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CHAPTER 4

Distributed processing in cultured neuronal networks

Steve M. Potter *

Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA

Introduction

Thanks to a number of recent technical advances, it will become increasingly popular to study the very basics of distributed information processing using cultured neuronal networks. Most researchers studying population coding are working with intact, living animals. Clearly, cultured neuronal networks lack many features of real brains, but they retain many others. They develop organotypic synaptic connections and exhibit a rich variety of distributed patterns of electrical activity. Progress in multi-electrode array technology, optical recording, and multi-photon microscopy, has made it possible that every cell in a cultured monolayer network can be observed, monitored, stimulated, and manipulated with temporal resolution in the submillisecond range, and spatial resolution in the submicron range, in a non-destructive manner. At present, such detailed and complete analysis of neural circuits is not feasible in living animals, or even brain slices. It is an open question, however, whether any of the 'processing' done by cultured neurons is relevant to that carried out by intact brains. This chapter serves to present efforts from a number of groups that lay the groundwork for an in vitro approach to studying population coding. I will suggest what it might take to advance the state of the art to the point where we can consider studying learning, memory, and distributed information processing in vitro.

* Corresponding author: S.M. Potter, Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA. E-mail: spotter@gg.caltech.edu

Dissociated neuronal networks

Mammalian neurons can be mechanically and enzymatically dissociated from brain tissue and grown in culture for months, with the proper attention to maintaining sterility, temperature, pH, osmolarity, oxygenation, and providing a supply of nutrients and growth factors. This technology was worked out years ago (reviewed in Banker and Goslin, 1998), although improvements continue to be made. During the first week in culture, the neurons extend many neurites, form synapses, and begin to develop spontaneous activity (Habets et al., 1987; Corner and Ramakers, 1991; Gross et al., 1993a; Basarsky et al., 1994). These activity patterns, including complex sequences of action potentials in isolation and in bursts (rapid barrages), continue to develop over the course of a month in vitro. Underlying these activity changes are morphological changes of the neurons, as they grow elaborate dendritic and axonal arbors and form numerous synaptic connections (Corner, 1994). Usually (but not always) the neurons are terminally differentiated at the time they are plated onto a culture dish. The glial cells, if present in the dish, continue to divide and proliferate until limited by contact inhibition or exogenous inhibitors of cell division (Banker and Goslin, 1998). Glial cells provide necessary trophic factors for cultured neurons (Meyer-Franke et al., 1995; Banker and Goslin, 1998), and there is evidence that direct contact between neurons and glia is also crucial for neuronal survival, if not synaptic processing as well (Pfrieger and Barres, 1997).

Multi-electrode array history

Traditionally, the excitable properties of neuronal cultures are studied using glass micropipet electrodes. Because each electrode must be held and tediously positioned by a bulky mechanical micromanipulator, it is very difficult to record from or stimulate more than a couple cells at a time. This limitation has not prevented neurophysiologists from learning much about single-cell properties, ion channels, pharmacology, and synaptic plasticity in vitro (Cotman et al., 1988; Misgeld et al., 1998). However, like observing the world through a drinking straw, these approaches miss many of the collective properties of neuronal networks.

Multi-electrode array culture dishes allow simultaneous recording from and stimulation of over a hundred neurons, greatly expanding our field of view, while keeping the single cell in sharp focus. These wired Petri dishes are most often referred to as MEAs (multi-electrode arrays or micro-electrode arrays), but have also been called multi-microelectrode plates, planar electrode arrays, and multi-electrode dishes. MEA technology enables the study of distributed patterns of electrical activity in cultured networks via non-invasive extracellular electrodes built into the substrate. These electrodes can also be used to stimulate neurons extracellularly and non-destructively (Regehr et al., 1989; Gross et al., 1993b), allowing a long-term two-way connection between a cultured neuronal network and a computer.

MEAs have been around for a while. Thomas and co-workers first described multi-electrode arrays for monitoring activity in electrically excitable cells in 1972 (Thomas et al., 1972). They recorded field potentials from spontaneously contracting sheets of cultured chick cardiac myocytes, but could not record activity from single cells. A few years later, Pine (1980) and Gross et al. (1982) independently developed arrays for chronic multi-single-cell recording and electrical stimulation of cultured neuronal networks. Until recently, custom-made MEAs, hardware and software were created by each of the labs that dared to get involved in this technically demanding field (Pine, 1980; Israel et al., 1984; Novak and Wheeler, 1986; Connolly et al., 1990; Eggers et al., 1990; Janossy et al., 1990; Borroni et al., 1991; Jimbo and Kawana, 1992; Martinoia et al., 1993; Gross and Schwalm, 1994).

