish electrodes 1 and 2 (Fig. 5b). Waveforms recorded

from neurites were generally similar to those shown in Fig. 5b. By comparing the recordings made by these two dish electrodes, we see that the seal provided by neuron B over electrode 2 resulted in

a significant improvement in the signal-to-noise ratio. Simultaneous measurements of cell membrane potential, using conventional microelectrodes, of cells A and B revealed that the sponta-

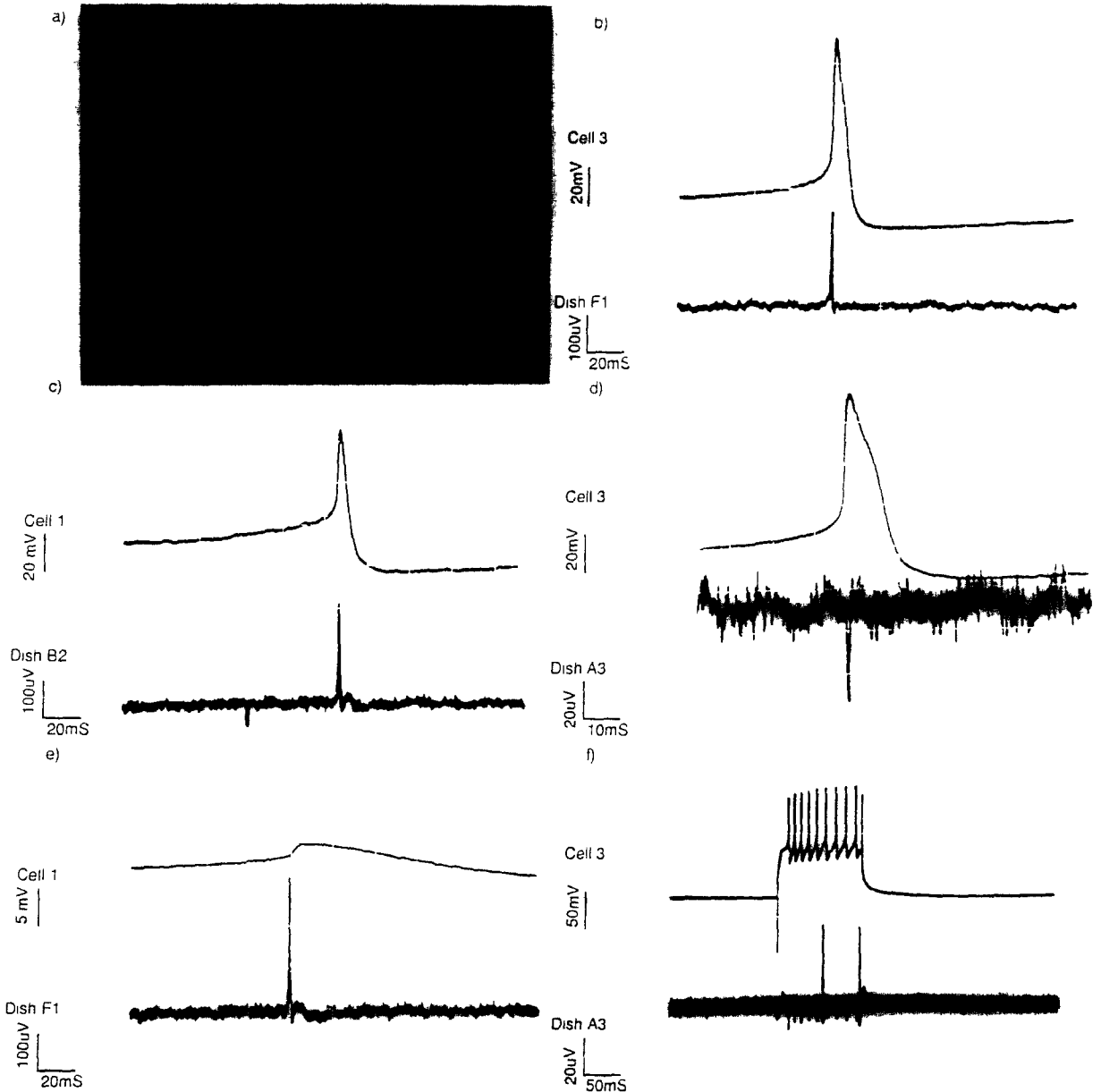


Fig 7 a: three LUQ neurons on a multielectrode array coated with air-dried concanavalin A (24 h after plating) b: intracellular penetration of cell 3 and recording with dish electrode F1 c: intracellular potential of cell 1 and waveform recorded by dish electrode B2 d: intracellular potential of cell 3 and waveform recorded by dish electrode A3. e: intracellular potential of cell 1 and waveform recorded by dish electrode F1. f: intracellular potential of cell 3 and waveform recorded by dish electrode A3

neous activity recorded by the dish electrodes was due to cell A. Dish electrode 2 also recorded waveforms associated with action potentials in cell B which were characteristic of those recorded by electrodes beneath *Helisoma* B5 somata.

