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I hereby declare that all the work contained herein is my own, unless otherwise stated.

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Executive Summary

This thesis reports on work undertaken as part of an ongoing project at Edinburgh University to develop an array of planar patch-clamps on a single substrate (a silicon chip), for the purpose of acquiring high-quality multi-channel recordings from networks of cultured neurons. The advancement of the project depends on successfully achieving high-resistance seals with neurons by using a chip with a single patch aperture, a goal which has already been repeatably achieved by some commercial companies, albeit in different context of pharmacological screening.

This work involved testing the ability of the chips to form seals compared to the standard patch-clamping technique and evaluating alternative paradigms for the final use of the chips: cells cultured directly over the patch aperture versus cells cultured on a separate substrate which is lowered onto chips with protruding patch nozzles.

Four experimental methods were used: culturing AtT-20 cells directly on chips, lowering AtT-20 cells cultured on glass coverslips onto patch nozzles, lowering AtT-20 cells cultured on acetate strips and positioning dissociated snail neurons onto patch nozzles. These methods have been refined. All methods have practical difficulties, however no method can yet be eliminated. There are indications that the latter paradigm of cells cultured on a separate substrate and lowered may prove superior.

The chips under test do not perform as well as standard glass electrodes in terms of maximum seal resistances achieved. Seals of > GΩ have not been achieved and no whole-cell recordings have been attained. There are good indications of the most promising type of chip – boron-doped thermally oxidised chips with apertures of diameter 1.5um or under. More of these chips particularly with smaller aperture diameters should be manufactured and tested.

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1 Introduction

Patch-clamping and the related technique of whole-cell recording were introduced in the late 70s and early 80s and since then have been widely used in the investigation of the electrophysiological properties of nerve cells (Sakmann *et al*. 1995). In this technique, a glass micropipette is manoeuvred to the surface of a cell and then suction is applied to form an electrical seal and yield direct electrical contact to the interior of the cell. In a separate line of technological development, multi-electrode arrays are used to make extracellular recordings from acute brain slices and neuronal cultures in order to investigate the network behaviour of neuronal cultures (Taketani *et al*. 2005).

Since the mid-90s there has been speculation about using MEMS technology to create closely packed arrays of planar patch-clamps for detailed multichannel electrophysiological recordings from cultured neural networks and a number of research groups have worked towards this goal. Such a system would effectively bring together the advantages of the two approaches mentioned above, whole-cell recording and multi-electrode array recording. A project was initiated at Edinburgh University in 2000 to achieve this goal, and is ongoing. This thesis reports on work carried out as part of that project.

The overall project has generated original protocols for the design of silicon chips with patch apertures (hereafter "devices") and investigated surface properties of various silicon-based materials. There has also been original work on the patterning of surfaces to achieve guided cell growth. However the devices so far produced have not shown the ability to form seals of the quality required for detailed intracellular recording. This is the necessary next step for the project to advance, and it is a goal which has already been repeatably achieved by some commercial companies, albeit in the different context of pharmacological screening.

The work detailed herein was carried out by the author in conjunction with Dr John Curtis and doctoral candidates Keith Baldwin and Evangelos Delivopoulos, in the period April to August 2005.

During this period four experimental methods were under test: culturing AtT-20 cells (a mouse pituitary tumour line) on devices, lowering AtT-20 cells cultured on glass coverslips onto devices, lowering AtT-20 cells cultured on acetate strips onto devices,

and positioning dissociated snail neurons over patch apertures.

Section 2 reviews the history and practice of patch-clamping, the development of planar patch-clamping systems, and the use of multi-electrode arrays for multichannel recordings from neural networks.

Section 3 presents the objectives of the work described in this report against the background of the overall project at Edinburgh University.

Section 4 describes attempts to achieve whole-cell recordings from AtT-20 cells with standard glass electrodes.

Section 5 details work aimed at culturing cells directly on devices so they would grow over the patch aperture.

Section 6 presents the complementary approaches of lowering cultured networks of cells onto the devices, with new and untried “nozzle” devices.

Section 7 details experiments where dissociated snail neurons were positioned over patch nozzles.

2 Literature review

2.1 Patch-clamping technique

Much research in neuroscience focuses on the electrophysiological properties of nerve cells and the way in which these properties emerge from their component parts, at various levels e.g. synapses, cell membrane proteins. Electrical recording from nerve cells or networks of nerve cells has been practised for many years.

The value of electrical recordings is limited both by the spatial resolution with which signals can be recorded and by the fidelity of the signal which can be achieved. The patch-clamp technique, which was introduced by Neher *et al.* 1976, significantly pushed back the boundaries, making it possible for the first time to record single ion channel events. The technique was improved as described in Hammill *et al.* 1981. Now a major use is in the testing the effect of candidate drug substances on ion channel behaviour. It has also spawned variations for purposes other than recording single ion channel events. Whole-cell recording is of particular interest in this project. In this, a fine glass pipette containing an electrode forms a seal with a cell membrane giving the electrode a low-resistance pathway to the interior of the cell and a very high-resistance seal from the extracellular fluid. Previously it was possible to use a much sharper pipette to puncture the cell membrane and thus record intracellularly, however whole-cell recording is superior in two respects. Firstly, the larger pipettes used have much lower resistance and capacitance is reduced so that voltage clamping can be carried out at a higher rate leading to more accurate recordings. Secondly, patch-clamping does not rupture the membrane (except for the access of the electrode) so the current through ion channels across the whole of the cell membrane can be accurately recorded.

The various techniques of patch-clamping are well documented, for example in Ogden *et al.* 1987 and Standen *et al.* 1992. Here follows a brief description of whole-cell recording. The electrodes used are fine glass pipettes with tip diameters in the region of 1-5μm. The pipettes are created by heating a glass capillary in the middle and pulling from both ends, extruding the heated region until it breaks leaving a fine circular end. This end is then typically, though not necessarily, fire-polished to leave a smoother tip. The pipettes are filled from the rear with a saline solution containing approximately the

ionic composition of the cytosol of the type of cell being used (typically with a much lower sodium and higher potassium concentration than the extracellular fluid). The pipette is connected to the recording system via a silver wire coated with silver chloride. An earth wire in the extracellular fluid surrounding the cells being patched completes the circuit. The pipette tip is positioned against the surface of the cell, at which point there is a noticeable rise in the resistance of the circuit between the electrode and earth. The rise in resistance is called the "deflection" and this state is called "loose-cell" (Figure 1A). At this point the pipette is resting against the membrane but is not attached, so if gentle positive pressure is applied the cell membrane is pushed slightly away from the mouth of the pipette resulting in a brief reduction in resistance. This is called a "bounce" and it is common practice to check for this as a confirmation that the technique has been successful up to this point. If the resistance doesn't reduce, or if having reduced it doesn't return to its initial level, this may indicate that the rise in resistance was not due to contact with the cell membrane but with other debris. Suction is applied and a portion of the cell membrane (a "bleb") is sucked inside and forms a tight seal with the inside wall of the pipette. This seal prevents most ionic flow between the inside of the pipette and the extracellular solution, greatly increasing the resistance of this circuit. This state is known as "cell-attached" (Figure 1B). The gold standard for the resistance of the seal is at least 1GΩ (hereafter a "gigaseal") though it can occasionally go much higher still. Where a gigaseal is not achieved the data from subsequent recording is usually rejected due to the possibility of current leaking through pathways other than through the cell membrane. Once the gigaseal is achieved, further suction or current pulses are used to break the cell membrane, making the solution in the pipette contiguous with the cytosol. This state is called "whole-cell" (Figure 1C) and high-quality recordings of the membrane potential and of the current across the membrane can then be achieved. The quality is good enough to detect post-synaptic potentials entering the soma which do not result in spikes.

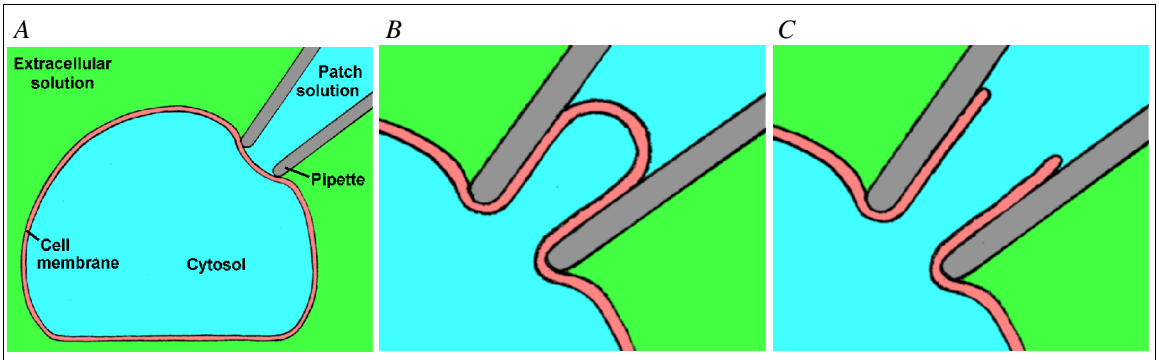

Figure 1 Three stages of whole-cell recording. A: Loose-cell, with pipette tip touching cell membrane. B: Cell-attached, with bleb sucked inside pipette. C: Whole-cell, with ruptured bleb. Diagrams not to scale.

Alternatively voltage clamping can be performed. This is where current is alternately measured and then injected to hold the membrane at a certain voltage. This enables particular membrane currents to be isolated, essentially "dissecting" the whole-cell current into its component parts. The faster the electronic switching can be performed the more accurately the time courses of various membrane currents can be recorded, and the low resistance and low capacitance of patch-clamping electrodes brings this technique into the speed range at which it is useful for investigating events with fast time courses.

2.2 Planar patch-clamps

The patch-clamping technique as described above has certain limitations. Firstly, there can be a great deal of variability in the success rate, with the skill of the experimenter and variations in the shape of extruded and fire-polished pipette tips being two major sources of variation. Secondly, it is very labour intensive. Patch-clamping can be used for testing the physiological effects of candidate drug compounds on ion channel behaviour, however the number of potential compounds is vast and higher throughput testing methods would cut time and costs. These issues all point to the desirability of automating the technique to as high a degree as possible.

Automation has been approached by switching from glass micropipettes to a new class of devices based on planar substrates with similar sized patch apertures which can potentially act in the same way. In fact there is a precedent for the idea of planar patch-clamping from 1975, where neurons were sucked into conical apertures in polythene membranes, from which whole-cell recordings were made (Kostyuk 1975). Success with modern approaches using silicon-based microfabrication techniques began to be reported in 2000. Researchers at the Swiss Federal Institute of Technology used a thin silicon nitride layer with an aperture bored through it (hereafter a "patch aperture"), coated with a layer known to promote surface adhesion of cell membranes, e.g. Poly-D-Lysine (PDL). Large artificial lipid vesicles containing ion channels were positioned over the aperture as suction was applied, gigaseals were formed and single channel recordings were achieved (Schmidt *et al.* 2000).

A group at Ludwig-Maximilians University detailed a similar microstructure intended for use with a living cell over the patch aperture, though they did not achieve success with this structure (Fertig *et al.* 2000). They then reported on improvements in their design

including the use of a quartz glass substrate (Fertig *et al.* 2002a). Gigaseals and single channel recordings were achieved with this structure, again with lipid vesicles (Fertig *et al.* 2002b). The same structure was used to achieve whole-cell recordings with CHO (Chinese Hamster Ovary) cells, though gigaseals were not achieved (Fertig *et al.* 2002c). With further refinements, gigaseals were achieved with HEK (Human Embryonic Kidney) cells (Bruggemann *et al.* 2003) and with CHO cells (Bruggemann *et al.* 2004).

In a separate line of development at Yale University silicone rubber (Polydimethylsiloxane - PDMS) moulds of glass pipette tips were created and stuck over apertures in a glass substrate. The PDMS was then exposed to oxygen plasma to temporarily increase its hydrophilicity, and this device was used to achieve gigaseals with Xenopus Oocytes (Klemic *et al.* 2002). They have since improved their fabrication technique to use a low-cost air moulding process (Klemic *et al.* 2005).

In 2002 there was speculation that the difficulties of using silicon nitride membranes were due to sharp sidewalls and insufficient contact area for proper seal formation. Surface roughness due to chemical etches was also blamed for low seal resistances (Sigworth *et al.* 2002). Following this, a group at UCLA reported on the use of a thicker membrane, of silicon. (Matthews *et al.* 2003). The Edinburgh project has followed a similar progression. Other academic groups working on planar patch-clamping are Lehigh University and the University of California. It is not clear from the literature that researchers at Lehigh University have yet been successful at achieving high resistance seals or recordings (Pandey *et al.* 2004). Researchers at the University of California used a PDMS moulded device and reported single channel recordings with the highest seal resistances achieved so far of 200MΩ, using HeLa cells (human tumour cell line). (Seo *et al.* 2004).

The above lines of research and other commercial endeavours have led to the situation today where automated planar patch-clamping for pharmacological assays has been achieved and the technology made commercially available. A summary of the main systems currently available is given below.

Nanion Technologies (www.nanion.de) have their “Port-a-Patch” system, developed from the work of Fertig *et al.* as described above. Their disposable single-aperture chips are made of borosilicate glass – the usual glass used for standard patch-clamping. Further details of the manufacturing process are however unavailable.

