A new approach to neural cell culture for long-term studies

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Abstract

We have developed a new method for culturing cells that maintains their health and sterility for many months. Using conventional techniques, primary neuron cultures seldom survive more than 2 months. Increases in the osmotic strength of media due to evaporation are a large and underappreciated contributor to the gradual decline in the health of these cultures. Because of this and the ever-present likelihood of contamination by airborne pathogens, repeated or extended experiments on any given culture have until now been difficult, if not impossible. We surmounted survival problems by using culture dish lids that form a gas-tight seal, and incorporate a transparent hydrophobic membrane (fluorinated ethylene–propylene) that is selectively permeable to oxygen (O_2) and carbon dioxide (CO_2), and relatively impermeable to water vapor. This prevents contamination and greatly reduces evaporation, allowing the use of a non-humidified incubator. We have employed this technique to grow dissociated cortical cultures from rat embryos on multi-electrode arrays. After more than a year in culture, the neurons still exhibit robust spontaneous electrical activity. The combination of sealed culture dishes with extracellular multi-electrode recording and stimulation enables study of development, adaptation, and very long-term plasticity, across months, in cultured neuronal networks. Membrane-sealed dishes will also be useful for the culture of many other cell types susceptible to evaporation and contamination. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Dissociated primary neuron cultures have been a popular research tool for decades (Dichter, 1978; Mains and Patterson, 1973; Murray, 1965) because they allow easy access to individual neurons for electrophysiological recording and stimulation, pharmacological manipulations, and high-resolution microscopic analysis. It would be informative to follow individual cultured neurons for months, electrophysiologically and morphologically, to help understand how neural activity and morphology interact. In vivo or in vitro, it is technically difficult to record from and stimulate more than three cells using standard intracellular microelec-

trodes, and those cells usually die within minutes or hours. Given that neural systems use distributed codes to process and store information (Nicolelis et al., 1993; Wu et al., 1994), much of their dynamics is missed without a multi-unit approach. Multi-electrode arrays (MEAs) provide a means to record from and stimulate many individual cultured neurons non-destructively (Gross et al., 1982; Pine, 1980; Regehr et al., 1989). Because the array substrate consists of transparent glass, neuron morphology can be easily monitored using an inverted phase-contrast microscope, or using vital labels and a fluorescence microscope such as a confocal or 2-photon laser-scanning microscope (Potter et al., 1996, 2001).

A prerequisite to our program of studying long-term plasticity in cultured networks was to devise a culturing method that keeps neurons alive for many months. Why do neuron cultures die? One obvious cause of neuron death is infection, usually by mold. A less obvious, but equally prevalent one, is hyperosmolality

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due to medium evaporation (Maher and McKinney, 1995). Our method addresses both of these problems, and has allowed us to repeatedly study primary neuron cultures for > 9 months, in one case for well over 1 year (Fig. 1). It involves growing cells on multielectrode arrays that are sealed by a selectively permeable membrane, in a non-humidified incubator.