Fortunately, MEA technology is now accessible to labs that do not care to delve into the subtleties of computer programming, array microfabrication and electronics development. Complete MEA systems capable of recording from at least 60 electrodes are produced by MultiChannel Systems of Germany (the 'MEA60' [1]), and Panasonic of Japan (the 'MED System' [2]). Guenter Gross (U. of N. Texas) supplies MEAs that can be used with multi-electrode processing hardware and software made by Plexon Inc. [3] Only very recently has computer and data storage technology made it feasible to be able to record continuously from 60 electrodes, at sampling rates over 20 kHz/channel. We will continue to see rapid advances in the capabilities of commercial MEA systems, propelled by advances in microfabrication, computer speed, and data analysis. The in vivo multi-electrode probe community is also helping to advance the state of the art, since they share many of the same hardware and data analysis problems with the in vitro community. [4]

MEA fabrication

MEAs consist of a number of cell-sized electrodes (10–100 μm) arrayed across the bottom of a cell culture dish. The substrate is usually glass, with leads made of gold or the transparent conductor indium–tin oxide, that carry signals from electrodes to external electronics, and carry stimuli to the electrodes (Fig. 1). (Indium–tin oxide electrodes and

[1] http://www.multichannelsystems.com

[2] http://www.panasonic.com/medical_industrial/medsys.asp

[3] http://www.plexoninc.com/

[4] I set up an internet mailing list, The MEA-Users, to facilitate interaction within and between these groups and provide a clearing-house for rapid dissemination of relevant information. To subscribe, send the message (no subject line, no quotation marks, no signature) 'subscribe mea-users' to majordomo@its.caltech.edu. To receive a description of the group, send the message 'info mea-users' to the same address.

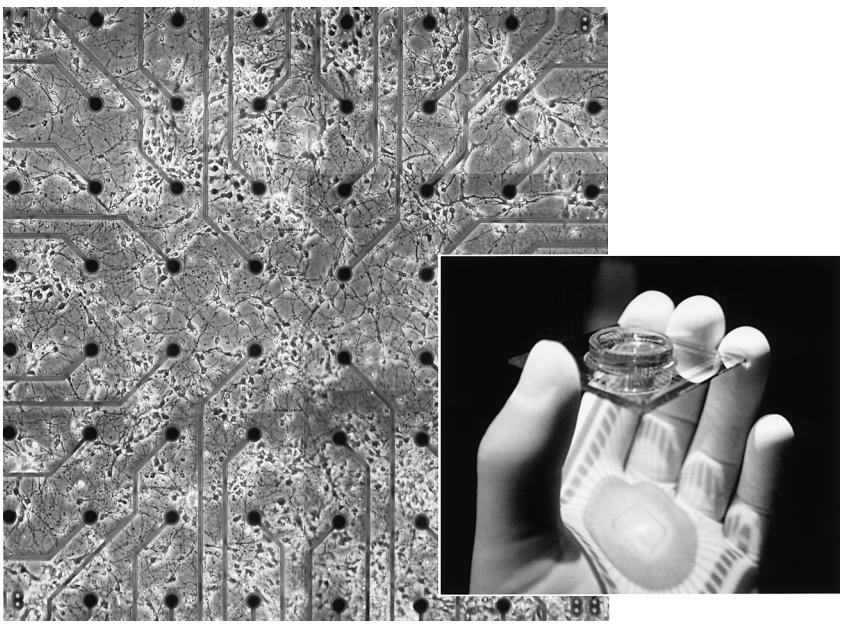

Fig. 1. 60-electrode MEA from MultiChannel Systems, with developing rat cortical culture after 6 days in vitro. The 10-μm diam. electrodes (not visible in the center of a 30-μm gold disk) are 200 μm apart, with sputtered titanium nitride to reduce impedance. The gold leads (beneath an insulating layer of silicon nitride) travel under a glass ring, containing cell culture medium, to contacts around the perimeter of the 50-mm glass plate (inset). These are connected to preamplifiers, analog-to-digital converters, and to a computer. They can also be connected to stimulation circuitry, allowing long-term two-way communication between the neurons growing over the electrodes and the computer.

leads are commonly used for liquid–crystal displays on digital watches and other consumer electronics.) MEA electrodes must be biocompatible, durable, and have a reasonably low impedance (less than 500 kΩ at 1 kHz) to allow the detection of small extracellular signals (from 10 to 100 microvolts). The low impedance also allows sufficient stimulation current to be passed without exceeding the electrochemical breakdown voltage of water and other components of the medium (usually around one volt). The electrodes on MEAs have traditionally been electroplated with porous platinum (‘platinum black’). This is not very durable, and thus the impedance rises unacceptably when the MEAs are re-used and even during long-term culturing. This problem can be greatly reduced by electroplating while sonicating, which allows only durable platinum crystals to form (Marrese, 1987). Recently, relatively tough, low-impedance electrode coatings have been created by sputtering iridium oxide (Blau et al., 1997) or titanium nitride (Egert et al., 1998). The surface of the MEA and the electrode leads are coated with some biocompatible insulator (usually polyimide or silicon nitride/oxide) that prevents electrical shorting to the bath, and allows cell adhesion after coating with traditional cell culture substrates such as polyamino acids and laminin. MEAs have also been fabricated out of silicon (Pancrazio et al., 1998; Maher et al., 1999), and there has been some success recording from and capacitively stimulating neurons growing on the insulated gates of silicon field-effect transistors (Fromherz and Stett, 1995; Offenhausser et al., 1997; Vassanelli and Fromherz, 1997). The Pine group has produced a 16-well silicon ‘neurochip’ designed to hold 16 neurons in close apposition to electrodes at the bottom of the wells (Maher et al., 1999). It has been difficult to design an effective grillwork on the well that keeps the cell soma in the well, yet allows neurites to grow out and make contacts. I and my Pine lab colleagues have observed that neurons persistently escape from the wells, especially if there are glial cells nearby for them to adhere to.