We found that multielectrode arrays can be used with considerable success with *Hirudo* neurons. The culturing technique for these neurons resulted in extremely clean neuronal somata that immediately adhered when placed on dish electrodes coated with concanavalin A (Ross et al., 1988). Despite the smaller size of the leech neurons it was possible to record from a single cell from two electrode sites by careful cell placement, as was the case in the experiment shown in Fig. 6. The neurons shown here are Retzius neurons after 24 h in culture. It was possible to record from cells 1, 3 and 4 simultaneously with good signal-to-noise ratio, and to record the spontaneous electrical activity from cells 1 and 2. Figs. 6c and e show examples of spontaneous action potentials recorded by array electrodes. Intracellular penetrations confirmed that dish electrodes B8 and C8 were recording from cell 1, dish electrodes A2 and B2 recorded from cell 3, and dish electrodes C3 and F3 recorded from cell 4. These recordings demonstrate the tremendous variation in the size of signals recorded from array electrodes even though all of the neurons were Retzius cells. Also, as spontaneous activity was recorded over an extended time, the signal size would sometimes change in amplitude. Differences in seal resistances were probably responsible for these variations in signal size, but the seal resistances were not measured in these experiments. We were unable to detect a signal from electrodes that did not appear to be completely covered by a neuron.

Based on our experience with *Helisoma* neurons and leech neurons, it was clear that obtaining large seal resistances would lead to improved recording with multielectrode arrays. Thus, the large identified *Aplysia* neurons, R2, LUQs, and the MCC, seemed particularly suited to study with the array. In the experiment of Fig. 7, 3 LUQ neurons were grown on a multielectrode array coated with air-dried concanavalin A. It was possible to record from two of the neurons with good signal-to-noise ratio (cell 1, Fig. 7c, cell 3, Fig. 7b, d), and the

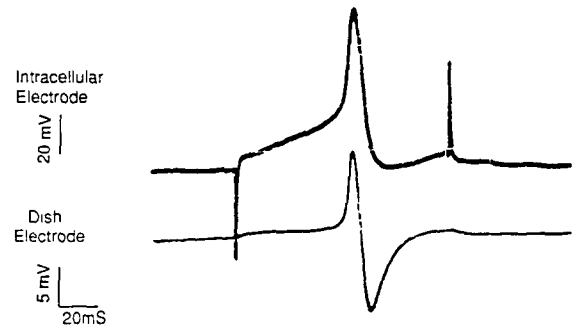


Fig 8 Top trace shows the intracellular potential of a MCC *Aplysia* neuron 24 h after plating Bottom trace shows a simultaneous dish electrode recording

two neurons were synaptically connected (Fig. 7e, f). However it was not possible to record activity from any electrode sites other than A3 and F1. In

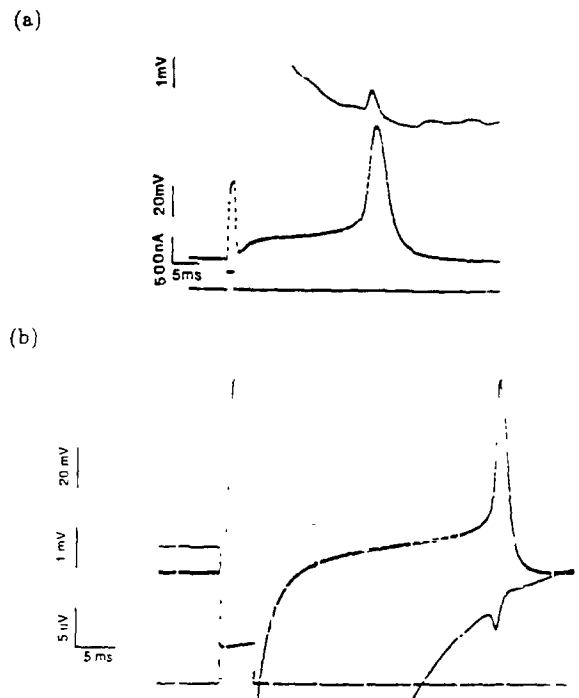


Fig 9 a a current pulse of 0.5 ms duration (bottom trace) passed through a dish electrode stimulates a cell to fire an impulse The same dish electrode is used to record the resulting action potential (top trace), and an intracellular electrode is used to verify the response (middle trace) b a current pulse passed through one dish electrode (bottom trace) stimulates the cell, and another dish electrode is used to record the resulting action potential (middle trace) An intracellular electrode records  $V_{cell}$