2. Membrane-sealed multi-electrode culture chambers

Gas-tight culture chambers have previously been used in order to provide a more controlled environment for cultured cells (Kleitman et al., 1998; Morrison et al., 2000). It is necessary to fill such chambers with the proper mix of gases (usually air with 5% CO_2) before sealing. Our approach is to let the incubator control the desired gas mixture, as it does in most labs, and use chambers that are permeable to the gases necessary for cell metabolism, but impermeable to microbes and water vapor. We developed culture chamber lids (Fig. 2, patent pending) that incorporate a thin membrane of fluorinated ethylene–propylene (FEP Teflon® film). This membrane is unusual in its permeability to O_2 (see Materials and Methods), and is often incorporated in dissolved-oxygen probes. Unlike the expanded polytetrafluoroethylene (PTFE) membrane used in 'breathable' (Gore-Tex®) rain gear, this hydrophobic FEP membrane has no pores and is impermeable to water vapor and microbes. The FEP membrane is also completely transparent and optically flat, which allows microscopic imaging. Because of its transparency, O_2 permeability and water impermeability, it has been used for embryo time-lapse imaging chambers (Kiehart et al., 1994; Kulesa and Fraser, 2000).
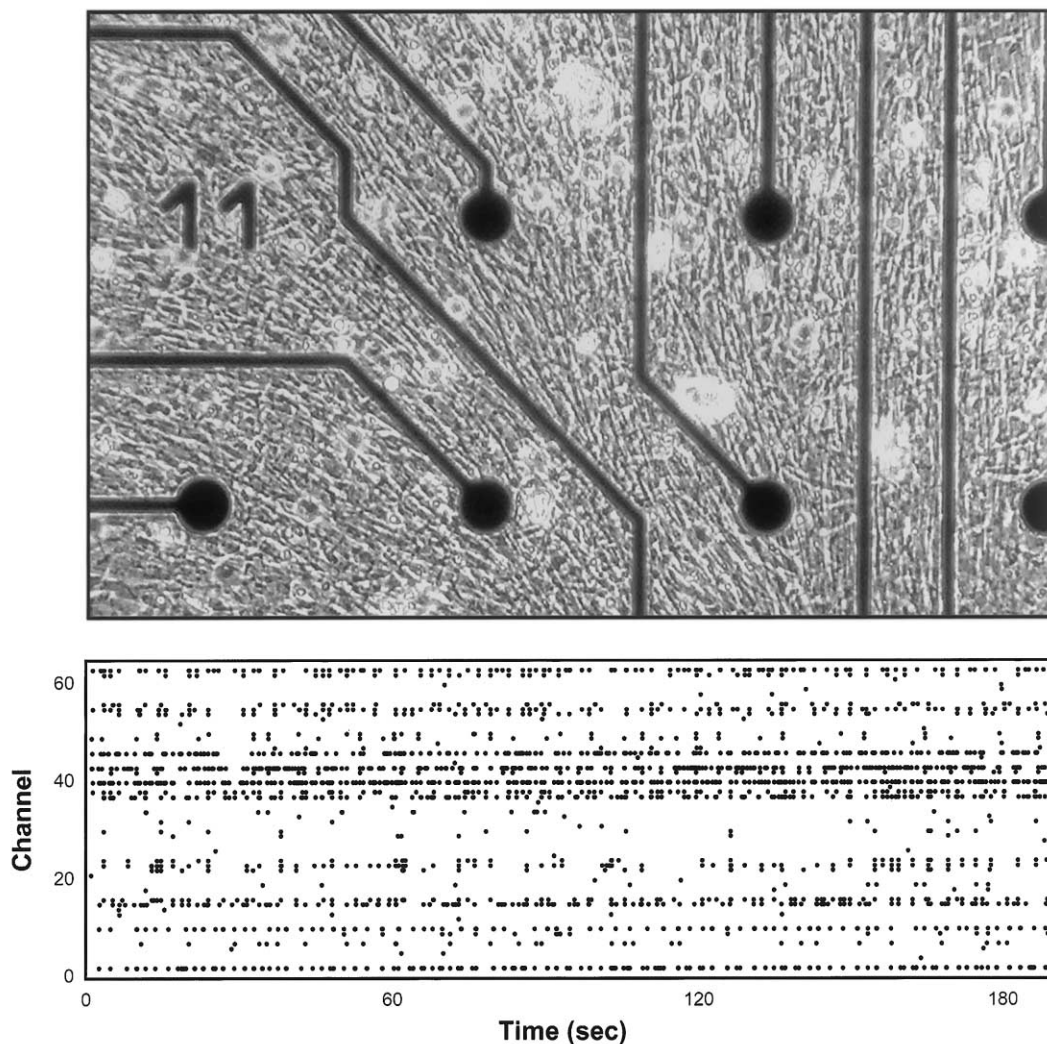