MEAs for studying neural coding

Retina researchers have provided fruitful examples of the directions we may wish to take with dissociated cultured networks. The 61-electrode Pine-style MEAs have been used quite successfully by several groups studying processing in the retina (Wong et al., 1993; Warland et al., 1997; Nirenberg and Latham, 1998). Explanted retinas are laid down on the MEAs, and exposed to various types of light stimuli, while recording ganglion cell responses (Meister et al., 1994). For the retina, the appropriate inputs are reasonably well-defined, that is, spatial patterns of light; and the sole output of the retina is sequences of action potentials in retinal ganglion cells. Nirenberg and Latham (1998) suggest that knowing the input–output relationship of the retina is equivalent to knowing how it *encodes* a visual stimulus. The words 'encoding' and 'processing' suggest some sort of non-trivial transformation of information. What would it take to believe that a dissociated cultured network had performed a non-trivial transformation of information? Sakurai (1996) as well as contributions in this volume demonstrate that intact brains rely on population coding. To verify that neurons can also make use of population coding in vitro, we must first devise a system in which there is any coding at all. Inputs, outputs, and a non-trivial transformation must be defined.

Recent progress with MEAs

Understanding the relevant parameters for coding in cultured networks is likely to require long-term monitoring and stimulation. MEAs make this possible because unlike glass micropipets, MEA electrodes are non-invasive. For example, Welsh and co-workers demonstrated the usefulness of MEAs for chronic recording from cultured networks of cells from the rat suprachiasmatic nucleus (SCN) (Welsh et al., 1995). The circadian activity intrinsic to these neurons was followed continuously for weeks, and it was demonstrated to be a single-cell, not a network property, by reversibly blocking synaptic transmission with tetrodotoxin. After washout, the activity of individual neurons resumed in phase with their pre-treatment activity. This, and more recent studies (Herzog et al., 1997, 1998; Honma et al., 1998) have shown that SCG neurons in culture exhibit a variety of circadian frequencies and phase relationships, and the mechanisms by which they are synchronized in vivo are now being tested in vitro (Liu et al., 1997).

Potential 'outputs' of cultured networks might be the recurring patterns of action potential firing they spontaneously exhibit. The Gross lab has pioneered the analysis and categorization of the "bewildering variety of spatio-temporal spike and burst patterns" (Gross and Kowalski, 1991, p. 66) in MEA cultures prepared from mouse spinal cord (Droge et al., 1986; Gross and Kowalski, 1991; Gross et al., 1993a; Rhoades et al., 1996). Of 120 cultures surveyed for spontaneous activity between 3 and 12 weeks in vitro, 60% showed "predominant bursting with an ever-changing sequence of random, patterned (possibly chaotic), and short periodic burst sequences" (Gross and Kowalski, 1991, p. 66). 10% of the cultures were silent (but activity could be induced pharmacologically), 20% exhibited mostly isolated action potentials and little bursting, and 10% exhibited periodic bursting. This activity is usually synchronized across all active electrodes. They demonstrated that cultures could be switched between different modes of bursting (e.g., periodic vs. random) by washing in and out various pharmacological agents (Gross et al., 1993a). Activity on each electrode was summed using a 'leaky integrator' process in which each action potential causes the pen of a chart recorder to rise a fixed, tiny increment, while it descends exponentially with a slow time constant (e.g., 300 ms). This process facilitates burst analysis, with each burst appearing as a large peak on the chart. However, it discards the subtle timing information of individual action potentials within and between bursts. Recurring patterns of action potential firing with precise timing have been observed in a number of brain circuits, such as the hippocampus (Nadasdy et al., 1999), respiratory centers (Frostig et al., 1990) and cortex (Abeles et al., 1994). It is clear that the arrival time of individual action potentials carries a lot more information in animals than does the mean firing rate (reviewed in Gerstner et al., 1997 and Rieke et al., 1997). If such activity patterns exist in cultured networks, their dynamics might be overlooked using the 'leaky integrator' approach.

Evidence that such subtle, recurring action potential patterns do exist in cultured networks comes

from the Kawana lab at Nippon Telegraph and Telephone in Japan. Using their own custom MEA hardware and software, they have pioneered the study of plasticity in spontaneous and stimulated activity patterns in dissociated rat cortical cultures (Jimbo and Kawana, 1992; Robinson et al., 1993a,b; Maeda et al., 1995, 1998; Kamioka et al., 1996; Kawana, 1996; Watanabe et al., 1996; Canepari et al., 1997; Jimbo et al., 1998, 1999; Konno et al., 1998; Tateno and Jimbo, 1999). Jimbo and co-workers used MEAs to reveal distributed changes in the network properties of cortical cultures as a result of extracellular stimulation via the substrate electrodes. They elegantly demonstrated that they could induce both potentiation and depression of network activity in a pathway-specific manner (Jimbo et al., 1999). They used cultures that had been growing on 64-electrode MEAs for at least one month. After this time, the cultures have reached a developmentally stable period (Jimbo et al., 1999), exhibiting a complicated pattern of spike-firing and bursting (Kamioka et al., 1996). They monitored network response to a single probe pulse stimulus (biphasic: 100 μs +0.6 V, 100 μs −0.6 V) applied to each electrode in succession at 3-s intervals. The responses at each electrode (the activity of one to five neurons near it) were averaged for 10 scans of this probe pulse across all channels. The response of the whole network to probe pulses on any given electrode was quite reproducible for the first 50 ms after the pulse (Fig. 2).