general, it was more difficult to get a good seal when working with *Aplysia* neurons: they tended to be coated with glia, and *Aplysia* saline has extremely high ionic strength. Without a seal the signal was shunted to ground. Even for an electrode beneath the soma, if the cell did not completely seal over the dish electrode, the resulting signal was extremely small. It was not the soma size that was important, but rather the area of the

neuron in contact with the substrate surrounding the electrode.

It is possible to obtain large seals and excellent signals from *Aplysia* neurons. An example of such a recording is shown in Fig. 8 in which a MCC was grown on the array for 24 h. An intracellular electrode was used to simultaneously stimulate the cell and to record the resulting action potential. The signal-to-noise ratio of the potential measured

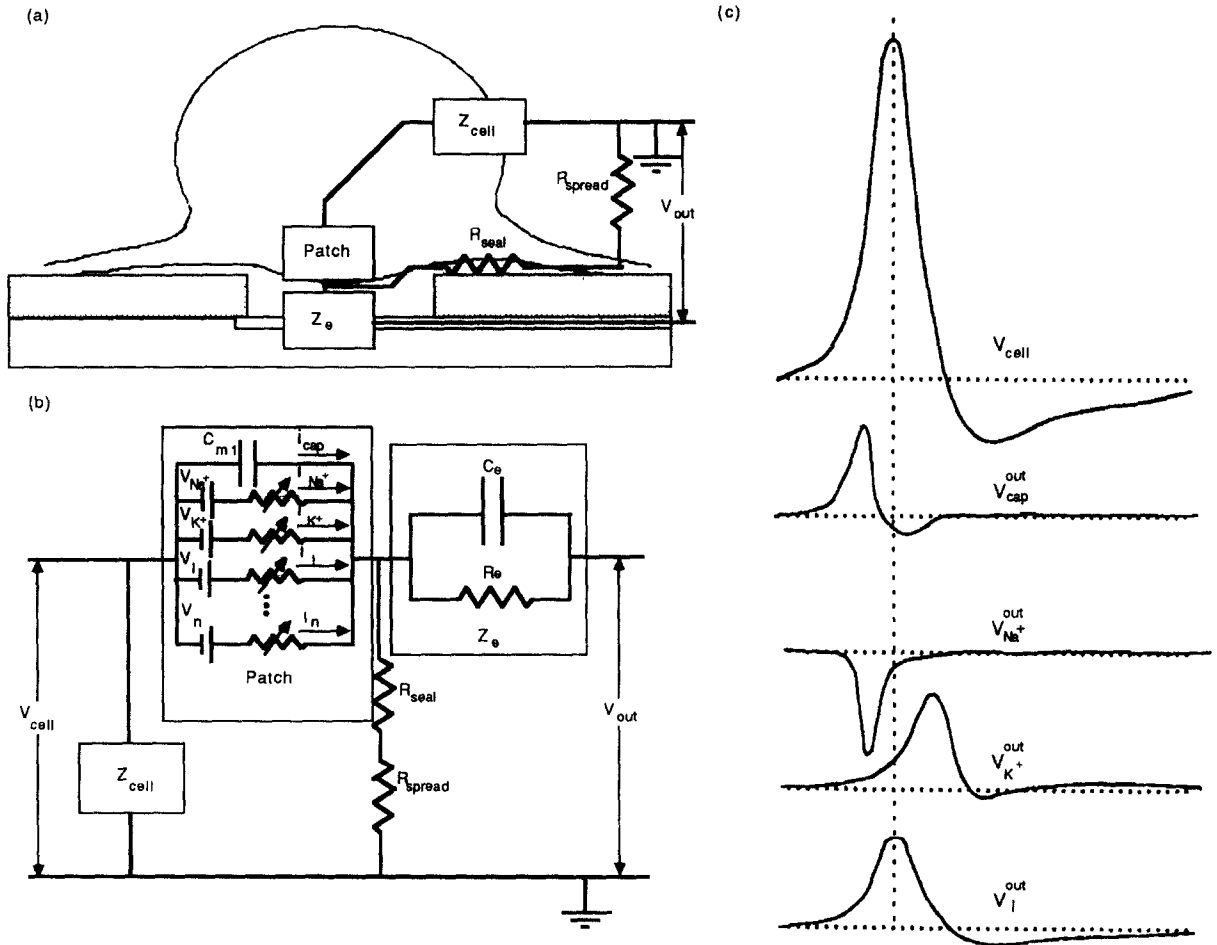


Fig. 10. a: schematic of a neuron sealed to an embedded dish electrode. b. approximate equivalent circuit for a neuron sealed in this manner. This equivalent circuit also applies to a patch-type electrode. c: a graphical representation of the various contributions to the potential recorded by the dish electrode during an action potential  $V_{cap}^{out}$  is the contribution due to capacitive current through the membrane above the patch electrode. During an action potential, the intracellular potential is given by  $V_{cell}$ . During the rising phase of the action potential, Na<sup>+</sup> enters through sodium channels giving a contribution  $V_{Na^+}^{out}$  at the dish electrode. Potassium channels then open and as potassium leaves the cell, there is a positive contribution to the potential  $V_{K^+}^{out}$  recorded by the dish electrode.  $V_l^{out}$  is the contribution due to current flow through non-ion-selective channels

with the array electrode was 500:1. While such signal-to-noise is much larger than can normally be obtained with *Aplysia* neurons, if such seals could be reliably obtained it would be possible to measure postsynaptic potentials.

### Stimulation

For neurons sealed to dish electrodes, it was possible to use the multielectrode array to both stimulate a neuron and record the resulting action potential. In the experiment shown in Fig. 9a, a dish electrode is used to stimulate a *Helisoma* neuron and to record the resulting impulse. In the experiment shown in Fig. 9b, a current pulse passed through a dish electrode was used to stimulate a *Helisoma* neuron to fire an action potential which was recorded by another array electrode (see Fig. 5a with electrode 1 stimulating cell 1 and electrode 2 recording the resulting action potential). A large stimulus current is required to elicit an action potential because the stimulating electrode is not sealed beneath the cell.

Similar results have been obtained for *Hirudo* and *Aplysia* neurons. However, due to smaller seal resistances and low input impedance for *Aplysia* neurons, it was often impossible to stimulate them. Dish electrodes are promising for chronic stimulation experiments in which a means of non-invasively verifying stimulation is required. It is important to monitor the seal resistance and adjust the stimulus current to avoid exceeding the membrane breakdown voltage.