Fig. 1. Top: Phase-contrast image of our oldest living dissociated rat cortical culture, taken at 15 months in vitro. Neuron somata are mostly obscured by abundant glia and fascicles. Scale: 200 μm between electrodes. Bottom: Spontaneous activity of this network, recorded after 1 year in culture using MultiChannel Systems MEA60 (see Section 4). Each dot is an action potential recorded by one of the multi-electrode array channels.
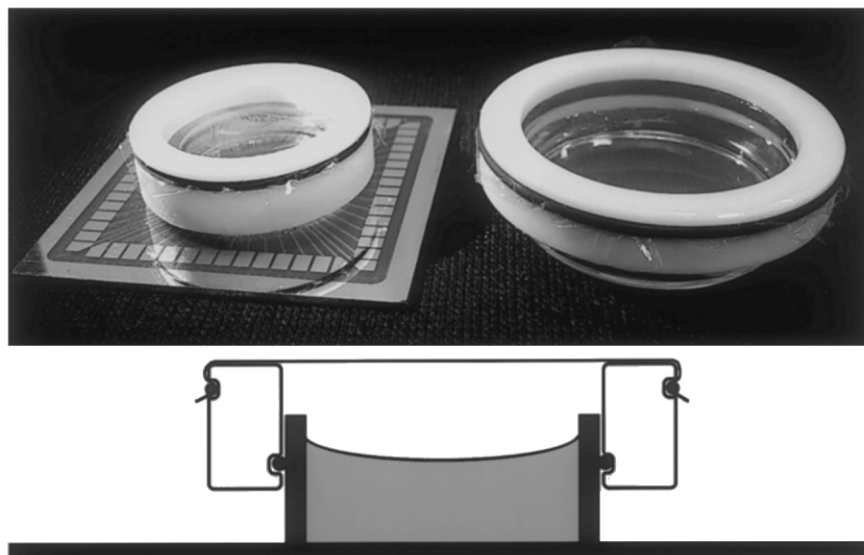
Fig. 2. Multielectrode array and plastic culture dish with FEP-membrane-sealed lids. Lids are machined PTFE rings, with grooves for O-rings that are used to hold on the FEP membrane and to form an airtight seal with the MEA (cross-section diagram) or disposable culture dish. The materials (PTFE, O-rings, and FEP membrane) are autoclavable and re-usable.

2.1. Preventing infection of cultures

It is common practice to discard neural cultures after recording from them once, because putting the dish on the microscope and introducing micropipets, objective lenses and electrodes in a nonsterile lab environment is likely to cause infection of the culture by ubiquitous mold spores, bacteria, or other microbes. By using extracellular electrodes embedded in the culture dish substrate, instead of individually manipulated microelectrodes, it is possible to avoid this source of contamination; sealed MEAs can remain covered and sterile during the experiment.

For standard cell culture, the threat of infection does not end once the cells are returned to the incubator. All commonly used cell culture chambers, whether individual dishes or multi-well plates, have a design that incorporates an 'air gap' between the lid and base, through which gases can pass freely. This gap allows O_2 and CO_2 in the incubator to equilibrate with the cell culture medium, and maintain cell metabolism and the proper pH. Unfortunately, the warm, humid environment found in incubators is ideal for the proliferation of microbes. A problem experienced by every cell culture lab at times is the contamination of cultures in the incubator by airborne pathogens passing through the chambers' air gap. One neurobiology lab with four incubators that we monitored for several weeks suffered the loss of $\sim 4\%$ of its cultures per day due to infection. Once one culture dish becomes contaminated, it is likely that neighboring dishes and eventually the incubator itself will become contaminated. The standard solution to this problem is to clean and sterilize the incubator, which is time-consuming and difficult or impossible to do completely. Moreover, there is no guarantee that a recently cleaned incubator will remain sterile for long, especially if latently infected cultures are returned to it after cleaning.

Using FEP-sealed culture dishes helps to eliminate the infection problem in several ways. The membrane presents a barrier to pathogens that would pass through the air gap of standard dishes. It also prevents an infected culture from infecting nearby cultures by sealing microbes in. But most importantly, the low permeability of FEP to water vapor allows the cultures to be grown in a non-humidified incubator, one that does not tend to encourage the proliferation of microbes. We maintain ours at an ambient relative humidity (RH) of $\sim 65\%$ (henceforth referred to as 'dry'). A dry incubator full of sealed dishes never has to be cleaned and disinfected. Another benefit of a dry incubator is that, without condensation on the glass door, it is easier to see the dishes inside.