Sophion Biosciences (www.sophion.dk) have their "Qpatch" system, which uses disposable silicon-based substrates. Again, further details are not available, however it is understood through personal communication that the surfaces of the chips are of thermally oxidised silicon.

Cytocentrics (www.cytocentrics.com) have a different system with a reusable quartz glass microstructure with a nozzle-like protrusion. There are two suction channels, one for the patch channel itself, and one to clamp the surrounding cell membrane around the patch-nozzle while seal formation takes place.

Molecular Devices (www.moleculardevices.com) have their "PatchXpress" system, which uses "SealChip-16" chips from *Aviva Biosciences Corporation* (www.avivabio.com). These are (an array of) disposable flat chips with single patch apertures. In 2003 they presented results demonstrating a 75% success rate at forming gigaseals with CHO cells (Guia 2003). They state on their website that the substrate is "transparent" and is "chemically modified to engineer a surface that is specifically designed to interact maximally with chemical species present on a cell membrane", however no more details are available.

Of particular note is the "IonWorks" system also from *Molecular Devices*, which uses their "PatchPlate" chips. These chips have multiple patch apertures in a single substrate, and it seems to be possible to use it not just with cells in suspension but also by plating cells on the substrate prior to use. Two publications give sparse details about the chip design, namely that it is "plastic" (Kiss 2003) and that the apertures are bored by laser (Schroeder 2003) however detailed specifications are not available. Although there are multiple patch apertures, only one signal is recorded from each set of apertures, i.e. this is not a multichannel recording system.

2.3 Multi-electrode arrays

The behaviour of biological neural networks can be investigated by the use of multi-electrode arrays. For *in vitro* recordings these are typically flat glass substrates with an array of individually addressable platinum electrodes embedded in the surface. Slices of neural tissue are laid over the arrays (DeMarse *et al.* 2001) or alternatively cells are

cultured on the devices (Fromherz 2003) and patterns of activation can then be recorded and analysed. The network can also be stimulated from the same electrodes. Fromherz demonstrated the ability to create a signalling pathway from a silicon chip through a biological neural network growing on the surface and back to the chip. A stimulus from one electrode invoked a burst of spiking in a cell which triggered a spike in a neighbouring post-synaptic cell. This cell's membrane acted as a gate for a field effect transistor on the surface of the chip and thus the spike was detected (Fromherz 2003). Based on this type of work there is speculation about using biological neural networks as elements of artificial computing systems. For example Potter *et al*. at Georgia Tech has trained cultured neural networks to control computer simulations of robots (Bakkum *et al*. 2004).

The investigation of network behaviour by this method is limited by the quality of the recording. An extracellular electrode picks up currents not just from the nearest cell but all other cells. The proximity of the cells to the electrodes is often limited (Neher 2001) and it is also speculated that in cultures where cells grow on a surface they do not form ion channels in the membrane touching the surface but on the topside only (Fromherz 2003). Attempts to increase the proximity of cells to electrodes have included printing patterns of cell-adhesion-promoting chemicals, e.g. PDL, which guide the growth of cells towards the recording sites (Peterman *et al.* 2003) and creating chips with wells which trap cells against electrodes (Maher *et al.* 1999). Fromherz also speculated about optimising the structural and electrical properties of the cell membrane with recombinant methods (Fromherz 2003). The effect of this limited quality is that only spiking events can be recorded. The electrical effects of post-synaptic potentials outside the cell are too weak and are masked by noise, and this may be a fundamental limitation. It should also be noted that passage through a membrane also alters the apparent time course of intracellular currents.

Multi-electrode arrays and accompanying recording systems are available from commercial companies including *Plexon* (www.plexoninc.com), *Multichannel Systems* (www.multichannelsystems.com), and *Panasonic* (www.med64.com).

2.4 Multiple planar patch-clamps

To summarise, neural network behaviour can be investigated by recording from arrays of

extracellular electrodes, but the major limitation of these recordings is quality. By contrast whole-cell recording by the patch-clamping method is noted for its high signal to noise ratio and its ability to record sub-threshold synaptic events. Attempts have been made to investigate synaptic transmission, the basis of neural network behaviour, using the conventional patch-clamping technique. In one study three cells were recorded from simultaneously (Fitzsimonds *et al.* 1997). However this approach cannot be scaled up because the micromanipulators which hold the pipettes are too big to allow access to many more than 3 electrodes within a confined area. The difficulty of positioning pipettes and the compounding of inherent failure rates for patch-clamping also limit this approach.

It is these considerations that lay the foundations for the current project. The goal is an array of patch-clamps closely packed in a single planar substrate for obtaining multichannel whole-cell recordings from cultured biological neural networks. This would combine the advantages of multiple recording sites with the recording quality of the patch-clamp technique.

3 Objectives of current work within the overall project

3.1 Introduction

The ongoing project at Edinburgh University has focused on the use of silicon substrates and standard silicon manufacturing techniques. A progression of device designs has been generated, as will be described below in section 3.3. Prior to the current work, none of the devices produced showed the ability to form gigaseals and this is the necessary next step for the project to advance. Complexities in device testing beyond those inherent in the patch-clamping technique reduced experimental throughput; the latest devices had not been extensively tested.

Different paradigms are envisaged for the use of the end product and these imply variations in device design and in the methods used for testing. In one paradigm, the neurons will be cultured directly on the surface of the device to form a network. The surface of the device will be patterned to guide cell growth so that they grow over the patch apertures. Suction will be applied as necessary at each of the patch apertures to achieve gigaseal formation and whole-cell breakthrough. Thereafter multi-channel whole-cell recordings can be obtained. This approach is illustrated in Figure 2.

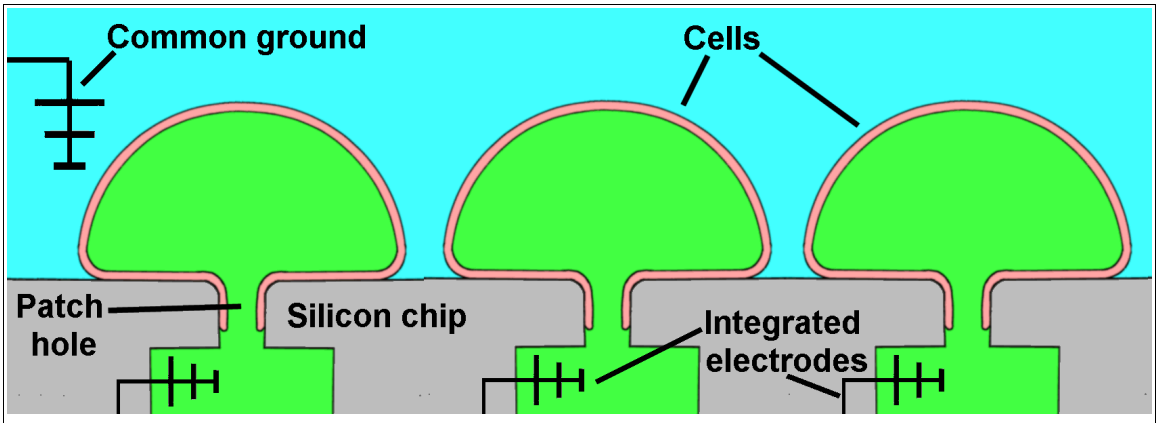

Figure 2 Cells cultured directly on surface of device are patch-clamped. Not to scale.

In an alternative paradigm, the neurons will be cultured on a separate substrate which is patterned to guide cell growth so that they grow over pre-determined sites and form a

desired network. This substrate will then be lowered onto the device with the cells aligned to the patch apertures. This approach is illustrated in Figure 3.

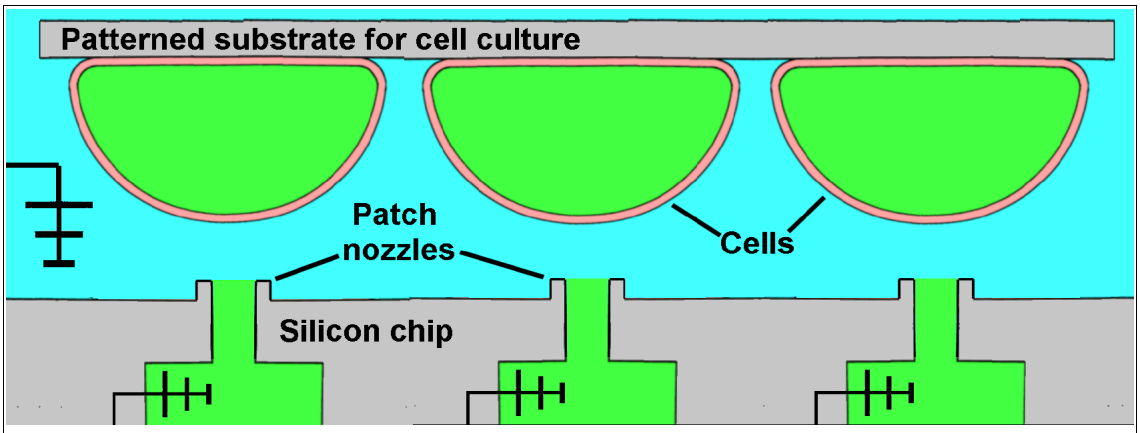

Figure 3 Cells cultured on a separate substrate are lowered onto the device. Not to scale.

3.2 Statement of intention

It was the primary goal of the current work to use the devices to achieve gigaseals with neurons and to subsequently obtain whole-cell recordings, as is possible with standard glass electrodes. Formally then, the work presented hereafter was an investigation of the hypothesis that **there is a difference between the planar devices and standard glass electrodes in the ability to perform patch-clamping with gigaseals for whole-cell recording.**

This work was also an investigation of the alternative paradigms envisaged for the use of these devices, i.e. the culturing of neural networks directly on the surfaces of devices versus the culturing of neural networks on separate substrates which are then lowered onto the devices. An additional hypothesis therefore was that **these paradigms have different respective advantages and disadvantages, such that one will be easier to apply successfully than the other.**

The investigation of the second hypothesis was through applying test methods based on these paradigms and attempting to improve these methods. The investigation of the primary hypothesis was by applying promising methods for attempting patch-clamping

with devices alongside control experiments where patch-clamping was performed by the standard technique.

3.3 Device designs

The two main types of device which were to be tested are labelled type II and type III devices. Type I devices are obsolete and will not be reviewed here. The interested reader is referred to Dworak 2004 pages 194-206.

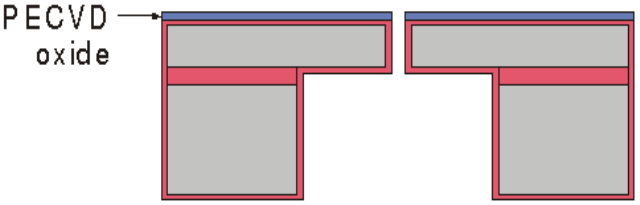

Figure 4 Type II device. Diagram from Dworak 2004 page 137. Not to scale.

Type II devices were created starting with silicon chips with an embedded oxide layer (Figure 4). This allowed the patch aperture to be etched from two sides with different dimensions. The topside aperture is of similar dimensions to a standard glass patch pipette, ≃2μm diameter. Aperture sizes will be discussed in section 3.5 below. The channel down from the topside is of sufficient length (≃10μm) for a bleb to form. This aperture was etched using a photolithographic mask constructed of rectangular shapes to form a very approximate circle. The laser beam was then defocused and this allowed a reasonably smooth circular hole to be bored. For the remainder of the depth of the chip (≃300μm) the aperture was etched from the rear side, a square of dimensions ≃80x80μm. These dimensions were chosen based on the need to integrate electrodes and fluid channels at a later stage.

These devices were given a surface of silicon dioxide by Plasma-Enhanced Chemical Vapour Deposition (PECVD) and some were subsequently doped with boron. Surface types will be discussed in section 3.4 below.

Type III devices had four improvements.

1) A circular template was created, resolving the problem of slightly non-circular apertures in the previous design.

2) An alternative etching technique, "Bosching", was used for the topside and this resulted in an aperture which tapers outwards away from the mouth, similar to a patch pipette.

3) The topside surface was formed by thermal oxidation (TO). TO was by this point considered to be a better option than PECVD for reasons explained in section 3.4 below.

4) For the paradigm where cultured cells are lowered onto the device, some devices were given a nozzle (hereafter "nozzle devices") which protrudes ≃2μm above the surface of the chip (Figure 5). This is similar to the system used by *Cytocentrics* and was inspired by the similarity to conventional glass pipettes, where the pipette is typically pushed slightly into the membrane prior to suction. This slight pressure is thought to minimise leak pathways around the tip of the pipette so that when suction is applied extracellular solution is not drawn into the patch aperture.

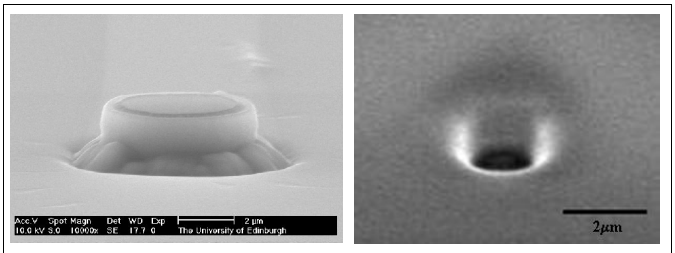

Figure 5 SEM images of patch apertures. Left: A protruding nozzle. Right: A sunken hole. Images taken by Keith Baldwin.