## Theory

### Equivalent circuit

By considering a simple equivalent circuit, it is possible to understand the principles involved in stimulation and recording using sealed array electrodes and similar types of electrodes (Regehr et al., 1988a). Fig. 10a is a schematic circuit of a neuron sealed to a dish electrode, and Fig. 10b is an electrical equivalent circuit of a large diameter electrode sealed to a cell. The electrode impedance  $Z_e$  depends upon the type of electrode used. In the case of a glass loose-patch electrode it is essentially resistive, and varies between 100 k $\Omega$  and 1 M $\Omega$ . For metal electrodes, the impedance varies as

the area of the exposed metal and is primarily capacitive. It is desirable to have a low electrode impedance to facilitate measuring the seal resistance, to reduce the Johnson noise, and to allow larger stimulus currents without gas evolution. The seal resistance  $R_{\text{seal}}$  is the resistance between the electrode and bath due to medium between the cell and the insulation. It can be measured and is typically on the order of megohms. When the membrane is closer to the insulation, or the area of membrane-insulation contact increases, the seal resistance increases. The spreading resistance,  $R_{\text{spread}}$ , is the resistance due to the extracellular solution. The magnitude of this resistance can be estimated by considering a spherically symmetric electrode of radius  $r$  in a medium of resistivity  $\rho$ , with the bath ground far away,  $R_{\text{spread}} = \rho/4\pi r$ . This approximation is qualitatively correct for both glass-patch electrodes and dish electrodes (although the details differ due to differences in the geometry). The resistances  $R_{\text{spread}}$  and  $R_{\text{seal}}$  are linearly proportional to the medium resistivity.

### Recording

The patch region beneath the electrode is made up of a layer of membrane with a capacitance estimated by assuming a membrane capacitance of approximately 1  $\mu\text{F}/\text{cm}^2$ ; therefore  $C_1$  is 1 pF for a 100  $\mu\text{m}^2$  patch. Spanning this membrane are  $N$  different channel types with electrochemical driving forces  $V_1 \dots V_N$ . The resistances  $R_1 \dots R_N$  corresponding to each particular channel type are voltage and time dependent, with  $R_{\text{chan}} = (g_{\text{chan}}n_{\text{chan}})^{-1}$  and  $g_{\text{chan}}$  the conductance of a particular channel type, and  $n_{\text{chan}}$  the number of open channels of particular channel type in the patch. The net resistance depends upon the channel densities, which are generally non-uniform over the cell and unknown in the patch region. The whole cell impedance  $Z_{m2}$  is also voltage and time dependent. At the resting potential of the cell,  $Z_{m2}$  can be approximated by a capacitor and a resistor in parallel.  $i_1 \dots i_N$  are the current passing through each particular type of channel, and  $i_c$  is the current through the membrane capacitance.