2.2. Preventing hyperosmolality

The air gap present in standard culture chambers causes not only an infection problem, but also a problem with evaporation of water from the medium, resulting in hyperosmotic conditions that are responsible for the slow death of many neural cultures after a few weeks (Maher and McKinney, 1995). It is generally believed that this problem is solved by using a humidified incubator. However, we have noticed that the mean humidity level is often substantially $< 100\%$ RH, especially in a busy lab in which the incubator is

opened several times per day. Even for an open-time of < 1 min, almost the entire volume of warm, humid air rises and escapes, and is replaced by drier room air. It takes a surprisingly long time for the air to re-humidify after the door is opened. For example, after a 30-s opening of a standard incubator (volume, 153 l, with a 33 × 30 cm warm water pan and circulating fan), it took 26 min to go from 32 to 85% RH, and 77 min to reach the original value of 95% RH.

It is likely that the tradition of only replacing half the medium during feeding arose, in part, by the benefit of reduced osmotic shock, compared to complete medium replacement. One worker in our group was able to maintain healthy hippocampal cultures in standard dishes for > 1 month, without any feeding, by carefully weighing the dishes and replacing evaporated water every few days with an equal amount of sterile water (McKinney, unpublished).

In culture dishes sealed with FEP membranes, evaporation and concomitant hyperosmolality problems are dramatically reduced. We compared increases in osmolality of medium due to evaporation in sealed dishes in a dry incubator to those in standard polystyrene 35 mm culture dishes (Corning; with air gap) in a humidified incubator, and in a laminar-flow hood (Fig. 3). The tonicity of the medium in standard dishes kept in a humidified incubator increased 10.5 mOsm/day, while that in sealed dishes in the dry incubator increased by less than half that rate, 4.0 mOsm/day. In our experience, a rise of > 50 mOsm is lethal to neural cultures (Maher and McKinney, 1995).

By using FEP-membrane-sealed dishes, and weekly feedings with a complete medium change, osmolality issues are no longer a concern, even in a non-humidified incubator. Without fear of humidity fluctuations or infection from the influx of room air from door openings, one is free to transfer cultures into and out of the incubator at a more leisurely and careful pace.

2.3. Maintenance of pH

Like osmolality, the acidity of culture medium is a crucial parameter for neuron survival (Banker and Goslin, 1998; Maher and McKinney, 1995). Mammalian cell cultures, especially neural cell cultures, are healthiest when maintained at physiological pH, around 7.3. To accomplish this, most culture methods employ a buffering system similar to that found in blood, in which there is an equilibrium between dissolved CO_2 (bicarbonate anion) and a well-regulated (usually 5% (v/v)) CO_2 atmosphere in the incubator. When brought into room atmosphere (< 0.05% CO_2), bicarbonate leaves the medium as gaseous CO_2 and the pH drifts up to a lethal alkaline value of over 8.5. Some media designed for the low ambient CO_2 levels (such as Hibernate, Brewer and Price, 1996) use additional organic buffers, such as MOPS or HEPES, to maintain proper pH. However, HEPES (and perhaps other synthetic organic buffers) is highly phototoxic, even under standard fluorescent ceiling lights (Lepe-Zuniga et al., 1987; Spierenburg et al., 1984). Phototoxicity of medium components or fluorescent labels (Potter et al.,
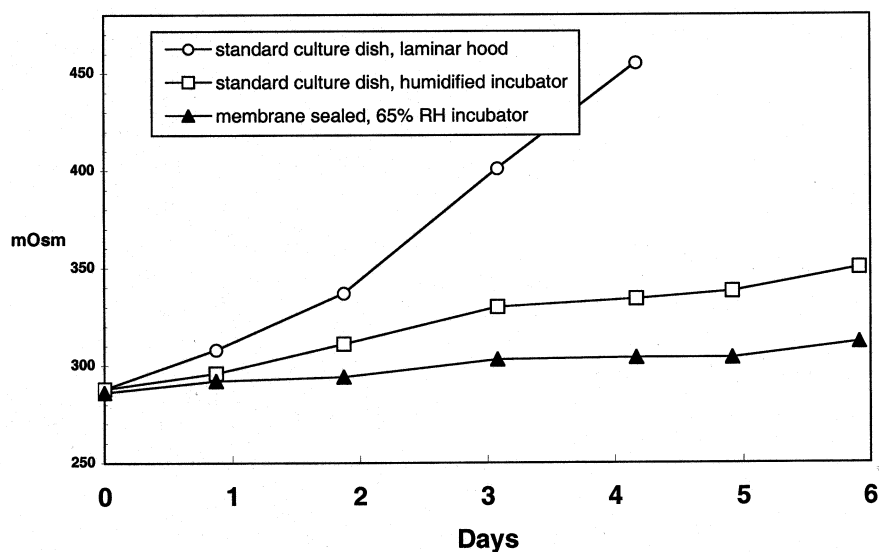