Type II devices had previously been tested by positioning dissociated snail neurons onto the apertures. Although the investigation of the success of devices is based on a number of factors, ultimately it is necessary to see results in terms of seal resistances achieved and whole-cell recordings obtained. Whole-cell recordings obtained without first achieving a gigaseals are considered unreliable and usually rejected, therefore it is important for this project to progress by achieving higher resistance seals. However seals obtained without then achieving the whole-cell state are ultimately worthless and furthermore may not indicate proper seal formation since it may be that the high resistance is caused by cellular or other debris blocking the patch aperture. Given this caveat, the highest seal resistances achieved prior to the current work were a 600MΩ seal and two other seals in the 100-200MΩ range. No whole-cell recordings had been achieved. The precise number of tests carried out in order to achieve these results is not known and therefore there is no proper indication of the ability of the devices to perform whole-cell recording, since the standard patch-clamping technique also has an inherrent

failure rate which can be high in certain experimental conditions.

In the current work Type II devices were to be tested by culturing cells directly on their surfaces. Type III devices had not previously been tested. Their testing was to be focussed on the lowering of cells onto nozzle devices, as this was thought to be a promising approach. Thus the current work is not an investigation of the performance of different device types *per se*, but rather an investigation of the ability of devices to form seals and of the methods used to achieve this, with the type of device used being the most appropriate in the particular context. The proliferation of different device models (see also the following sections regarding aperture sizes and surface types) has acted as a confounding factor in some of the experiments.

3.4 Surface types

Different surfaces are available from standard silicon manufacturing processes. The ideal surface material for the mouth and interior of the patch aperture was sought, being either that closest in character to the borosilicate glass used in standard patch-clamping or else that which could be established to have the most favourable properties for seal formation. Two factors are likely to be important in seal formation, the surface charge and the surface roughness. Although the exact nature of patch-clamping seals is not completely understood, surface charges may affect seal formation in either of the following ways. A net positively charged surface may be electrostatically attractive to the net negative charge expressed at the cell membrane. Alternatively, charges of either polarity may provide sites for hydrogen bonding with the membrane's hydrophilic phospholipid heads or with membrane proteins. Whilst membrane proteins may play a role in seal formation, the ability to patch-clamp artificial lipid vesicles demonstrates that any role they play is not essential. Another proposed bonding mechanism was salt bridges where ions in the solution form ionic or dipole-dipole bonds with both the membrane and the surface. Surface roughness is thought to reduce the quality of the seal by providing pathways between the surface and the membrane through which ions can “tunnel”. It is also possible that a surface with sharp discontinuities could mechanically damage a cell membrane during seal formation.

A series of experiments were carried out to analyse surface characteristics for different available surface types.

1) Contact angle experiments were carried out where droplets of water were dropped on different surfaces and measurements were made of the angle at which the droplet contacted the surface. This gave a measure of "wettability" – the extent to which the water will form polar bonds with the surface in preference to other water molecules. This is an indicator of the extent to which the surface is charged.

2) Surfaces were scanned with an Atomic Force Microscope (AFM) and the scans analysed to give measures of surface roughness. The output of an AFM is a measure of height for each point on a surface. The measure of surface roughness then was based on the standard deviation of the heights of the sampled points on each surface. The use of this measure is questionable, because the type of roughness which is more likely to be problematic for seal formation is that where there are sharp discontinuities in the height of the surface; the measure chosen takes no account of this. A better measure of roughness might be the standard deviation of the first derivative of the surface height across the scans.

3) Rat hippocampal cells were cultured on the different surfaces. The extent to which the cells spread out over the surface as opposed to clumping on top of each other was measured. This was taken as a measure of the compatibility of the cells and the surfaces, and it may indicate a surface charge or smoothness, or a combination of these.

The main surface coverings tested were as follows.

- Silicon dioxide, deposited by Plasma-Enhanced Chemical Vapour Deposition (PECVD)
- Silicon dioxide, thermally grown (Thermal Oxide - TO)
- PECVD, Boron doped (PECVD+B)
- TO, Boron doped (TO+B)

The main findings from this work were as follows.

- As layers of TO are grown the aperture becomes less round and more square, because the oxide grows preferentially in the same planes as the silicon's crystal lattice.
- Coating with PECVD avoids this problem, however it has a rough surface.
- Boron doping the PECVD leads to a smoother surface.

- TO has a smoother surface than PECVD.
- TO+B has the smoothest surface, with the added advantage of being hydrophilic.
- Cells grow preferentially on positively charged surfaces. TO+B performed better than TO for cell growth.

Therefore there is potentially a design trade off between the larger scale irregularities produced with TO and the smaller scale irregularities produced by PECVD. PECVD devices were initially favoured, because this was thought necessary to minimise a squaring of the aperture caused by a limitation of the initial technique used to bore the aperture. This consideration had been eliminated in the design of type III devices and therefore TO(+B) surfaces were created. The potential utility of TO surfaces was confirmed by the success of *Sophion Biosciences* at using this type of surface in their "Q-Patch" planar patch-clamping system.

The type II devices used in this work were PEVCD(+B) whereas the type III devices were TO(+B). Direct comparisons between these surfaces were not drawn in the work presented here; rather this factor was investigated concurrently by another researcher. Nevertheless the surface type was a factor in the current work and in some cases was a confounding factor in experimental design.

3.5 Aperture sizes

For standard patch-clamping, there is a body of knowledge regarding the ideal diameter for the tips of the glass electrodes. Ogden gives the range 1-3μm with the condition that larger pipettes should be made of thin-walled glass (typically 0.35mm thick) (Ogden 1987). Other sources state that tip diameters of ≃1μm are desirable (Standen *et al.* 1992 page 69; Penner 1995), and this seems to be a consensus. For example, patch apertures of approximately double this diameter (≃2μm) and cited as a possible reason for the low success rate of planar devices developed at Yale University (Klemic *et al*. 2005). However it is thought that in general terms smaller cells require smaller tips whilst larger cells can be patched with larger pipette tips. It is also thought that while achieving gigaseals is easier with smaller tip diameters, subsequently achieving whole-cell breakthrough is easier with larger tip diameters.

Given this uncertainty the previous lead researcher considered that pipettes in the range

of 1-5μm diameter may be suitable for whole-cell recordings (Dworak 2004). It was thought that larger aperture diameters may prove beneficial by compensating for a lack of aperture circularity introduced in the manufacturing process. For type II devices this lack of circularity was due to the use of photolithographic templates based on rectangles (for a detailed discussion the interested reader is directed to Dworak 2004 pages 79-103), and for type III devices this was due to a squaring effect introduced as silicon dioxide is thermally grown. The type II devices were manufactured with aperture diameters of ≃2μm. For the manufacturing of type III devices, a range of aperture diameters were created in order to explicitly test this factor. The target aperture diameters for 5 silicon wafers were 1, 1.5, 2, 3, and 4μm. However due to an unfortunate error in the manufacturing process the resulting apertures were ≃1.5, 3.5, 4, 4, and 4μm respectively. This made 1.5μm apertures a rare commodity, with low yield on the wafer with the smallest aperture size compounding the problem. Aperture diameters of ≃3.5μm and 4μm were subsequently brought down to ≃2.5μm and 3μm respectively by further thermal oxidation at the expense of further squaring these nozzles.

In the current work the suitability of different aperture diameters with AtT-20 cells was investigated, as will be described in section 4. For tests of planar devices with snail neurons, the type III devices with aperture diameters of ≃2.5μm and 3μm were used, and efforts were made to eliminate this confounding factor in the experimental design, as will be described in section 7.

4 Glass electrode patch-clamping on AtT-20 cells

4.1 Introduction

In both paradigms for the use of the devices (as described in section 3.1) it is desirable to be able to guide the growth of cells so they are positioned at patch sites. Otherwise, in all but the densest cultures, the chance of achieving a seal at any given patch aperture may be so low as to eliminate any advantage from developing a multi-aperture device. There is a good deal of understanding on how to pattern surfaces for cell growth; the common approaches involve stamping surfaces with biological substances, typically PDL (James *et al*. 2000). This may be problematic where cells are to be grown directly on the surface of devices, as this macromolecule would bond to the surface of the patch aperture and may prevent seal formation. An alternative approach is envisaged where patterning is achieved with different non-biological surface treatments available in standard silicon manufacturing processes.

This work is ongoing, however for the purpose of testing the current batch of devices a different approach was taken. Previous device testing had been carried out with snail neurons. For experiments where cells are cultured directly onto the surfaces of devices it was decided to switch to using the AtT-20 cell line. This is a cell line originally extracted from a mouse pituitary tumour. The advantage of using a standard cell line in general is that a high homogeneity in characteristics can be expected. The advantage of AtT-20 cells in particular is that they have some of the characteristic properties of neurons, i.e. formation of dendrites and synaptic junctions, membrane potential of approx -65mV and ability to spike, including spontaneous spiking behaviour (Adler *et al.* 1983). AtT-20 cells are known to adhere well to many surfaces including glass and it was expected that they may do likewise on other silicon-based surfaces. As they are cancerous, they divide and so unlike normal neuronal cultures they will multiply *ad infinitum* to cover any given surface. This means that if cells are allowed to grow on a device for long enough then a cells should eventually grow over the patch aperture. Compared to the alternative of plating dissociated rat hippocampal cells on the device and accepting a certain probability that one will be positioned over the patch aperture, it was hoped that this approach would reduce time and cost overheads for the testing phase.

It was first necessary to establish the basic properties of AtT-20 cells under local experimental conditions. In particular it was necessary to establish a success rate of whole-cell recording from AtT-20 cells by the standard patch-clamp technique in order to be able to draw any conclusions from attempts to patch on these cells with devices. In this respect the following advice from the *Nanion* researchers is pertinent "Of course some cells are easier to patch with the chip than others, as is the case with patch pipettes. The 'patchability' is very much correlated, i.e. a cell that is easy to patch with a pipette will most likely work fine with the chip as well." (Bruggemann *et al.* 2004).

Given the need to test devices with apertures of various diameters in the range $\simeq$1.5-4μm, pipettes of varying tip diameters in this range were used. It was expected that pipette resistance could be used as an indicator of the size of the tip. To test this assumption a separate experiment was carried out where pipette resistances and internal tip diameters were measured.

4.2 Materials and methods

Cell culturing

AtT-20 cells were a generous gift from Dr Ian Rowe. The age and *passage* history of the cell line was unknown. The cells were grown at 37C in 5% CO2. The growth medium contained: 1 part Foetal Bovine Serum (FBS):10 parts Dulbecco's Mod Eagle Medium (D-Mem), glucose 25mM, glutamine $5x10^{-4}$mM. 1:200 penicillin/streptomycin (pen/strep) was added to some batches.

Borosilicate glass coverslips (10mm diameter, manufactured by Marienfeld, Germany) were placed individually in cell-culturing wells, and covered with a solution of 70% ethanol for 12 hours. They were rinsed three times with Milli-Q filtered water. Then they were sterilised under UV light in a biosafety cabinet for at least 30 minutes.

To *passage*, the following procedure was carried out in a biosafety cabinet. Old growth was removed and the flask was rinsed with Hanks Balanced Salt Solution (HBSS). 0.5ml of trypsin (a protease) 0.25% in HBSS ask was gently agitated regularly for about 1 hour until a good proportion of the cells had detached from the flask. New growth medium was then added and the mixture applied to cell-culture wells and a new flask. The amount

of growth medium and the proportion of growth medium to trypsin was varied. *Passage* was repeated every 1-2 weeks.

Patch-clamping

Glass capillaries (thick-walled, external-diameter 1.5mm, internal-diameter 0.86mm, manufactured by Harvard Apparatus Ltd, Kent) were pulled with horizontal electrode puller (P87, Sutter Instruments, Novato, CA) in a range of sizes.

Pipettes were back-filled with filtered intracellular solution containing (in mM): 130 D-gluconic acid, 20 N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), 10 ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), 5 magnesium chloride (MgCl2). The solution was buffered to pH 7.2 with potassium hydroxide (KOH).

Coverslips were used after culturing for 4-11 days. Coverslips were removed from growth medium and placed in a Petri dish filled with extracellular solution at room temperature. The extracellular solution contained (in mM): 130 sodium chloride (NaCl), 40 potassium chloride (KCl), 25 HEPES, 10 glucose, 1 calcium chloride (CaCl2). The solution was buffered to pH 7.4 with sodium hydroxide (NaOH). Patch-clamping attempts were made within 2 hours of the removal of the cells from the incubator.

To create silver/silver chloride (Ag/AgCl) electrodes, Ag wires were cleaned with an abrasive then placed in 3M KCl solution and 4V D.C. was passed through them with the wire as the cathode for ≃10 minutes.