Fig. 10c shows qualitatively the contributions of different channels and the capacitive current to the signal recorded by the loose-patch electrode.

$V_{\text{cap}}^{\text{out}}$  is the differentiated intracellular voltage seen through the capacitance of the membrane under the electrode. For this signal to be large, the action potential must be fast.  $V_{\text{Na}^+}^{\text{out}}$  is the voltage drop across the seal resistance due to current flowing through tetrodotoxin (TTX)-sensitive sodium channels in the patch (Fig. 10c). The sodium conductance  $g_{\text{Na}^+}$  is voltage dependent, and the number of open channels  $n_{\text{Na}^+}$  is both voltage and time dependent. The shape of the contribution of current flowing through the sodium channels is given by,  $(g_{\text{Na}^+})(n_{\text{Na}^+})(V_{\text{cell}} - V_{\text{Na}^+})$ . The contribution per ion channel can be approximated by assuming a conductance of 10 pS per sodium channel and a driving force of  $-50$  mV, so the maximum voltage contribution per open sodium channel is about  $-1 \mu\text{V}$  for  $R_{\text{seal}} = 2 \text{ M}\Omega$ . Similarly, the voltage drop across the seal resistance due to current flowing through inward rectifying potassium channels in the patch is given by,  $V_{\text{out}}^{\text{K}^+} = (g_{\text{K}^+})(n_{\text{K}^+})(V_{\text{cell}} - V_{\text{K}^+})$ .

$V_{\text{out}}^{\text{leak}}$  is the voltage drop across the seal resistance due to current leakage through the patch. For a leakage resistance  $R_1 = 5 \text{ G}\Omega$  and  $R_{\text{seal}} = 2 \text{ M}\Omega$ , a 100 mV action potential gives rise to a signal  $V_{\text{out}}^{\text{leak}} = 40 \mu\text{V}$ . This signal is the intracellular signal attenuated by an amount  $R_{\text{seal}}/(R_{\text{seal}} + R_1)$ . By applying a large current pulse to the patch electrode it is possible to temporarily make this signal very large by putting holes in the patch beneath the membrane and greatly decreasing the leakage resistance. Ideally one would like this signal to be as large as possible, so that patch recording would make it possible to record post-synaptic potentials. But, for seal resistances less than several times the cell's resting input impedance,  $R_1$  cannot be made too small or the cell may be damaged due to the resulting low conduction path to the bath. For any given cell type, an acceptable maximum leakage resistance  $R_1 + R_{\text{seal}}$  that will not harm the cell can be empirically determined.

The recorded voltage  $V_{\text{out}}$  is given by.

$$V_{\text{out}} = \left( i_c + \sum_{\text{chan}=1}^N i_{\text{chan}} \right) (R_{\text{seal}} + R_{\text{spread}})$$

$$V_{\text{out}} = \left( C_{\text{ml}} \frac{dV_{\text{cell}}}{dt} + \sum_{\text{chan}=1}^N \frac{(V_{\text{cell}} - V_{\text{chan}})}{R_{\text{chan}}} \right) \times (R_{\text{seal}} + R_{\text{spread}})$$

$$V_{\text{out}} = V_{\text{cap}} + V_{\text{ionic}}$$

$$V_{\text{ionic}} = V_{\text{Na}^+}^{\text{out}} + V_{\text{K}^+}^{\text{out}} + V_{\text{out}}^{\text{leak}} + \sum_{\text{chan}=4}^N i_{\text{chan}} (R_{\text{seal}} + R_{\text{spread}})$$

While the contributions of sodium channels, potassium channels, and non-selective holes have been shown explicitly, there may be significant contributions from other unspecified channel types contained in the patch membrane.