Fig. 3. Change in osmolality of culture medium due to evaporation of water. Each dish (containing culture medium, but no cells) was measured only once and discarded, and all data points are averages of triplicate measurements. (Standard deviation error bars would be covered by the plot symbols.) Least-squares linear regression gives daily osmolality increases of 10.5 mOsm for normal dishes in the humidified incubator (open squares), 4.0 mOsm for the FEP-sealed dishes in the dry incubator (filled triangles), and 58.6 mOsm for normal dishes in the laminar flow hood (open circles). Variability in slope (standard deviation of daily change) of 3.8 mOsm for the sealed dishes and 6.1 mOsm for the standard dishes is due to daily variability in ambient humidity and incubator usage.
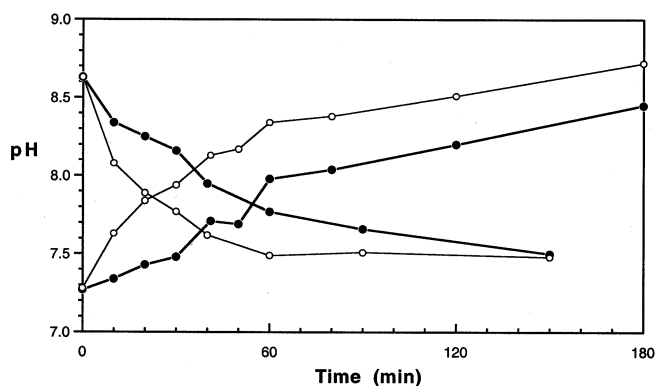
Fig. 4. Equilibration of pH in sets of sealed dishes (filled symbols) and standard culture dishes (open symbols). Falling curves: Dishes were filled with medium equilibrated with room air (pH 8.6), and placed in the same humidified 5% CO_2 incubator. Diffusion of carbon dioxide through the FEP membrane is sufficient to allow equilibration of carbonate-buffered media from alkaline to physiological pH in ~2 h. Rising curves: a separate set of incubator-equilibrated dishes were brought out to the lab bench and equilibration with room air over time was measured. The somewhat slower time-course of pH increase for sealed dishes is beneficial for electrophysiology sessions in room air, since it allows recording without a medium change, for at least 30 min, before the upward drift of pH affects cell physiology. For both sets of curves, each dish was measured once, immediately after removing the lid, and discarded.

1996) can be a large factor in the survival of neuron cultures.

It is not widely known that FEP membranes are permeable to CO_2, a fact that makes them ideal for sealing cultures with carbonate-buffered media. In order to quantify pH equilibration rates in sealed dishes, CO_2-buffered medium was left out in ambient air overnight, until its pH leveled off at 8.6. It was then placed in standard culture dishes or sealed dishes in a standard, humidified 5% CO_2 incubator (Fig. 4, falling curves). The equilibration rate is slower in sealed dishes, than with unsealed disposable culture dishes, but is acceptably rapid.

We also compared the time-course of increasing pH in standard versus FEP-sealed dishes brought out into ambient air after complete equilibration in the incubator (Fig. 4, rising curves). The slower pH equilibration of FEP-sealed dishes is actually an advantage for multielectrode array electrophysiology, because we can record from sealed dishes at ambient CO_2 levels for at least 30 min without experiencing detrimental pH drift in CO_2-buffered media. Thus, no medium change is necessary for most recording sessions, reducing the chance of infection, and eliminating the problem of electrophysiological transients that ensue after a medium change (Gross et al., 1993).

3. Discussion

We have developed a method that enables the survival of primary neuron cultures for over a year in vitro. By sealing culture chambers with a membrane that is permeable to CO_2 and O_2, and relatively impermeable to water vapor, and by keeping these chambers in a non-humidified incubator, we have greatly reduced or eliminated problems with infection and hyperosmolality, while maintaining pH and O_2 homeostasis. By combining this technique with multielectrode array dishes, it is now possible to follow optically and electrophysiologically, the development and plasticity of many individual cells in cultured neuronal networks for many months.