The experiments were carried out on an air-cushioned table in a Faraday cage. The Petri dish was placed on the stage of a microscope (BX50WI Olympus), fitted with a camera (OCRA-ER, Hamamatsu), for on-screen viewing and image capture (SimplePCI software, Compix Inc.). Pipettes were mounted on headstages (Axon Instruments, Foster City, CA). Headstages with amplification ratios of x0.1 and x1 were alternately used. The signal electrode went in the rear of the pipette and the

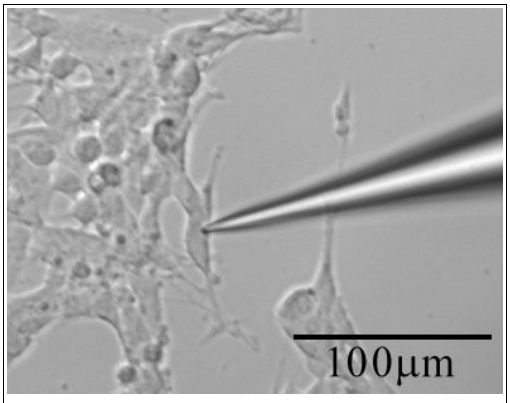

Figure 6 Pipette touching the membrane of an AtT-20 cell.

ground electrode went in the extracellular solution in the Petri dish. The headstages were mounted on micromanipulators (MS314, Märzhäuser Wetzlar, Germany). An intermittent current pulse (2 Hz, duration 100ms, amplitude variable, usually in the range 1-5nA) was delivered from a current clamp amplifier (Axoclamp2B, Axon Instruments), driven by a pulse generator (Master-8 AMPI, Intracell Ltd, England). Circuit resistance as indicated by voltage response to current pulses was observed on an oscilloscope (OX 8020, Metrix). The pipette tip was immersed in the extracellular solution and circuit resistance was measured (this being primarily the resistance of the pipette). The pipette tip was then brought close to the membrane of a selected cell (Figure 6), until an increase in resistance was observed. A brief positive pressure was applied so that a "bounce" could be observed. Suction was applied, the sealing progress monitored, and current pulse amplitude reduced as deemed appropriate. Where seal resistance peaked, whole-cell breakthrough was attempted with further suction or with brief voltage pulses. Current and voltage were sampled at 62.5kHz and recorded for off-line analysis by a standard P4 PC (Software Axoscope 9.0 and Clampex, Axon Instruments), via an amplifier (LPF 202, Warner Instrument Corp).

Only one patch-clamping attempt was made on any one cell. Only one patch-clamping attempt was made with any one pipette.

Measurement of pipette diameters

In a separate experiment, pipette of various sizes were pulled, their resistances were measured by back-filling, mounting, immersion and recording as described above, and their tip diameters were measured as follows. The tip was brought into focus under a x60 immersion lens (Figure 24). Three measurements of the distance between the inside walls at the tip were taken using SimplePCI software, and the mean was calculated, using a pixel/µm ratio previously established by calibration with a graticule.

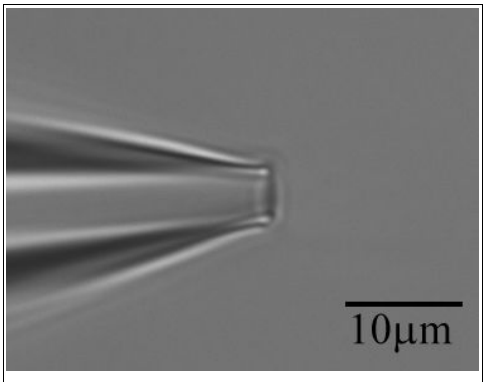

Figure 7 the tip of a 3.4µm diameter glass pipette.

4.3 Results

66 patch-clamping attempts were made in this phase of experimentation. The overall success rate at achieving a gigaseal was 12%. Table 1 summarises the results.

Table 1 Summary of patch-clamping results

Maximum seal resistance	**Number of attempts**	**Percentage of attempts**
< 100 MΩ	22	33%
≥ 100 MΩ < 1000 MΩ	36	55%
≥ 1000 MΩ	8	12%

Of the 8 gigaseals achieved only one subsequently went whole-cell and on this occasion the membrane potential measured was only -5mV, indicating either a problem with the recording set up or an unhealthy cell. In other cells where a sub-GΩ seal was achieved prior to going whole-cell, small membrane potential fluctuations were also observed. No membrane potential measured was below -30mV, a threshold for resting membrane potential at which AtT-20 cells are commonly considered healthy (Adler *et al*. 1983).

For the separate experiment where tip diameters were measured against pipette resistance, it was expected that resistance would be inversely proportional to the cross-sectional area of the pipette tip, in analogy to the formula for the resistance of a wire:

$$R = \rho \frac{l}{A}$$

where R is resistance, ρ is resistivity, l is length and A is cross sectional area.

This was indeed the case. Figure 8 shows a scatterplot of pipette resistance against 1 / tip area, where tip cross-sectional area is π(internal diameter / 2)^2.

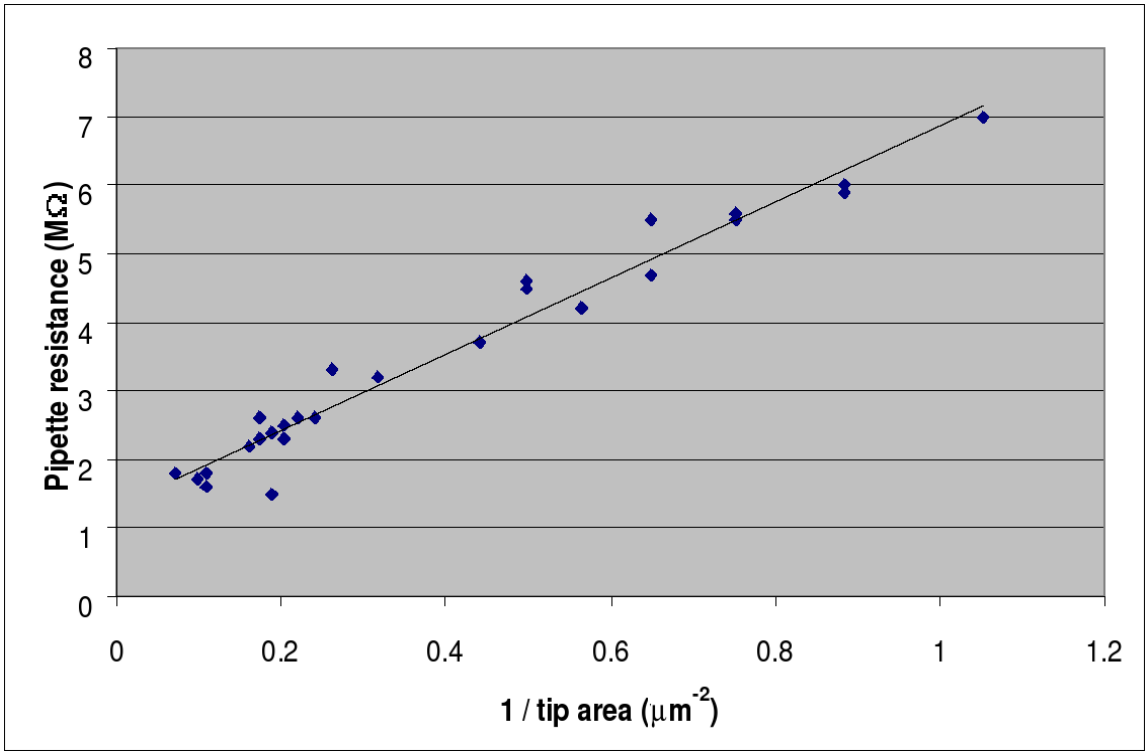

Figure 8 Relationship between pipette resistance and tip size.

A linear correlation gives a Pearson Correlation Coefficient r = 0.983, and a one-tailed significance test with d.f. = 26 gives $p<0.01$. A linear regression of pipette resistance on 1 / tip area, then substituting tip diameter (as a more intuitive measure) gives the following formula:

$$Pipette\ diameter\ (\mu m) = \sqrt{\frac{7.07}{(pipette\ resistance\ (M\ \Omega) - 1.31)}}$$

Figure 9 is a scatterplot of the 66 patch-clamping attempts showing the resistance of the pipette as an indicator of tip size and the maximum seal resistance achieved as a measure of the success of the patch-clamping attempt. Note that the maximum seal resistance does not include the pipette resistance; this has been subtracted.

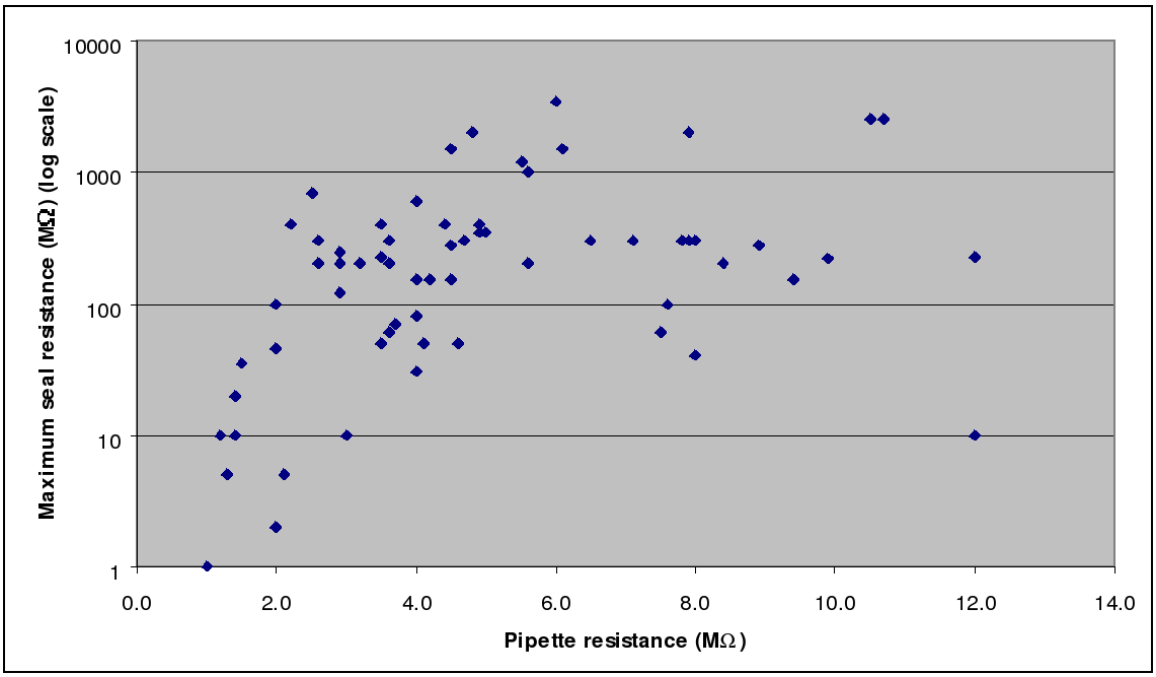

Figure 9 The effect of tip size (as indicated by pipette resistance) on seal resistance.

Although the results are highly variable there appears to be a relationship between tip size and the seal resistance achieved; the lack of success in achieving gigaseals when tip diameters were large (i.e. when pipette resistance was low) is immediately apparent. In particular, the lowest pipette resistance at which a gigaseal was achieved was 4.5MΩ, which by the above formula would be a pipette of diameter $\simeq$1.5µm.

In preliminary testing of devices with 4µm diameter apertures with AtT-20 cells it was observed that entire cells could be sucked into the apertures without seals forming. For comparison, two pipettes were pulled, with diameters $\simeq$3.5µm. In attempting to patch with these pipettes on both occasions the entire cell was sucked into the pipette. An example of this is shown in Figure 10. The same effect was seen with a pipette of $\simeq$3.0µm diameter (as judged by pipette resistance).

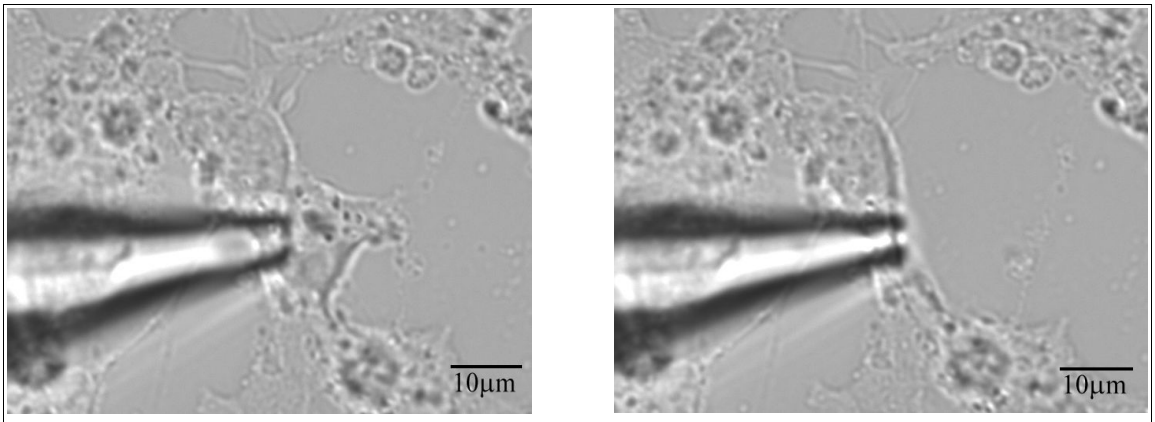

Figure 10 patch-clamping on AtT-20 cell with 3.5μm diameter pipette. Left: before suction. Right: after suction.

4.4 Discussion

There are many factors that may influence the success of patch-clamping, and many of the following factors were varied during the course of experiments, intentionally or otherwise:

- Experience of the experimenter.
- Age of the cell culture since *passage* and the density of cultures.
- Medium used to culture the cells, including whether antibiotics were applied, how often the medium was changed and the residual levels of trypsin since the previous *passage.*
- Ambient temperature.
- Length of time the cells had been out of the incubator upon patch-clamping.
- Stability of the air-cushioned table, and nearby vibrations.
- Shape, size and health of cells.