To induce synaptic weight changes, a strong stimulus was delivered to the network at a single site (tetanic pulse sequence of 20 trains (5-s intervals) of 10 pulses (20 Hz, as above)). Finally, the original 10 scans of network response to single probe pulses were repeated. Across 8 MEA cultures studied (41–53 days in vitro), an average of 22 electrodes (out of 64) per dish showed a potentiated response after single-site tetanus, while an average of 6 electrodes (out of 64) showed a depressed response. An analysis of cross-correlations between the tetanized electrode's activity, and the others was very revealing: those neurons that tended to fire in synchrony with the tetanized pathway were potentiated. Those whose correlation was poor gave a depressed response. Interestingly, both potentiated and depressed pathways showed enhanced synchrony with the neurons recorded on the tetanized electrode, after tetanus. They concluded that potentiation and depression of pathways in these cultures are two possible outcomes of the same process, whose details are still unknown. Tightly correlated pathways become potentiated, loosely correlated pathways become depressed.

This study represents a significant advance on the paired-cell recording and stimulation work that showed similar influence of relative spike timing on plasticity (Bi and Poo, 1998; Markram et al., 1998; Zhang et al., 1998), because the multi-electrode approach showed that the changes were synapse-specific and network-wide, not cell-specific. That is, looking at activity on a specific electrode, they saw

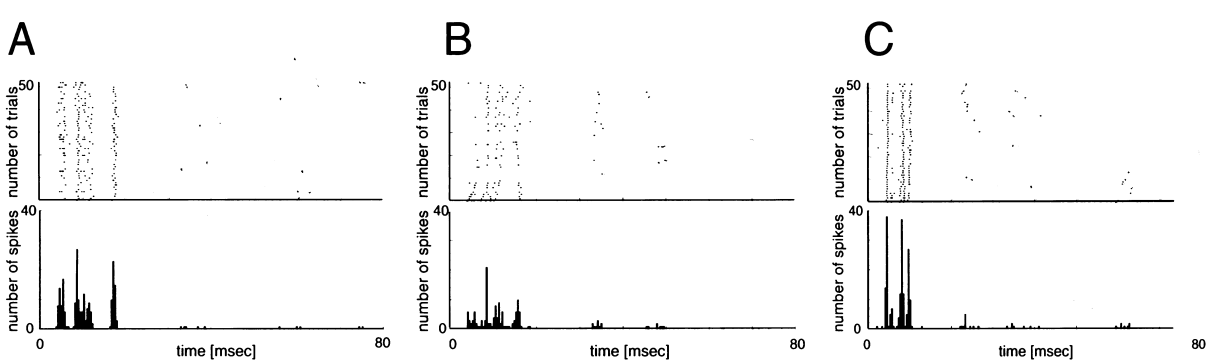

Fig. 2. Example of the reproducible response of an MEA culture to probe stimuli, adapted from Tateno and Jimbo, 1999 (with permission). Raster plots (top) and post-stimulus time histograms (bottom, 0.5 ms bins) of action potentials recorded from one MEA electrode are shown for three different blocks of 50 probe pulses applied to electrode 'C2,R2'. Between blocks A and B, a strong tetanic stimulus was applied to electrode 'C5,R6', and between blocks B and C, the tetanic stimulus was applied to both 'C5,R6' and 'C2,R2'. Note how the response timing generally shortens and sharpens up with stimulation, yet still contains reproducible patterns past 50 ms after the probe pulse.

enhanced responses to some probe stimuli (as they sent a single probe pulse to each electrode in turn), and depressed responses to others, all resulting from tetanus at a single electrode in the MEA. However, this conclusion is weakened by the fact that some electrodes contact more than one neuron, and the number of cells directly activated by the tetanus is not known. They showed previously that while intracellular tetanus to a single cell had no effect on network activity, extracellular tetanus (presumably exciting more than one cell near the electrode) evoked a large network response (Jimbo et al., 1993). In a separate study by Tateno and Jimbo, a similar tightening of synchrony was observed as a result of tetanic stimulation (Tateno and Jimbo, 1999, fig. 2). The authors hypothesize that "changes in synaptic efficacy enhance or reduce the reliability and reproducibility of spatially correlated neuronal responses in networks" (p. 45). In none of these studies did they monitor the changes in synaptic weight past one hour. It would be informative to carry out more long-term recording to determine how permanent are the changes induced by various types of stimulation.