The advantages of this system might also make it attractive for use with other cell types. For example, sealed dishes greatly reduce health risks to lab personnel working with dangerous cell lines, cells transfected with viruses, or cultures loaded with radioisotopes or other dangerous chemicals that might be spilled. Furthermore, a dry incubator is much more hospitable than a humid one to electronic sensors or cell stimulation and recording equipment that researchers might wish to use with their cultures (e.g. in Welsh et al., 1995). The humid environment found in standard incubators causes electrical shorts, changes in component properties, and destruction of materials commonly used in electronic devices. We have conducted continuous recording for days at a time from our MEAs by placing the MEA60 preamplifier inside the incubator. We are using a custom-made in-incubator stimulation system to determine whether continual stimulation can be used to control the development of cultured dissociated networks (Wagenaar, unpublished). Although our demanding and unusual research requirements (DeMarse et al., 2000, 2001; Potter, 2001) led us to the idea of growing cultures in sealed chambers in a dry incubator, this simple, inexpensive technique will benefit many cell culture labs.

4. Materials and methods

4.1. Sealed dish fabrication

Rings were machined from solid polytetrafluoroethylene (PTFE) Teflon® round stock to fit the MEAs tightly when a rubber O-ring (EP75, Real Seal, Escondido, CA) is fitted in the inside groove (see cross-section diagram, Fig. 2). A grove on the outside of the PTFE ring accommodates a second O-ring that holds on the membrane of fluorinated ethylene–propylene (Teflon® FEP film, 12.7 μm thickness, specified permeabilities to CO_2, O_2 and water vapor of 212, 95, and 78 micromol/cm²/day, respectively, by ASTM D-1434 and

E-96 tests), manufactured by Dupont, Circleville, OH; supplied by American Durafilm, Holliston, MA. (Note that because water is in tremendous excess in culture dishes, its relative permeability as a fractional change per unit time is far less, compared to CO_2 and O_2.) Similar rings were fabricated to accommodate disposable 35-mm polystyrene culture dish bottoms, but were not used for these studies.

Custom-made glass 'control' dishes with the same dimensions as the MEA dishes consisted of a glass ring (internal diameter 20.3 mm, height 6.6 mm) glued to a glass microscope slide with silastic adhesive (MDX4-4210, Dow Corning). All components of the lids and control dishes can be repeatedly sterilized by autoclaving.

4.2. MEA preparation

We use 60-electrode glass MEAs from MultiChannel Systems (Ruetlingen, Germany, http://www.multichannelsystems.com) with 10-μm diameter electrodes, 200-μm interelectrode spacing (Egert et al., 1998). For electrophysiology using sealed MEAs, a reference electrode consisting of a fine platinum wire was threaded through a tiny hole in the PTFE cylinder, and glued in place with silastic adhesive. Newer MEAs made by MultiChannel Systems incorporate a large reference electrode in the substrate, obviating the need for a separate platinum wire. They are re-usable when cleaned as advised using the supplied '3% BM' cleaning solution. After cleaning they were soaked in ultrapure water overnight, sterilized in pure ethanol, and allowed to dry. On the day of use, a 100-μl drop of 0.05% (w/v) polyethylene imine solution in borate buffer (Lelong et al., 1992) (Clonetics CC-4195 in CC-4196) was applied to the center of the dish for 1 h, rinsed 4 × with sterile water, and allowed to dry. Twenty μl of 1 mg/ml laminin (Sigma # L2020, stored at − 70 °C) was diluted in 1 ml of medium (recipe below), and a 10-μl droplet was applied to the center of the MEA, covering the electrode region. Dishes were covered and allowed to sit at room temperature for at least 30 min to allow the laminin to attach to the substrate. Most of droplet was aspirated just before adding the cell suspension (described below) to the dish.