The initial approach taken was to have a phase of free experimentation where different factors were varied in order to get a feel for what may or may not work, and then to fix procedures to produce proper controls. However the lack of success in achieving whole-cell recordings meant that it was not possible to establish a good regime in which to fix factors in order to achieve a high quality of control data.

The patch-clamping technique is known to be difficult and somewhat unpredictable

(Standen *et al.* 1992 page 75). Furthermore much of the accumulated knowledge about how to improve success at patch-clamping remains anecdotal. Notwithstanding this, the success rate at achieving whole-cell recordings was so low as to raise suspicions about fundamental factors such as the health of the cell line being used. It may be better to procure a batch of AtT-20 cells directly from a cell bank and good whole-cell recordings achieved before continuing with device trials. On the other hand, it is clear that seals including gigaseals can be formed with these cells, and if seals in even the 100MΩ range could be reliably achieved with devices this would represent an advancement of the project. Therefore there is some scope in proceeding with device trials given this control data.

Regarding the diameter of the patch apertures, gigaseals were only achieved with 1.5μm diameter pipettes and below, whereas 3μm diameter pipettes are big enough to suck up an entire cell. These observations strongly indicate that of the devices available only those with aperture diameters of 1.5μm may be suitable for use with AtT-20 cells. These results are consistent with the apparent consensus that pipettes with tip diameters of ≃1μm are ideal (see section 3.5) The use of devices with larger apertures is not ruled out for other cell types, particularly snail neurons.

Other factors were varied. The use of pen/strep was initially avoided as it was thought that this may make the cell membranes less patchable. Antibiotics can repeatedly puncture cell membranes, indeed this is the basis for the alternative patch-clamping technique of "perforated-patch" (Standen *et al.* 1992 page 80-82). However the use of pen/strep was adopted after progress was hampered by infections. This did not lead to any obvious difference in the patchability of the cells, although this was not specifically tested for. Other patch-clamping studies where pen/strep was used with AtT-20 cells (e.g. Garcia 1998) support the opinion that the use of pen/strep is not problematic, however for such studies the exact success rate of patch-clamping attempts would not have been vital.

No other factors (e.g. days after *passage*, time out of incubator) made enough apparent difference to warrant further investigation. As stated in Section 4.2 above, cell use was ceased 2 hours after removal from the incubator.

5 Culturing directly on devices

5.1 Introduction

This section presents the results of experiments where cells were cultured on the surfaces of devices. A potential problem with culturing cells on devices is that the patch aperture needs to be filled with intracellular solution during the patch-clamping attempt. Devices cannot be easily back-filled as glass pipettes can. A method for filling devices to avoid air bubbles in the patch aperture had been developed and is described in section 5.2. The method however involves exposing the device to a vacuum and is not compatible with living cells. Clearly, a method of back-filling the patch aperture once a cell is growing over it cannot involve pressure as this would force the cell off the aperture. Therefore the patch apertures need to be filled prior to cell culturing. Two paradigms were envisaged.

1) The patch aperture is filled with intracellular solution and then the rear of the patch aperture remains exposed to a bath of intracellular solution whilst the top surface of the device is bathed in growth medium.

2) The patch aperture is filled with extracellular solution and then both sides of the device are exposed to growth medium. For patch-clamping the device is mounted with the rear of the patch aperture exposed to a bath of intracellular solution so that the solution diffuses into the aperture.

To choose between these paradigms a major factor is how quickly solutions will diffuse through the aperture. If diffusion is fast, then in paradigm (1) the intracellular solution will diffuse into the growth medium and ultimately kill the cells. If diffusion is slow then paradigm (2) may not allow experiments to be carried out on a reasonable time scale. Alternatively if solution is not given sufficient time to diffuse out then the patch aperture will contain growth medium, which may inhibit seal formation or may damage the cell once whole-cell breakthrough is achieved through the infusion into the cell of ionic concentrations which change transmembrane diffusion gradients.

To investigate this factor a set of junction potential experiments were carried out. This technique is described in Standen *et al.* 1992 page 72-73. These experiments will not be

reported in detail here as they were not primarily the work of this author, however the results were that the fluid in the ≃80x80x300μm rear aperture would intimately mix with the fluid in the bath to which it is exposed within 30 minutes, whereas the fluid in the ≃10μm length topside aperture with diameter ≃2μm would take a matter of days to diffuse out. This knowledge however does not resolve the issue and experiments with both of the above paradigms were carried out, based upon speculation on this issue. See section 5.4 for further discussion.

5.2 Materials and methods

Devices

Devices were of type II, as described in section 3.3. Some devices were mounted onto perspex frames in preparation for mounting on a custom-built rig for patch-clamping. The following procedure was carried out in level 10 cleanroom facilities.

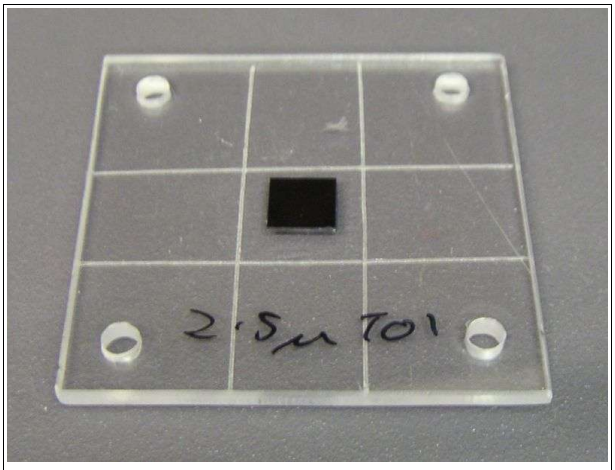

Figure 11 A device mounted on a perspex frame.

The frames were cleaned by wiping with iso-propan-2-ol. Sylgard 184 is a commercial brand of PDMS (Dow-Corning, Midland, MI). It is a two-part curing system. The parts were mixed according to the manufacturers instructions in the ratio 10:1. The mixture was degassed by placing under a vacuum for 5 minutes. The Sylgard was then lightly applied to the back of the devices with a brush, taking care to cover as much area as possible (for reduced capacitance) but to avoid covering the aperture. The devices were then applied one to each frame over a hole in the middle of the frame. The mounted devices were cured in an oven at 60^{o}C for 12 hours. Figure 11 shows a device mounted on a frame.

An alternative design of frame was as above but with an additional raised perspex ring around the device (adhered with Sylgard). This was to enable a separation of solutions

between the front and rear of the device. That is, the frame and thus the rear of the device could be bathed in intracellular solution whilst the front of the device was bathed in growth medium.

The apertures of the devices were filled with intracellular solution. To achieve this the devices were placed face down in a vacuum jar partially filled with intracellular solution which had been passed through a 0.2μm filter. A vacuum was then applied for ≃5 minutes. Then, still under vacuum, the jar was repeatedly shaken, to remove gas bubbles from the surfaces of the device. This was repeated for a further ≃5 minutes. After this the vacuum was gently released.

Where devices were to be reused, they were cleaned by submersion in "Piranha" (this is commonly used to clean substrates with organic contamination). This procedure was carried out in level 10 cleanroom facilities, in a fume hood with appropriate precautions. Piranha contained 3 parts sulphuric acid (H_2SO_4) 96% solution to 5 parts hydrogen peroxide (H_2O_2) 30% solution. As soon as the ingredients were mixed, the devices were submerged for 15 mins. Then they were submerged in de-ionised water for 2 minutes to rinse them and this rinsing step was repeated 3 times.

Cell culturing

AtT-20 cells were cultured on the surfaces of devices initially filled with intracellular solution. Devices which were stuck to frames were placed in Petri dishes, whereas unmounted devices were placed individually in cell-culturing wells. Devices were then sterilised by the same means as glass coverslips and thereafter plated, with all procedures as described in Section 4.2 above. AtT-20 cells were also plated onto glass coverslips at the same time so that culture density could be easily monitored (since the microscope available at the cell culturing facility was not suited to observing cells on the opaque silicon chip).

For experiments under paradigm (1) above, devices mounted in frames with additional raised perspex rings were filled and then bathed in intracellular solution whilst the front of the device was plated with cell growth medium. For experiments under paradigm (2) as described in section 5.2 the devices mounted on frames were initially filled with intracellular solution and then entirely bathed in cell growth medium. In a second alternative under paradigm (2) the devices were not mounted on frames but rather filled,

sterilised and then cultured in cell growth medium whilst free-floating. This was to increase experimental throughput.

Patch-clamping

After 6-13 days of culturing, device surfaces were examined under the microscope. Light from a monochromatic source (Polychrome IV, TILL Photonics) was applied via the microscope in order to observe cells on the surface of the device. Where cells were growing on or very near the aperture, the device was mounted on the rig and patch-clamping was attempted.

Figure 12 shows the rig used to mount devices for patch-clamping attempts where the

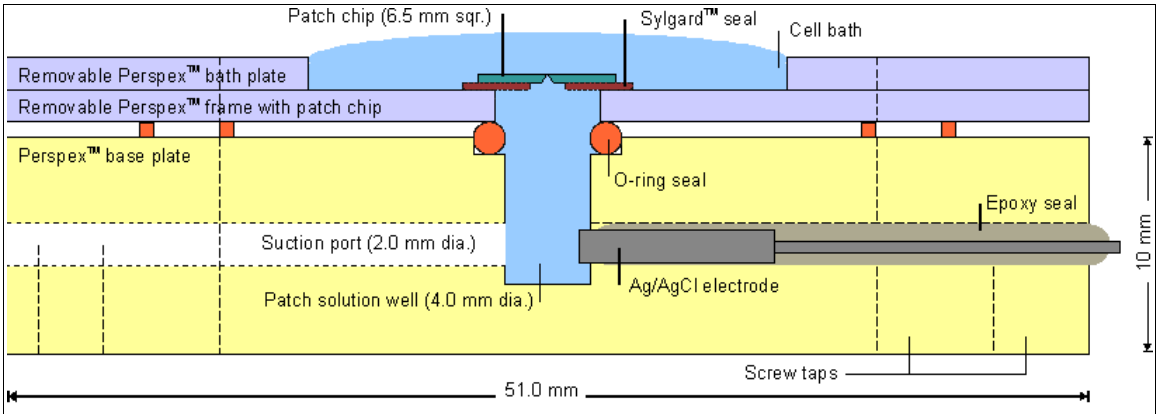

Figure 12 Rig for mounting framed device. Diagram modified from Dworak 2004 page 161.

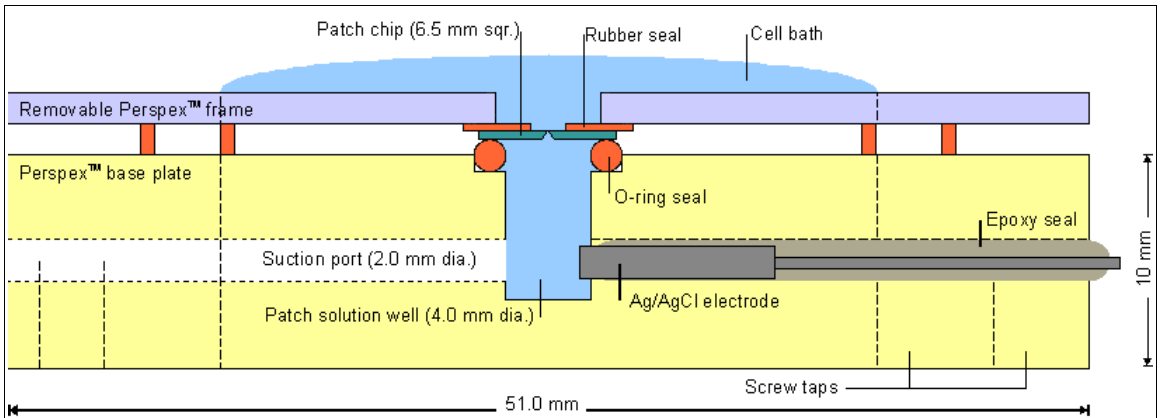

Figure 13 Rig for mounting free device. Diagram modified from Dworak 2004 page 161.

device was mounted on a perspex frame. The well in the centre of the device was filled with intracellular solution and then the framed device pre-filled with intracellular solution was placed over the well and screwed down tightly. A bath of extracellular solution was then gently pipetted over the surface of the device. The well under the device was examined under the microscope for air bubbles and if present the step would be repeated. Figure 13 shows an alternative configuration of the same rig for mounting devices where they were not previously mounted on frames. In this configuration a rubber ring clamps down around the edge of the device. The suction port led away via a flexible plastic tube to a syringe, from which positive or negative pressure could be manually applied.

Recording equipment was as described in section 4.2 above, with the rig replacing the glass pipette. The electrode in the well beneath the device was connected to the headstage, and an Ag/AgCl electrode was placed in the extracellular bath to complete the circuit. Patch-clamping was attempted following the procedure described in section 4.2, though no selection of cells or manoeuvring of the device with respect to the cell was possible and therefore the procedure started from the application of suction.

5.3 Results

Several attempts to culture on devices without pen/strep resulted in infection and the death of all cells, as indicated by acidification of growth medium and debris on the surfaces of devices.