Brain slices on MEAs

There are a number of groups applying MEA technology to brain slices, either acute (freshly cut) or maintained in organotypic culture (Wheeler and Novak, 1986; Novak and Wheeler, 1988; Borroni et al., 1991; Boppart et al., 1992; Heck, 1995; Borkholder et al., 1997; Stoppini et al., 1997; Thiebaud et al., 1997, 1999; Egert et al., 1998; Fejtl et al., 1998; Duport et al., 1999; Jahnsen et al., 1999). Slices have the advantage that their cytoarchitectonics and connectivity are closer to those of intact brains, compared to dissociated cultures. Thus their 'inputs' and 'outputs' might be more clearly defined. However, for analyzing networks and cells in great detail, brain slices have many of the same problems as whole animals, with too many cells packed too closely. MEAs record field potentials from slices, not single-cell activity. For acute slices, there is the concern that the electrodes are closest to a layer of dead or dying cells near the cut surface. To surmount this problem, some are experimenting with MEAs that have electrodes on the ends of small spikes (Thiebaud et al., 1999). This 'bed-of-nails' approach might allow recording and stimulation of more healthy cells within the slice. For cultured slices, the problem is that there is poor access of oxygen and nutrients to the cells at the bottom of the slice. Thus, the ones near the electrodes again are the least healthy. The creation of porous MEAs may eliminate this problem (Boppart et al., 1992; Stoppini et al., 1997; Thiebaud et al., 1999). Because of these difficulties, the slice-MEA field is still in its infancy, but we can expect some advances in the near future that will help fill the gap between intact brains and dissociated networks.

Optical imaging of cultured networks

Unlike slices, dissociated neural cultures form a monolayer on a clear substrate, lending themselves well to optical recording of activity in individual cells. By imaging the calcium signals in developing cortical cultures using the calcium-sensitive dye, Fluo-3, Voigt and co-workers showed that cells that fired bursts in synchrony with the rest of the culture (on the time scale of seconds) survived better (63% survival 4 days after optical recording) than those that did not (22% survival, asynchronous and non-bursting groups combined) (Voigt et al., 1997). This suggests that neural co-activation plays an important developmental role in network architecture, even in vitro. The temporal resolution of calcium imaging systems is usually not fast enough to see individual action potentials, only bursts of them. Jimbo and co-workers used simultaneous MEA and optical recording to verify that these optical calcium signals correspond to bursts of electrical activity (Jimbo et al., 1993). It remains to be determined whether subtleties in action-potential timing responsible for the synaptic weight changes observed by Jimbo et al. (as described above) are also involved in neuronal survival.

Optical recording of membrane voltage, in contrast to imaging calcium signals, can provide a direct, fast measure of electrical activity in many individual neurons of neuronal networks. In 1973, Davila, Salzberg, and Cohen presented the first optical recording of an action potential using a voltage-sensitive dye and a single photodiode, and proposed that:

> "An apparatus with a large number of photodiodes, arranged so that each detector would receive the

> light from an individual cell body, could, with a small computer, monitor the activity of, perhaps, a hundred cells at once. Such a large increase in the number of monitored cells could facilitate the determination of functional connexions between cells, and ultimately lead to an understanding of the neuronal basis of behaviour." (Davila et al., 1973, p. 160)

Since then, multi-single-neuron optical recording of voltage signals (as distinct from optical recording of field potentials or intrinsic signals in bulk tissue) has been used with great success in invertebrate ganglia (Wu et al., 1994, 1998), and recently in mammalian intestinal enteric plexus, which is naturally a monolayer network (Obaid et al., 1999). Thus, it is reasonable to expect that optical recording should allow the observation of distributed processing in small networks of cultured neurons, in even greater detail than using MEAs. Three things have impeded the realization of this goal: (1) the optical signals from cultured (especially mammalian) neurons are very small, usually less than 1% change during an action potential; (2) sensitive, fast imaging systems with submillisecond and single-cell resolution are not readily available; and (3) the potentiometric dyes used tend to be very phototoxic and photobleach (fade) rapidly.

It is well worth trying to overcome these difficulties, and to combine optical recording with MEAs. Because electrically recorded extracellular signals are approximately 100 μV or less, and extracellular stimuli are often 10,000 times larger, electrical recording from an MEA electrode during stimulation is not feasible. Optical recording during stimulation would make it possible to observe exactly which cells were stimulated by current injection through substrate electrodes. It would also allow us to observe activity in cells too far from substrate electrodes to record from electrically. Spike-sorting algorithms could be tested out on cases where the same cells are recorded optically and electrically. The optical recordings would provide the 'ground truth', that is, exactly where each cell is in relation to the electrode and when it fired. These tests would be of interest to the in vivo multi-electrode probe community, where the ground truth for the multi-unit activity picked up by the probe is not readily accessible to the experimenter.