The results predicted by this simple equivalent circuit were tested by two-electrode experiments. These experiments were based on the assumption that sealed dish electrodes behave similarly to glass-patch electrodes of the same diameter. Experimental results using array electrodes indicate that this assumption was justified. Patch pipettes with tip openings 5–14  $\mu\text{m}$  in diameter were used to cover a cell membrane patch similar in area to that covered by a dish electrode. An intracellular electrode was used to simultaneously record the intracellular potential. Using glass electrodes allowed accurate monitoring of the seal resistance, substitution of the ionic contents of the pipette, and recordings from different parts of the cell. Tests were conducted primarily with *Aplysia* neurons, although some tests were performed on *Helisoma* neurons.

Results from a representative experiment are shown in Fig. 11. In this experiment, the giant cell R2 from the *Aplysia* abdominal ganglion was stimulated with the intracellular electrode and the patch electrode was placed on different areas of the cell. The seal resistance was determined by injecting a current pulse of known amplitude through the patch electrode. Patch electrode recordings were then compared with the derivative of the intracellular potential ( $dV_{\text{cell}}/dt$ ). For a patch electrode containing ASW, the recordings from patches on the soma were essentially derivatives of the action potential indicating that the capacitive current was much greater than the

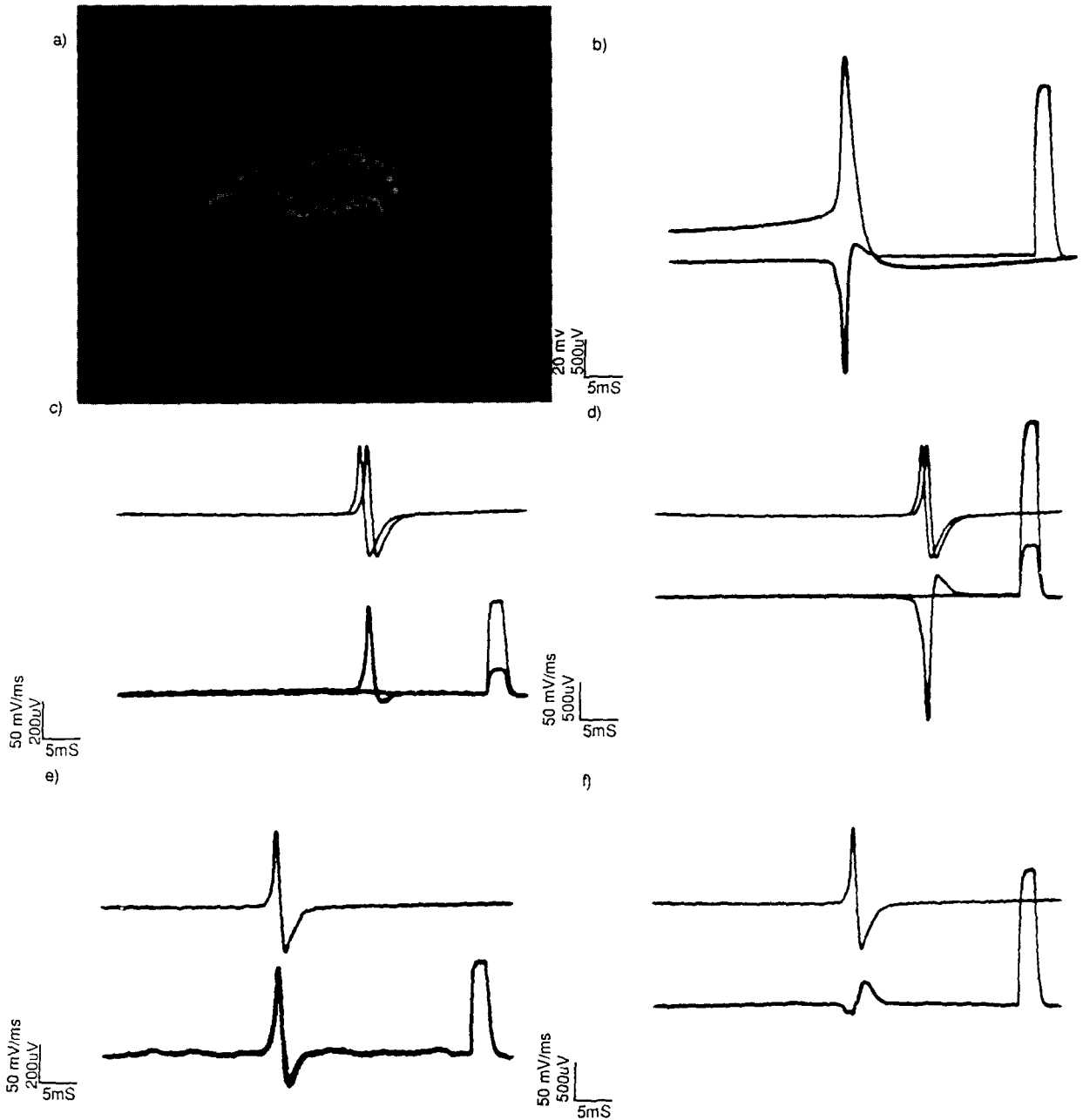


Fig 11 Example of a simultaneous two-electrode experiment performed using identified *Aplysia* neurons. A known current pulse was injected through the patch electrode to measure  $R_{\text{seal}} + R_e$  for traces b–f ( $R_e = 130 \text{ k}\Omega$ , and current injected for different traces was different). a) neuron R2 with circles to show where the patch electrode was placed (patch electrode diameter  $9 \mu\text{m}$  for b, c and d,  $11 \mu\text{m}$  for e and f). b) simultaneous recording of the action potential using an intracellular electrode and a patch electrode sealed to patch 2 ( $R_{\text{seal}} = 250 \text{ k}\Omega$ ): the top trace shows the  $dV_{\text{cell}}/dt$  for two trials. Trial A was conducted with the patch electrode just above the soma, and trial B with the electrode sealed to spot 1 (trial A:  $R_{\text{seal}} = 0 \text{ k}\Omega$ , trial B:  $R_{\text{seal}} = 300 \text{ k}\Omega$ ). d: top trace shows  $dV_{\text{cell}}/dt$  for two trials, and the bottom trace shows two successive recordings using the patch electrode, the first with the electrode just above the surface of the axon