4.3. Culture preparation and feeding

Dissociated cortical cell cultures were prepared by papain digestion of embryonic-day-18 rat whole cortex. Timed-pregnant Wistar rats were euthanized by CO_2 inhalation, according to NIH-approved protocols for the care and use of lab animals. Embryos were removed, chilled on ice, and the cortex was microdissected under sterile conditions. Papain solution was prepared according to Segal et al. (1998), quick-

frozen by immersion in liquid nitrogen in 2-ml aliquots, stored at − 15 °C, and thawed at 35 °C just before use. One-mm cortex pieces were digested in 2 ml papain solution for 30 min at 35 °C with gentle inversion every 5 min. The papain solution was aspirated and the pieces were triturated three times, three passes each with 1 ml of medium, using a P-1000 Pipetman. 20 000 to 50 000 cells were plated in a 20-μl droplet covering the 1.5-mm electrode region of the MEAs, forming a dense monolayer. The dishes were flooded with 1 ml of medium after the cells had adhered to the substrate (> 15 min), and stored with FEP membrane lids in a 65% RH incubator at 35 °C, 5% CO_2, 9% O_2 (by nitrogen purging Brewer and Cotman, 1989). The medium, adapted from Jimbo et al. (1999), was Dulbecco's modified Eagle's medium (DMEM, Irvine Scientific # 9024) with 10% equine serum (Hyclone). Antibiotics and antimicotics are reported to adversely affect neuron health and electrophysiology (Heal et al., 2000), and were therefore not used. The medium was used without glial conditioning, but was stored in an FEP membrane-sealed flask in the incubator to equilibrate the pH and temperature before feeding. No attempt was made to control glial cell proliferation with antimitotic drugs, because glial cells aid in neuronal survival and synaptic development (Pfrieger and Barres, 1997; Ullian et al., 2001). Feedings consisted of complete medium replacement once per week. Lids were removed in a laminar-flow hood and the medium was replaced using a P-1000 Pipetman. An in-incubator, continuous feeding system would be preferable, however our preliminary attempts caused problems with sterility (due to the large number of difficult-to-sterilize components) and leaks.

4.4. Evaporation study

The purpose of this study was to determine whether the FEP membrane would allow the use of a dry incubator, due to its low water vapor permeability. The new culture method (FEP-sealed dishes in a dry incubator) was compared to the method used in most cell culture labs (standard plastic dishes in a humidified incubator). Fresh standard medium (above) was placed in either 35-mm Corning polystyrene culture dishes with standard 'air gap' plastic lids (2 ml) or glass control dishes with our FEP sealed lids (1 ml). The Corning dishes (lids on) were placed in either a standard, humidified incubator at 35 °C (Fisher Model 1168710), or left out in the laminar flow hood. Mean conditions in laminar flow hood during measurement period, 26 °C, 58% RH. The sealed glass control dishes were placed in the 'dry' incubator (65% RH) at 35 °C (Napco model 7101 FC-0R). Both incubators were being used by others in the lab as normal, opened ∼ 12 times per day. Dishes were removed from each location

daily, osmolality was checked using a Wescor Vapro 5520 vapor pressure osmometer, and they were discarded (plastic) or cleaned for later use (glass) after measuring.

4.5. pH Study

The purpose of this study was twofold: (1) to determine whether the 12.7 μm thick FEP membrane has sufficient CO_2 permeability to allow proper buffering of our medium with an incubator atmosphere of 5% CO_2; and (2) to determine how quickly the pH drifts to unhealthy levels when sealed dishes are removed from the incubator. For (1), an open flask of medium was left out in the laminar flow hood overnight until its pH leveled off at ~ 8.6. This was aliquotted into either 35-mm Corning culture dishes with standard 'air gap' plastic lids (2 ml) or glass control dishes with our FEP sealed lids (1 ml). Both sets of dishes were placed on the same shelf of a standard humidified incubator with a 5% CO_2 atmosphere. At intervals up to 150 min, one dish of each type was removed, opened, and the pH was rapidly measured, Corning dish first, using a small-probe PH-Check® pH meter. For (2), a similar set of dishes was left in the same incubator overnight, to allow the pH to equilibrate to 7.3. The whole set was removed to the lab bench at the same time (25.5 °C, 58% RH), and one of each type was opened to check the pH at intervals up to 180 min. As with (1), all dishes were measured only once and then discarded (plastic) or cleaned for later use (glass).

Acknowledgements

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