Traditionally, optical recording is done using photodiode arrays, with 10×10 or 25×25 pixel resolution (Chien and Pine, 1991). To allow higher-resolution high-speed imaging, Pine and I designed and built a CCD (charge-coupled device) camera with 64×64 28-μm pixels capable of recording spontaneous and evoked action potentials (in a single trial) in cultured rat neurons (Pine and Potter, 1997; Potter et al., 1997b). This camera has the unique ability to digitize any arbitrary combination of pixels, and pass over uninteresting ones, to allow imaging at over 1000 frames/s. Bullen, Patel and Saggau created a functionally similar, but entirely original optical recording device that rapidly scans a laser beam from cell to cell using computer-driven acousto-optic deflectors (Bullen et al., 1997). This was used to record optical signals in single cultured hippocampal neurons with a 5 mV, 0.5 ms resolution (Bullen and Saggau, 1999) (Fig. 3). Such a device should be capable, as should our high-speed CCD, of detecting subthreshold spontaneous activity simultaneously in over a hundred neurons. However, the necessary light dose is quite damaging. In an effort to reduce photodamage, Obaid et al. (1999) bathed enteric neurons in a cocktail of the carotenoid pigment astaxanthin, and the enzymes glucose oxidase and catalase. Presumably by reducing oxygen concentrations and free-radical-mediated reactions, this mixture allowed continuous recording for up to 5 min. This is a tremendous improvement over the commonly accepted few seconds of potentiometric dye recording, but still a long way from being able to record for hours or days, as with MEA electrodes. Blau, Friedrich, and I are presently exploring new dyes, filter combinations, and voltage-sensitive fluorescent proteins (VSFPs) (Siegel and Isacoff, 1997; Blau, 1999; Friedrich et al., 1999), to enhance signal-to-noise ratios and reduce phototoxicity and photobleaching. Until significant progress is made in reducing the photodamage problems, the optical recording approach is limited to short-term, terminal experiments.

Flat monolayer cultures also lend themselves to detailed morphological analysis by imaging at much slower time scales. The advent of 2-photon laser-scanning microscopy (Denk et al., 1990) has made it possible to carry out time-lapse imaging of fluorescently labeled neurons continuously for many

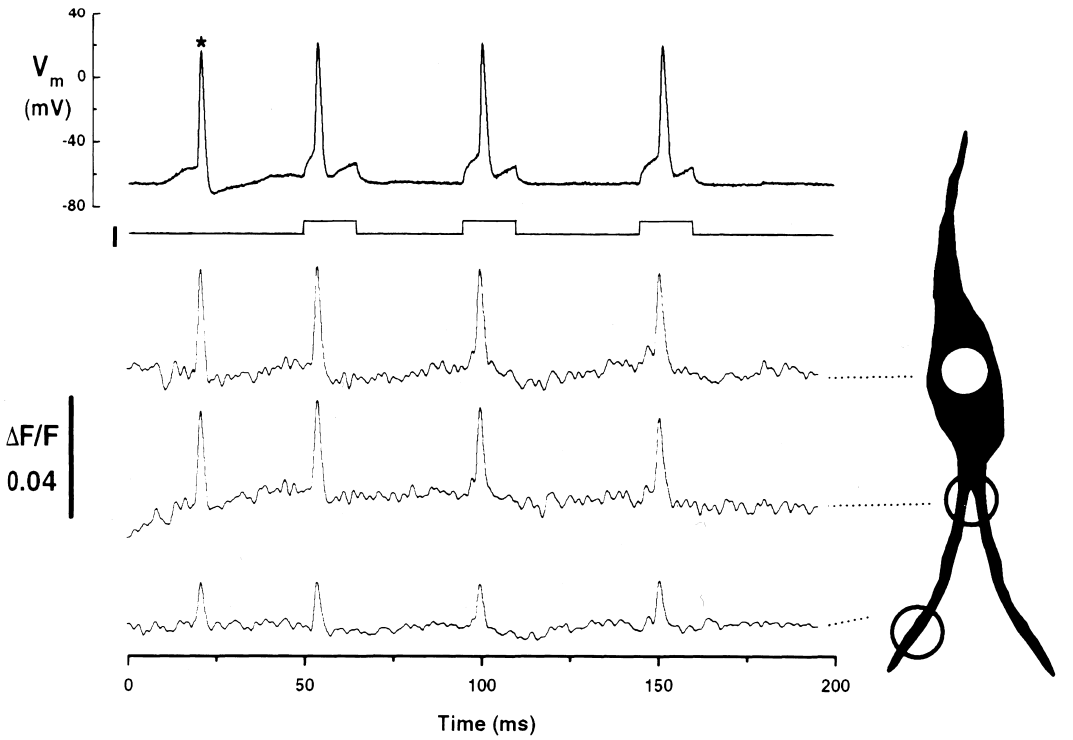

Fig. 3. Optical recordings made from different parts of a single cultured rat hippocampal neuron, using the laser-scanning system of Bullen et al., 1997 (reprinted with permission). The top trace is a standard whole-cell electrode recording, showing both spontaneous (marked with an asterisk) and elicited action potentials. The second trace shows current injected (100 pA). The bottom three traces show single-trial (no averaging) fluorescence signals from the circled regions of a neuron stained with the voltage-sensitive fluorescent dye, di-8-ANEPPS.

hours without concern about photodamage (Potter, 1996). Time-lapse imaging allows us to observe how changes in cellular and network morphology relate to changes in the electrical properties of the network. High-resolution (submicron) time-lapse imaging can also be carried out non-destructively using sensitive cooled scientific CCD cameras (Ramakers et al., 1998). However, mature MEA cultures can be quite complex, with many overlapping neurites. By labeling a subpopulation of the network with lipophilic dyes, one can follow changes in individual cells in crowded cultures (Potter et al., 1996; Potter, 2000). My colleagues at Caltech are developing more long-lasting labeling using viruses to infect cells with the gene for different colored fluorescent proteins (Okada et al., 1999; Nadeau et al., 2000). These should allow new lines of inquiry relating cellular morphology to electrical activity, which are difficult or impossible to carry out using living animals.