stump, and the second with the electrode sealed to spot 2 (trial A:  $R_{\text{seal}} = 0 \text{ k}\Omega$ , trial B:  $R_{\text{seal}} = 260 \text{ k}\Omega$ ). e. top trace shows  $dV_{\text{cell}}/dt$  and the bottom trace a simultaneous patch electrode recording with a patch electrode containing  $50 \mu\text{M}$  TTX in ASW sealed to the axon stump on spot 2 ( $R_{\text{seal}} = 300 \text{ k}\Omega$ ). f) top trace shows  $dV_{\text{cell}}/dt$  and the bottom trace a simultaneous patch electrode recording with the same TTX-filled patch electrode used in e but now sealed to the axon stump at spot 1 ( $R_{\text{seal}} = 200 \text{ k}\Omega$ ).

ionic currents. However, when sealed over the axon stump, the primary contribution was due to TTX-sensitive sodium currents. Fig. 11c,d show that a seal is required to record signals using a large diameter pipette. Without a seal, the current generated during an action potential was shunted to ground. For all of these experiments, no suction was applied to the patch pipette. Even without providing suction the patch would sometimes visibly deform. These cases were rejected. Even though there was no clear deformation of the patch membrane, the signals recorded via the patch pipette located on the soma were much larger than one would predict based on the equation  $V_{out} = R_{seal} C_{m1} (dV_{cell}/dt)$ , the measured area of the patch pipette, the seal resistance  $R_{seal}$  and the measured  $dV_{cell}/dt$ . For example, with a seal resistance of 300 k $\Omega$ ,  $dV_{cell}/dt = 80$  mV/ms, and a 9- $\mu$ m diameter patch electrode, one would predict the potential amplitude recorded by the patch electrode to be 15  $\mu$ V. Instead, as shown in Fig. 11c, the recorded signal was 400  $\mu$ V, a factor of 27 larger. For the dual electrode experiments, the patch electrode recordings were a factor of  $38 \pm 16$  ( $n = 16$ ) larger than one would predict from the capacitive contribution, using the area of the pipette tip to calculate  $C_{m1}$ . We interpret this to be a result of the area of the patch pipette tip being an underestimate of the area of the membrane within the patch due to invaginations of the membrane. Qualitatively, this result is consistent with anatomy (Graubard, 1975). For the 16 soma patches taken on *Aplysia* neurons, the wave forms were qualitatively similar to the derivative. However, on some traces there were small ionic components superimposed upon the capacitive signals as was sometimes observed when recording with array electrodes (see for example Fig. 7b,c lower traces). These were likely due to outward  $K^+$  currents, but this was not confirmed pharmacologically as was done for  $Na^+$  currents.