Two attempts to culture on devices resulted in uninfected cultures. For discussion, photos of all successfully cultured devices are presented here, in Figure 14 - Figure 16. In each photo there is a lighter coloured square. This is the $\simeq$80x80μm rear aperture showing through from rear illumination. In the centre of these squares is a spot which is the actual topside patch aperture.

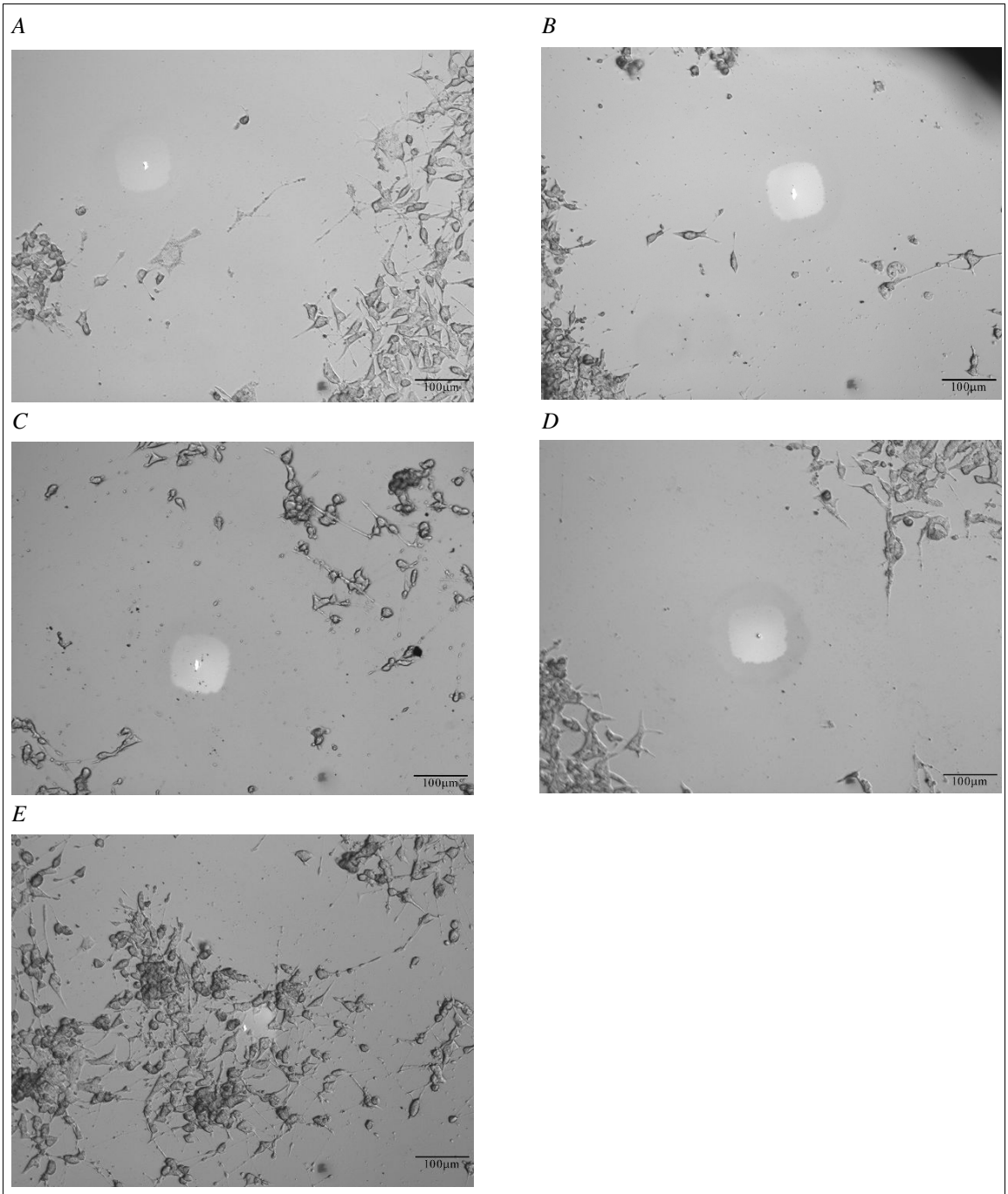

Figure 14 AtT-20 cells cultured for 7 days on PECVD devices.

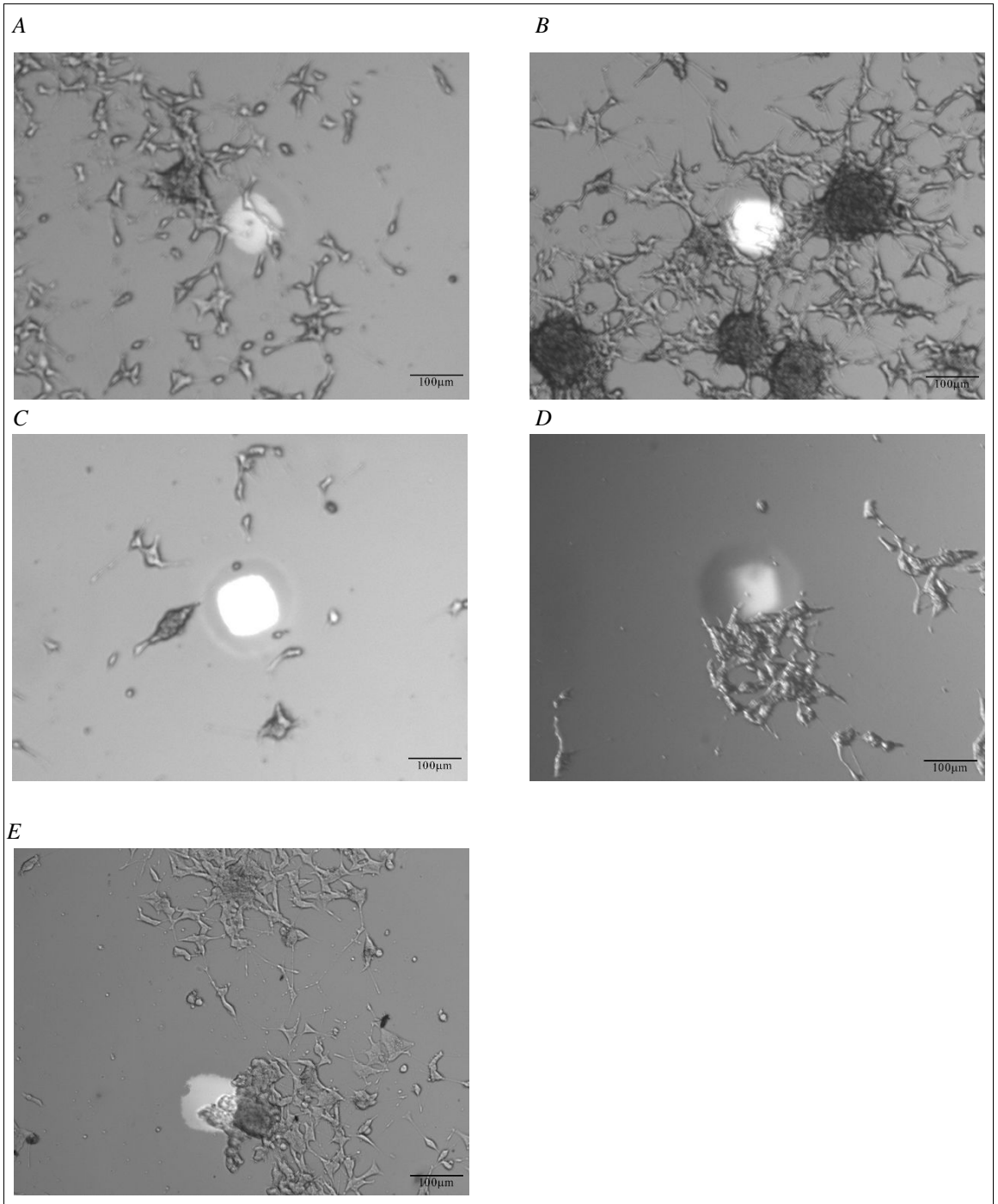

Figure 15 AtT-20 cells cultured for 6 days. A,B: PECVD devices. C,D: PECVD devices, details of subsequent surface treatment lost – either boron doped or annealed.. E: PECVD annealed devices.

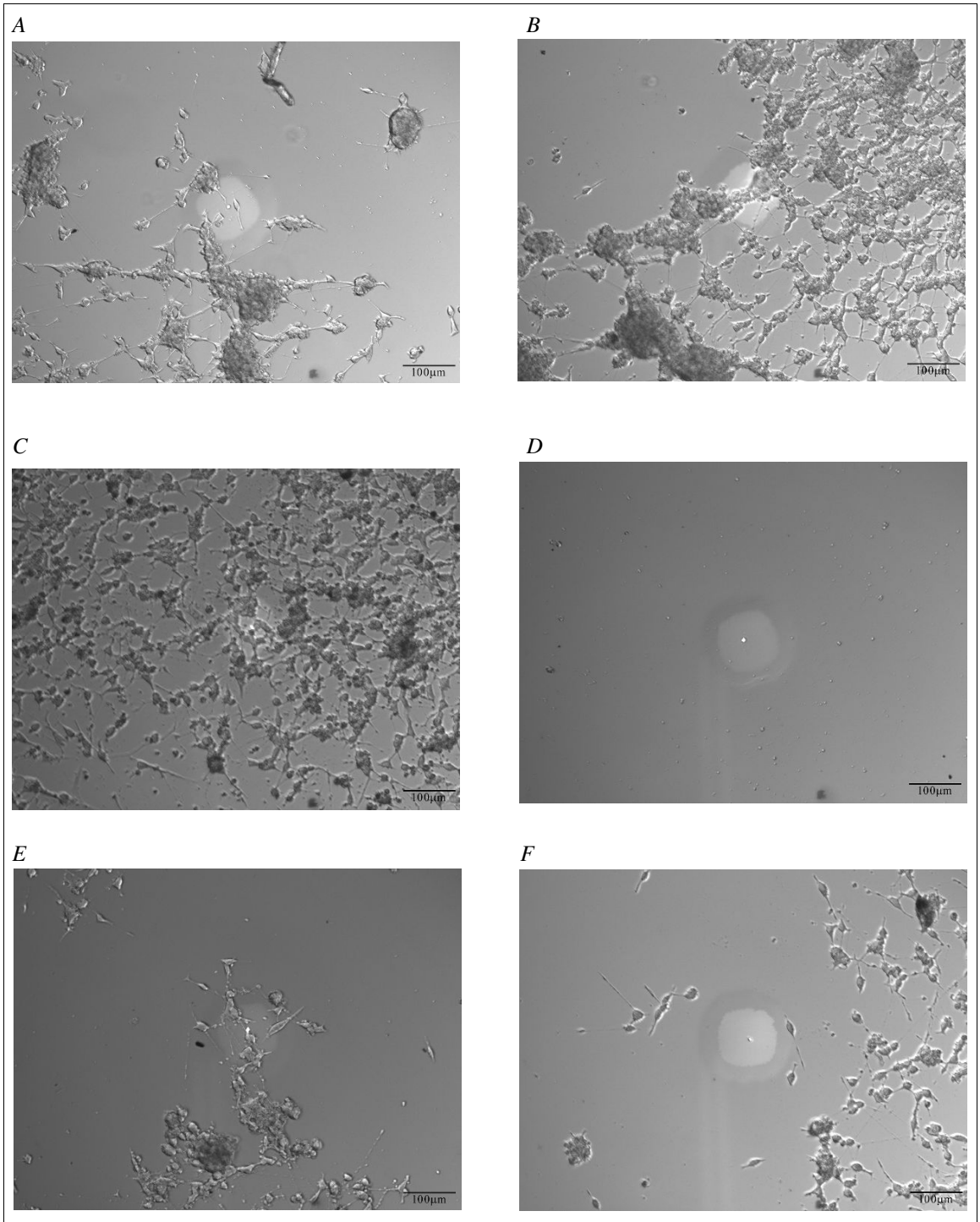

Figure 16 AtT-20 cells cultured for 13 days. A,B,C: PECVD devices. D,E,F: PECVD annealed devices.

The first successful attempt at culturing devices is shown in Figure 14. These devices were mounted in frames and pen/strep was not used. The second successful attempt is shown in Figure 15 - Figure 16. These devices were left free-floating and pen/strep was used. This batch of devices was divided in two for examination on two separate occasions. After 6 days glass coverslips plated during the same *passage* had a reasonable density. However the devices examined (Figure 15) had a noticeably sparser covering. The remaining devices were therefore left until they had been cultured for 13 days (Figure 16). Qualitatively, in many of these photos cells do not appear to be as well adhered to the surfaces as they normally are on glass coverslips.

Of 16 devices, only 5 had cells growing close to the aperture (Figure 15 B and E, Figure 16 B, C and E), and of these only 1 (Figure 16 B) had a cell growing unambiguously over the aperture. Where cells were growing close to the aperture all attempts to patch failed, as would be expected where full contact with the aperture was not made. Two attempts (Figure 15 E and Figure 16 C) ended with the cells being dislodged from the area of the aperture. Figure 17 shows this effect for the device in Figure 15 E. In both of these cases the devices had been free-floating and dislodging occurred when attempting to mount the device on the rig, probably during the application of extracellular solution.