Embodied, situated neuronal cultures

Even if optical and MEA technologies are capable of observing and influencing distributed patterns of activity in cultured networks, they will not allow us to say much about learning, memory, and information processing because these networks are removed from a body, and therefore isolated from the rest of the world. There is a movement gaining momentum that neural systems should not be studied in isolation (Clark, 1997). They evolved to serve a body, and that body interacts with an environment. They are described as *embodied* and *situated*. This notion has been promoted, at several conferences on the Simulation of Adaptive Behavior, as the 'animats approach' (Meyer and Wilson, 1991; Meyer and Guillot, 1994). An animat is a simulated animal. Animats, either software simulations or actual robots, have been used to develop more 'natural' artificial

intelligence, that is good at solving the types of problems real animals have to solve, such as locomotion, obstacle avoidance, finding food, or group behaviors such as flocking (Meyer and Guillot, 1994). The animats approach may solve the problem that unlike retinas, neural cultures lack obvious inputs and outputs. It may suggest candidate population *codings* or non-trivial transformations that could be carried out by a network of neurons growing in a dish suitably interfaced with a computer using MEAs.

In order to embody a cultured network, we are creating the first *neurally controlled* animat (Potter et al., 1997a, DeMarse et al., 2000), a culture of dissociated cortical neurons on an MEA whose electrical activity controls the behavior of a simulated animal on a computer. An embodied culture capable of behaving may then exhibit changes in behavior as a result of experience, that is, learning. The animat is situated within a computer-simulated environment, a sort of 'virtual reality'. Sensory input to the animat is fed back to the culture as patterns of electrical stimulation, in real time, allowing a sensory–motor feedback loop (Fig. 4). The behaviors and the environment give meaning to the patterns of activity within the culture. This meaning has been the key missing element in the study of population coding in cultured networks. Without it, we are merely studying the collective dynamics of a network of coupled excitable elements. But as soon as these dynamics are imbued with meaning by connecting the culture to a body and situating it within an environment, we can legitimately discuss the processing of in-