### Stimulation

Generally, neurons used in these experiments will fire an action potential when depolarized by 5–15 mV from their resting potential. Such a depolarization can be achieved by injecting less than 1 nA with an intracellular electrode. In order

to achieve such a depolarization, with large-diameter patch electrodes much larger currents are required. For a current pulse applied through the patch electrode, only a small fraction passes into the cell through the patch and then out through the rest of the cell. Most of the current flows through the seal resistance to ground. For a stimulus current pulse  $I_{stim}$ , the voltage applied to the patch is  $V_{patch} = I_{stim} R_{seal}$  assuming  $R_{seal}$  is much smaller than  $Z_{m1}$ . A positive current pulse thus hyperpolarizes the patch membrane and depolarizes the rest of the cell membrane. For stimulus pulses which are short compared to the time constant of the cell (which is typically 5–20 ms), the internal voltage change is approximated by (Regehr et al., 1988a):

$$\Delta V_{cell} = \frac{\Delta t C_{m1}}{C_{m2}} \frac{dV_{patch}}{dt} + \frac{\Delta t}{C_{m2}} \left( \sum_{chan=1}^v (V_{cell} - V_{patch} - V_{chan})(n_{chan} g_{chan}) \right)$$

Since channel type and density in the patch are not known, the necessary stimulus current must be empirically determined. There are two constraints limiting the stimulus current. First, if a voltage greater than about 1 V is applied to a metal electrode, gas may evolve which damages the cells. An electrode can be approximated by a capacitor with a capacitance  $C_e$ . A current pulse with amplitude  $I_{stim}$  and duration  $\Delta t$  results in an electrode potential  $\Delta V_e = I_{stim} \times \Delta t / C_e$  which limits the total charge  $I \Delta t$  useful for stimulating. Second,  $I_{stim}$  must be kept small to prevent  $V_{patch}$  from exceeding about 300 mV, or the membrane in the patch may be electrically broken down. This would damage the cell unless  $R_{seal}$  was much larger than has been reliably obtained.

### Discussion

Stimulating and recording with multielectrode arrays offers the ability to non-invasively monitor spontaneous activity simultaneously from a large number of neurons. Success for both stimulating and recording depends upon causing the neuron to seal over the dish electrode. The necessary seal

resistance was found to be dependent on cell type. For example, differences in recordings obtained from *Helisoma* B5 and B19 neurons can be explained in terms of differences in the electrophysiology of these two types of neurons (Cohan et al., 1985). For B19 neurons which have a very fast action potential,  $dV_{\text{cell}}/dt$  is large and the capacitive term dominates. However, for B5 neurons, the action potential is broad, the capacitive term is much smaller, and the contribution from leakage and ionic currents can be of comparable size. Consequently, to obtain a signal-to-noise ratio of 10:1 from an array electrode beneath the soma of a B5 neuron, a seal of about 1 M $\Omega$  is required, while a seal of only 200 k $\Omega$  is required for an electrode sealed beneath a B19 neuron. In general, higher seal resistances are obtained in lower ionic strength electrolytes. This would tend to favor *Helisoma* neurons over *Hirudo* neurons, which would in turn be favored over *Aplysia* neurons. However, success with the leech neurons demonstrated that a culturing technique that left the cell body extremely clean, when used with an appropriately sticky substrate, led to large seal resistances and allowed reliable stimulation and recording. Even for *Aplysia* neurons, it was possible to obtain large seals that facilitated recording with excellent signal-to-noise ratio. In fact, arrays could be used to measure subthreshold responses if high-resistance seals could be reliably obtained. Substrate preparation and the condition of the cell surface are crucial factors in reliably obtaining large seals. It may also be possible to extend this technique to smaller neurons, such as vertebrate neurons. Building arrays of smaller electrodes would not be difficult. The major difficulty is obtaining reliable seals to the electrodes, cell placement, and finding substrates sticky enough to prevent cell movement after plating.

These results suggest techniques that might be implemented to obtain improved recording. Once neurons have grown out and formed connections, a confluent layer of cells, such as glia, could be grown over the neurons. This may seal the neurons to the electrodes, preventing bath electrolytes from shunting the signal, and allowing recordings from processes as well as somata. It may be possible to apply these methods to smaller vertebrate

cells, since the 'sealing' need not be done by the neurons, but would be done by other cell types. Alternatively, a gas permeant electrical insulator (e.g. silicone oil) could be lowered over cells during recording sessions.

At present, no reliable technique exists to chronically electrically stimulate individual cultured neurons. While we have demonstrated that sealed dish electrodes can stimulate neurons, we did not perform long-term experiments. In the course of our experiments it became clear that great care must be taken to measure the seal resistance during long-term stimulation. A decrease in the seal resistance would result in the stimulus current dropping below threshold, and an increase in seal resistance could result in  $V_{\text{patch}}$  becoming large enough during stimulation to exceed the membrane breakdown-voltage of the cell and damage it. By carefully monitoring the seal resistance it should be possible to use array electrodes for long-term stimulation experiments.

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