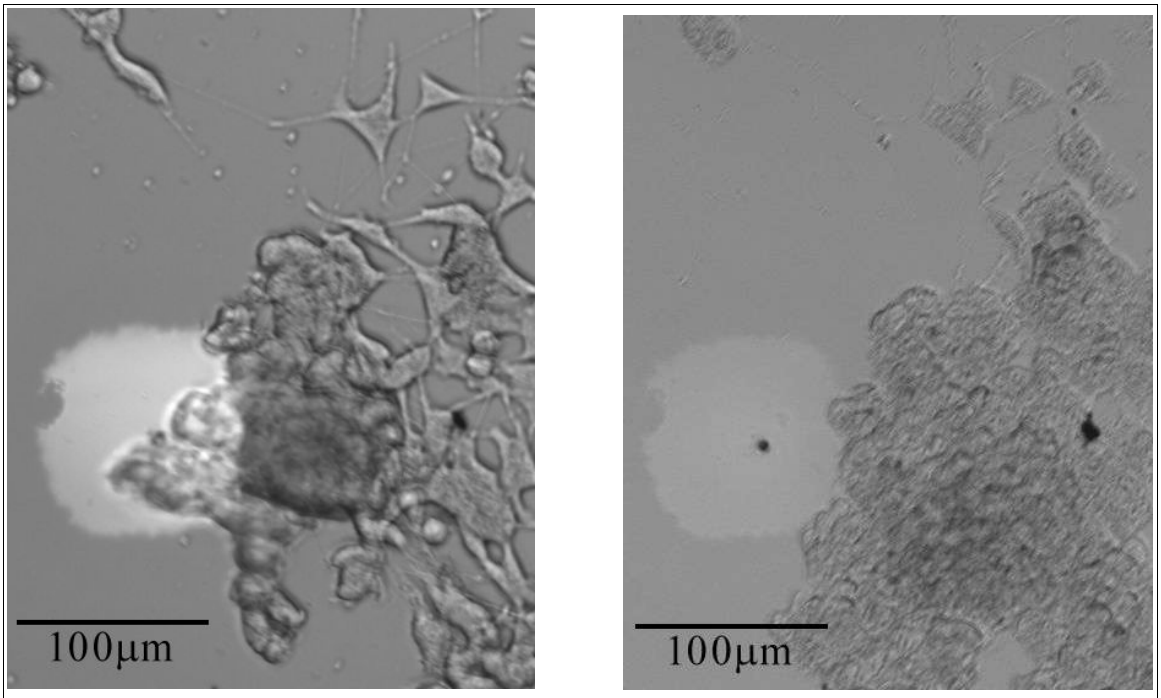

Figure 17 An abortive attempt to patch onto AtT-20 cells growing partially over the patch aperture. Left: before mounting. Right: after mounting.

In the case where cells were growing over the aperture (Figure 16B), applying suction resulted in a loose-cell seal resistance of $\simeq$5MΩ however further suction failed to initiate the process of sealing (the device in question had a aperture of diameter $\simeq$2μm).

5.4 Discussion

Much of the work at this stage of experimentation involved developing optimal procedures for cleaning and preparing cultures to the extent that the application of antibiotics would not be necessary, for the reasons stated in section 4.4. However repeated infections resulted. One possible explanation could be that the device aperture can hide bacteria from the sterilising effect of UV light, attempts to sterilise both sides of the device notwithstanding.

Other methods of sterilisation were considered. Bathing in 70% ethanol solution and 2M hydrochloric acid (HCl) solution were both attempted but abandoned due to concern about their effects on the integrity of the PDMS seals. Autoclaving or other methods of heating were not possible at least as a final sterilisation step as this may have evaporated the liquid in or otherwise created gas bubbles in the patch aperture.

The success rate of growing a cell over the aperture of 1 in 16 reported here is purely arbitrary, however it is clear that increasing the density of the cultures increases the likelihood of success in this respect. This was the main rationale for using AtT-20 cells i.e. their ability to multiply to cover surfaces. During this phase of experimentation attempts were made to increase the density of cultures by starting with higher concentrations, using more trypsin solution to increase contact time with cells (since 0.5 ml was not sufficient bathe the surface of the flask) in order to transfer more cells from the old flask during *passage* and by plating cells directly onto only the exact surface area of the device. All of these approaches are viable and could be improved in a further round of experimentation.

It had been assumed that AtT-20 cells would adhere well to PECVD and especially to PECVD+B surfaces. This was based on the anecdotal knowledge that AtT-20 cells will adhere well to glass coverslips whereas other cell types such as rat hippocampal neurons will not adhere to glass without the prior application of PDL. PECVD surfaces were

assumed to be similar to glass for the purpose of cell culturing and boron doping was thought to give the surface similar charge characteristics. However previous comparisons of between PECVD and glass on compatibility with rat hippocampal cells were not conclusive. The dislodging of cells during mounting of the devices together with the qualitative observation that cells did not appear to be well adhered to the surface in many cases and the apparent low densities of cultures compared to those on glass coverslip controls all indicate a problem with surface adhesion. This may be remedied by switching to boron-doped thermally oxidised surfaces, which were previously found to be the most "preferred" surface of those available. This observation led to further experiments where AtT-20 cells were cultured on the different available surfaces. These experiments will not be reported here as they were not carried out by this author.

The dislodging of cells obviously indicates that the greatest care should be taken when handling the devices, however the cells may also have been dislodged due to slight fluid pressure from the rear of the device as it was clamped down onto the rig for recording. This issue may require further investigation.

Regarding the issue of intracellular solution in the patch aperture during culturing as discussed in section 5.1, both paradigms have disadvantages. If the patch aperture contains intracellular solution then even though the diffusion from the patch aperture into the cell culture is known to be slow it may still be that the small amount of diffusion that occurs could create a gradient of increasingly unfavourable ionic concentrations in the direction of the patch aperture which would inhibit cell growth towards it or damage cells that grow towards it. Any cell which grows over the patch aperture would have a significant and prolonged exposure to intracellular solution and this may be sufficient to trigger apoptosis. If the patch aperture contains growth medium during culturing then in preparation for patch-clamping the rear of the device can be exposed to a bath of intracellular solution for 30 minutes allowing medium in the large rear aperture to be replaced. However for practical reasons the device could not be exposed as above for the days necessary to replace the solution in the narrow upper channel of the patch aperture. The $\simeq 30\mu m^3$ of fluid remaining in the narrow channel would be unlikely to cause damage to the cell, the approximate volume of an average AtT-20 cell being 2 orders of magnitude higher. However it is possible that the remaining fluid could inhibit seal formation. There is support for this possibility in the use of potassium chloride solution to achieve whole-cell break through (e.g. Kostyuk 1975). Some commercial planar patch-clamping systems use brief suction to attract the cell to the patch hole (e.g. the *Q-Patch*

system by *Sophion Biosciences*) and this implies some extracellular solution in the patch aperture, however it may be that the diffusion dynamics of these chips are more favourable.

Beyond this issue, the prolonged exposure of the surface of the device to growth medium may be problematic. Proteins in the growth medium may bond to the surface of the patch aperture. This may alter the charge properties of the surface to be bonded to during patching, or alternatively it may effectively make the surface less smooth. Under one model of seal formation a separation of 1 nm or less between the membrane and the surface is required for gigaseal formation (Corey *et al.* 1983) and the bonding of variously sized proteins to the surface would open leak channels of greater than 1nm. Cells may also deposit proteins as they grow, and this may inhibit seal formation where a cell has already grown over the mouth of the patch aperture.

No conclusions can be drawn from the failure of the attempt to patch where a cell was over the aperture, since only one data point was achieved. However the result achieved is not inconsistent with the majority of the results which will be presented in section 7.

It is hoped that these devices may be cleaned and re-used, as the manufacturing cost per unit may be high. However the effect of cleaning procedures has not been adequately addressed. In particular it is not known whether the use of Piranha affects the surface roughness. The devices shown in Figure 14 were used for the first time whereas the devices in Figure 15 - Figure 16 were re-used. This experiment however was not designed to highlight any differences between new and re-used devices. Experiments to establish the effects of Piranha are therefore required, of the types described in section 3.4.

6 Lowering cultured cells onto the patch aperture

6.1 Introduction

Patch pipettes benefit from blunter tips than intracellular electrodes, so as not to penetrate the membrane (Rae *et al.* 1992 page 68). However a planar patch-clamp could be thought of as an infinitely blunt pipette and therefore presents much more of its surface to a cell than a standard glass pipette does. In previous experiments where snail neurons were lowered onto the patch aperture, it was thought that the failure of seal formation may have been at least partly due to debris lying near the aperture on the surface of the device. This may allow suction leaks around the mouth of the patch aperture and result in failure to draw in a bleb.

Therefore an alternative type of device was created, as described in section 3.3 above, where a nozzle surrounding the patch aperture protrudes from the surface, making the profile presented to the cell more similar to a standard glass pipette (see Figure 5 left). Such a device is unlikely to work where cells are to be cultured directly on the surface of the device, as the nozzle would act as a barrier to a growing or migrating cell. However it may work in the alternative paradigm where a network of neurons is cultured on a separate substrate and then inverted and lowered on to the device.

A previous attempt to test this approach involved culturing AtT-20 cells on a glass coverslip and then attaching that coverslip to an arm held by a micromanipulator, as shown in Figure 18. However it had proven difficult to align a cell with the device due to obstruction of the microscope. It was also difficult to get the coverslip in the same plane as the device so that the cell would come into contact with the nozzle.

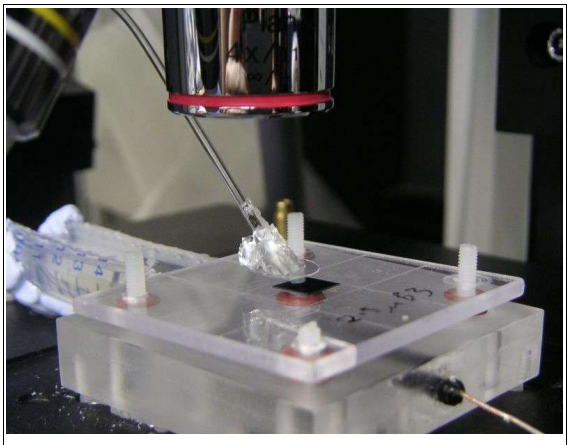

Figure 18 Apparatus for previous attempt to lower coverslip of AtT-20 cells onto nozzle device.

In this section the results of two alternative approaches are presented.

In the first approach, a PDMS (Sylgard) cone was created which would fit over a microscope lens, so that a glass coverslip could be attached to the end, held exactly in the focal plane of the lens, as shown in Figure 19.

In the second approach, cells were cultured on thin strips of acetate. These strips were gripped by a crocodile clip attached to a micromanipulator. Then the strip could be manoeuvred down to the surface of the device, flexing where necessary to achieve close contact, as shown in Figure 20.

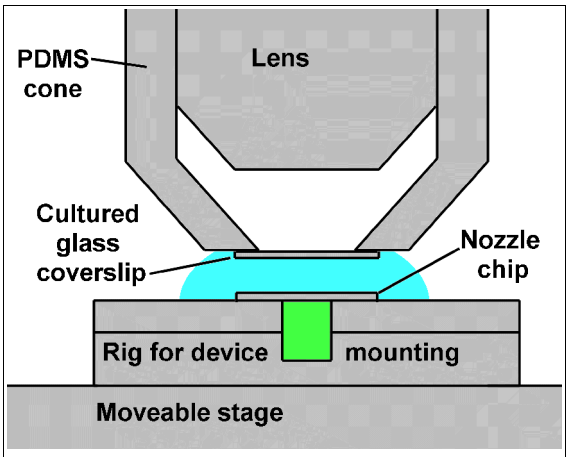

Figure 19 Apparatus for lowering glass coverslips onto nozzle device. Not to scale.

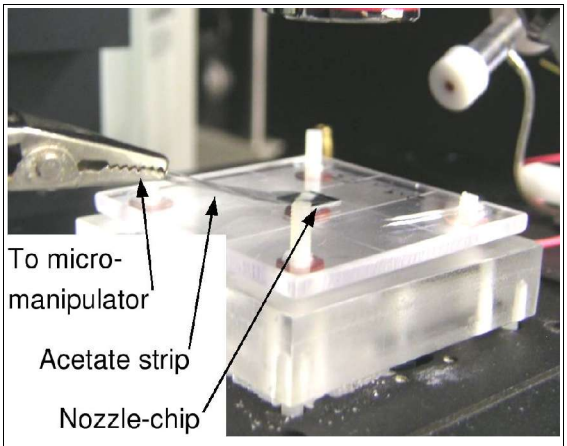

Figure 20 Apparatus for lowering cultured acetate strip onto nozzle device.

6.2 Materials and methods

Devices

Type III nozzle devices were used. For the coverslip method the devices were of aperture diameter ≃1.5µm and for the acetate method the aperture diameters were ≃4µm.

Chips from the wafer which was intended to yield the smallest aperture diameters had their apertures precisely measured. An SEM (Philips XL40 FEG) was used together with its bespoke software. For each aperture vertical and horizontal on-

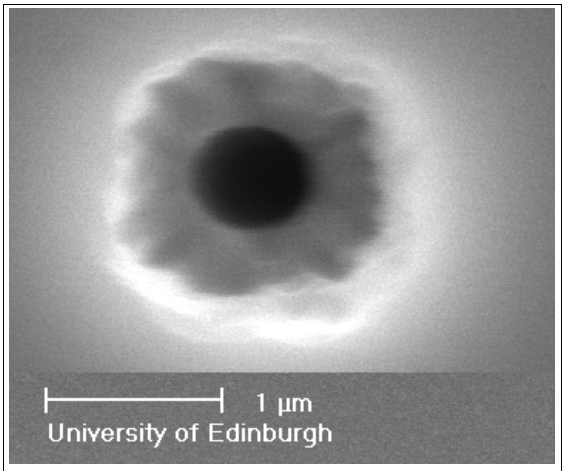

Figure 21 SEM image of device whose nozzle is malformed.

screen measurements of the diameter were made and the mean was calculated. Defective devices were also eliminated at this stage. For example, Figure 21 shows a device where the nozzle is malformed.