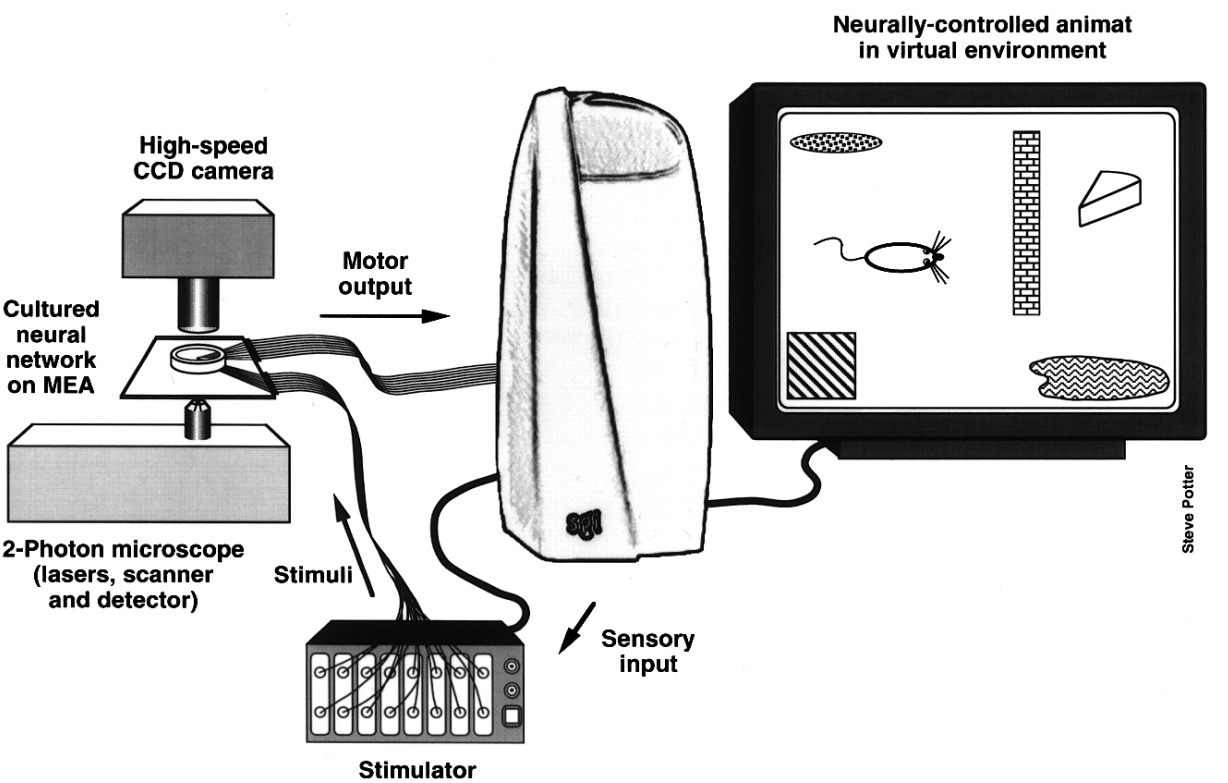

Fig. 4. Plan for an embodied cultured neuronal network. MEA technology allows us to create a long-term two-way communication between a small network of cultured cells and a computer. The computer uses patterns detected in the spontaneous neural activity to control the behavior of a simulated animal, the 'neurally controlled animat'. This animat is situated within a simulated environment, and its sensory inputs are fed back to the culture as spatio-temporal patterns of electrical stimulation. This allows one to do 'in vitro neuroethology'. Because MEA cultures are so accessible, we can follow changes in great detail at the millisecond time scale (with high-speed optical recording) and at the minutes or hours time scale (with two-photon microscopy), to make connections between the animat's behavior and the morphology and activity patterns of the neurons and supporting cells.

formation by cultured cells. Because the mapping from neural activity to the animat's behaviors is arbitrary, as is the mapping of sensory input to patterns of stimulation, we are much less constrained than those studying population coding in vivo. Distributed coding may exist at many different spatial and temporal scales. Rybka and I have begun to characterize the types of patterns that may be used to control the animat's behaviors (Rybka, 1999). DeMarse and I are exploring which parameters of patterns of extracellular electrical stimuli through the MEA substrate generate robust network responses (T. DeMarse and S. Potter, unpublished). These will be used as sensory inputs to the neurally controlled animat. Eventually, this system may help to bridge the gap between top-down (behavioral, cognitive) and bottom-up (molecular and cellular) neuroscience approaches.

Future innovations

Now that MEA hardware is readily available, multi-unit researchers are presently hampered most by the paucity of powerful software tools that allow spike detection, spike sorting, and recognition of dynamic spatio-temporal patterns of neural activity in real time. A number of other fields, such as satellite imaging or economics, are also generating very large data sets in need of automated analysis and this has resulted in a boom in the 'data mining' meta-field (Fayyad et al., 1996) that MEA researchers will certainly benefit from. Number-crunching of multi-neuron signals, recorded either optically or electrically, would seem to be a perfect application for parallel processing systems. The signal from each electrode, pixel, or neuron could be analyzed by a single microprocessor of a many-processor computer. Already a large bank of digital signal processors (Wheeler and Valesano, 1985), such as the system developed by Plexon Inc., is used by a number of labs to do real-time spike-sorting and analysis of MEA data.

Even for a thousand-neuron culture, to record with 60 electrodes is a vast undersampling of the net's activity. Assuming the hardware and software can keep up (a difficult task!), it would be useful to have MEAs with many more electrodes. Getting all those signals out to external electronics presents a significant wiring problem that might be solved using multi-layer fabrication, or on-chip multiplexing and analog-to-digital conversion. Progress is being made in this direction for both in vivo probes (Najafi and Wise, 1986; Ji and Wise, 1992) and in vitro MEAs (Pancrazio et al., 1998). 2-Photon uncaging of neurotransmitter receptor agonists (Furuta et al., 1999) allows stimulation at more sites than presently possible using electrodes, and it is likely that it will be used on cultured networks in conjunction with MEA- or optical recording.

Until MEAs with many electrodes are realized, the 60 or so electrodes presently available should be used optimally. It would be helpful if companies that supply MEAs could rapidly fabricate custom electrode geometries to suit the specific needs of each researcher. Such a personalized fabrication service for in vivo silicon probes at the University of Michigan has been quite successful. [5]

Conclusion

The nascent field of population coding in networks of cultured neurons is poised for rapid expansion, thanks to advances in a number of key technologies. Neural cell culture, long-term multi-electrode recording and stimulation, and multi-single-unit optical recording are now accessible to many labs. Recent studies show that these networks exhibit a variety of recurring activity patterns that can be modified by electrical stimulation. Computers are fast and cheap enough to allow real-time spike analysis and stimulus generation, which will make it possible to give cultured networks a simulated body to behave with, and an environment to interact with. By allowing the culture to behave and receive sensory input (even if artificial), meaning can be ascribed to the patterns of electrical activity it produces, and persistent changes in network activity can be thought of as learning. Simultaneous high-resolution time-lapse imaging using 2-photon or video microscopy will enable the study of the morphological correlates of this learning. Artificial neural networks, with only a few tens or hundreds of computer-modeled neurons so simple they are usually called 'units', have accomplished many interesting and useful learning,

[5] http://www.engin.umich.edu/facility/cnct/

pattern recognition and processing tasks (e.g., Dowla and Rogers, 1996). Thus, I suspect that a network of a few thousand real, living neurons, with all their intracellular complexity and prolific interconnectivity, is capable of quite a bit of distributed information processing.

Abbreviations

animat	simulated animal
CCD	charge-coupled device
MEA	multi-electrode array
SCN	suprachiasmatic nucleus
VSFP	voltage-sensitive fluorescent protein

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