Cell culturing

Glass coverslips were cultured with AtT-20 cells, as described in section 4.2. Acetate sheets were partially cut into strips and placed in Petri dishes. Thereafter they were treated as glass coverslips in all respects for the culturing of AtT-20 cells.

Patch-clamping

These experiments used the rig configuration shown in Figure 12 with devices pre-mounted onto perspex frames. The light source was as described in section 5.2. In other respects recording equipment was as described in section 4.2.

For manoeuvring coverslips a PDMS cone was moulded as shown in Figure 19 following standard procedures for the use of Sylgard (section 4.2). The cone has a smooth lower surface with a circular hole in the middle of diameter $\simeq$5mm, so that the outer edge of the coverslip contacts the PDMS surface whilst the central area of the coverslip is exposed to the lens and held exactly in its focal plane. Glass coverslips were attached to the end of the cone either with surface tension or with the use of Vaseline. The cone was placed over the microscope lens, and the coverslip was quickly lowered into the extracellular bath taking care to keep the cultured side moist with extracellular solution at all times. The coverslip was brought close to the surface of the device so that the patch aperture could be located. A promising cell was identified and the platform holding the rig was manoeuvred so that the patch nozzle was directly underneath the cell. Then the microscope was lowered bringing the coverslip closer to the nozzle until a deflection was observed. Thereafter the patch-clamping attempt proceeded as described in section 4.2.

Cultured acetate strips were cut from the cultured sheet as required, gripped in a crocodile clip attached to a micromanipulator and quickly manoeuvred into the extracellular bath, taking care to keep the cultured side moist with extracellular solution at all times. The microscope was used to locate the patch aperture and a promising cell on the acetate strip. The micromanipulator was used to manoeuvre the cell over the nozzle and to lower the cell until a deflection was observed. Thereafter the patch-clamping

attempt proceeded as described in section 4.2.

Statistical analysis

Maximum seal resistances obtained from each patch-clamping attempt with a device were compared to data from standard AtT-20 patch-clamping (presented in section 4.3). Data points were drawn from the control data which matched the devices used by patch aperture diameter. Seal resistances obtained from patch clamping do not appear to be normally distributed (see Figure 9, note the log scale). Therefore data points from the two methods (standard versus planar) were ranked by maximum seal resistance. These two samples were compared with a two-tailed Mann-Whitney Rank Sum test.

6.3 Results

Upside-down coverslip

Several attempts were made and 4 devices were used. In most cases it was not possible to bring the cell down close enough to the surface of the device to contact the nozzle. Figure 22 left shows such a situation. The cell is in the focal plane however the surface of the device is out of focus indicating that it is several microns removed from the cell, with the microscope and thus the coverslip already lowered as far as possible. This could have been either because the coverslips were slightly concave by manufacture or because

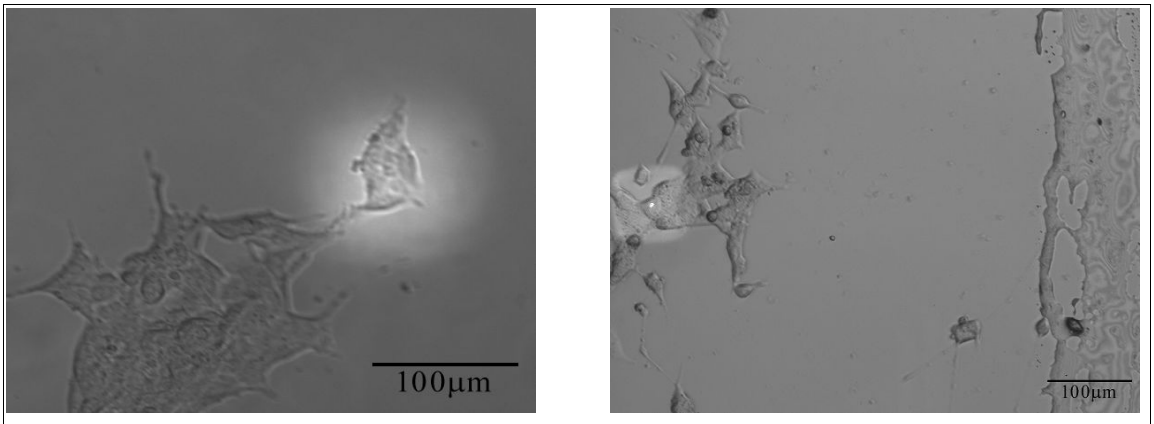

Figure 22 Left: Cell on coverslip (in focus) positioned over patch nozzle (out of focus). Right: Cell in closer proximity to nozzle (to the right is the Vaseline used in adhering the coverslip to the cone).

surface debris or perhaps PDMS lips around the edges of the devices prevented the coverslips from hinging down further, however no such problems were apparent to the best inspection available.

In four cases however, deflections were achieved. In one of these cases the cell detached from the coverslip and therefore this data was rejected. In the remaining cases the resulting seals achieved were 5, 25 and 50MΩ, (mean: 27MΩ; s.d.: 23MΩ) with devices of aperture diameters 1.55, 1.65 and 1.7μm respectively.

These three data points were compared with data from standard AtT-20 patch-clamping (presented in section 4.3). Given the aperture diameters of the devices used, the results of standard patch-clamping experiments where the resistance indicated (by the formula presented in section 4.3) that the pipette tip diameter was within two standard deviations of the mean device aperture diameter were taken (12 data points). These data points gave mean 143MΩ and s.d. 167MΩ. These two samples were compared with a Mann-Whitney Rank Sum test, ranking results according to the maximum seal resistance achieved, and found to be significantly different (U_1=2.5, n_1=12, n_2=3, two-tailed, $P<0.05$).

Upside-down acetate strips

On the first attempt to culture cells on acetate the culture was sparse in comparison with coverslips cultured at the same time. Most cells did not seem to be well adhered to the surface Figure 23 right. The cells in Figure 23 left seem healthy and are extending neurites. These were exceptional however they demonstrate that it is possible for AtT-20 cells to grow on this surface. On two subsequent attempts to culture on acetate, gas bubbles formed on the acetate and it floated to the surface of the growth medium while in the incubator. The top surface of the acetate dried out so that any cells growing on it would have died.

Positioning of cells over nozzle could be repeatably achieved providing that cells were close to the edge of the strip. Two attempts at patch-clamping were made, corresponding to the two sets of cells shown in Figure 23. Deflections were observed but on suction the cells disappeared into the patch apertures. On this occasion devices with aperture diameters of ≃4μm were being used because the conclusion in section 4.4 that these apertures were too big had not been reached at the time.

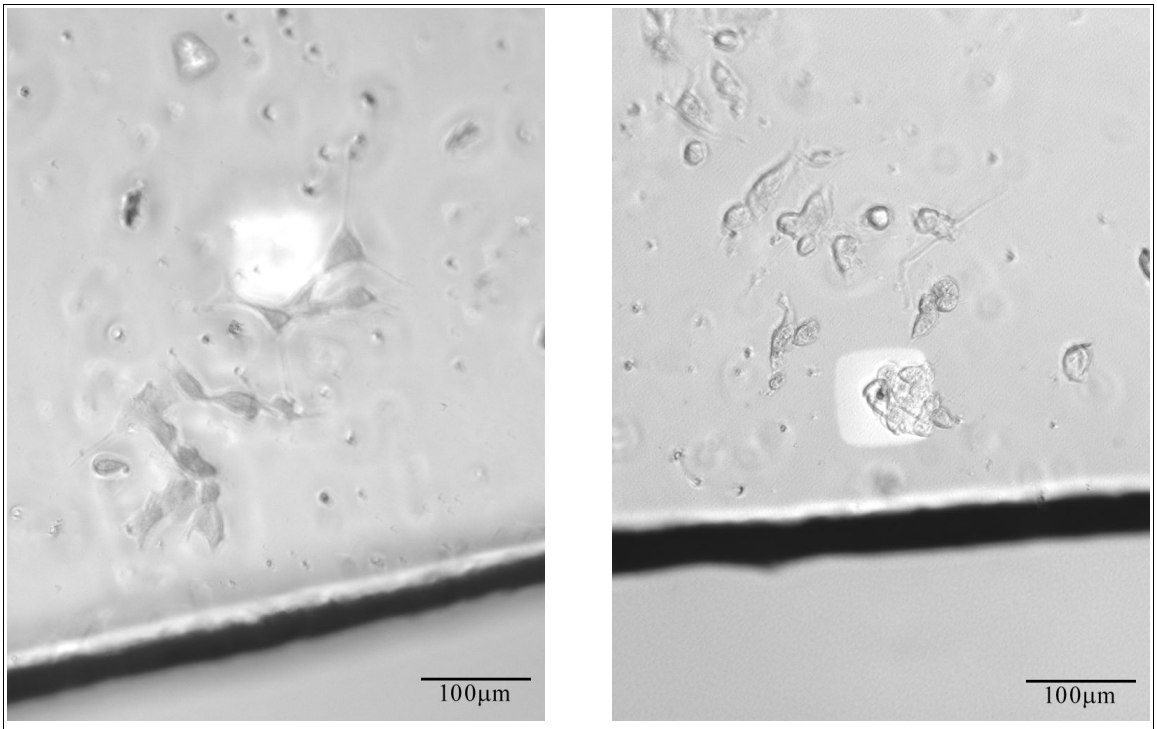

Figure 23 Cells cultured on acetate strips. The dark lines are the edges of the strips. Left: the cells seem healthy and are extending neurites (this was exceptional). Right: The cells do not seem well stuck down. A clump of cells is positioned over the aperture.

6.4 Discussion

Upside-down coverslip

Trying to bring these flat hard surfaces together is difficult in practice, and this difficulty is predictable. Given that the minimum distance from the nozzle to the edge of the device is 3.25mm and that the height of the nozzle is 2μm, and assuming that an average AtT-20 cell stands ≃10μm high off the surface it is growing on, the coverslip cannot afford to be more than 0.21° off parallel with the device if the nozzle is to contact the cell. The use of a PDMS base for the coverslip is an improvement over the previous method (section 6.1) because if one side of the coverslip touches the device we would expect the PDMS to give and the coverslip to hinge down over the device. However surface defects or debris could easily introduce the few microns of error necessary to prevent contact.

Even if this technique can be made to work for a single cell it is unlikely to scale to a multi-nozzle device, because cells will have different heights and will contact their intended patch nozzles at different points in the lowering process. Cell membranes have a certain amount of "give" but can easily be damaged if their thresholds for being manipulated are exceeded. One interesting possibility is that neurons could be grown on a bed of glial cells. Then any compression the cells experience may be shared with the glial cells below, possibly reducing the damage caused.

Where cells have been brought into contact with the nozzles the initial results have been promising with some sealing being achieved beyond initial deflections. Quantitatively these results are inferior to the control data from patch-clamping with glass pipettes on AtT-20 cells. However the actual number of trials is very low and further testing would be warranted.

Upside-down acetate strips

The problem of the acetate sheet floating to the surface during incubation may be remedied by somehow weighing it down in the Petri dish. The origin of the gas is a cause for concern as it could be a by-product of the type of acetate used. Trials with a range of different acetates should be considered. This may also help to resolve the problem of sparse cultures and low adhesion. Another possibility would be to coat the acetate with PDL prior to culturing to improve cell adhesion.

For ability to manoeuvre cells over the aperture, this technique had advantages over the coverslip technique. However although the acetate will flex at a macro scale, local deformities in the surface of the acetate may still prove problematic. Greater problems are envisaged when attempting to scale up to the use of a multi-nozzle device, as for the coverslip technique. Furthermore in this system the surface the cells are on bends and thus the cells are distorted, albeit only slightly. The possible effects of this on the health or behaviour of the cells has not yet been researched.

The use of devices with large aperture diameters confounded these results. Further testing with devices with small apertures is required.

7 Lowering dissociated cells onto the patch nozzle

7.1 Introduction

Previous attempts to patch-clamp with devices had used dissociated snail neurons individually positioned over the patch aperture. Given the need to test type III devices with nozzles, and given the availability of a large number of these devices with aperture diameters of above the size deemed necessary for using AtT-20 cells it was decided to use snail neurons for testing the new devices.

These experiments were designed to give better control data than all previous attempts, by the following means. Firstly the glass pipettes used were fire-polished. This was partly to improve success (Rae *et al.* 1992, page 69) and partly because the smoothness of fire-polished glass was considered a better control for the TO(+B) surfaces being tested. Secondly, standard patch-clamping was attempted on each day alongside planar patch-clamping experiments and using neurons from the same dissection, in order to remove variation from the health of the snail and the treatment of the cells, and other ambient factors which may vary from day to day. The pipettes used were matched by tip diameter to the devices used. Thirdly, cells were labelled with a fluorescent marker to indicate those which were alive following dissociation, in order to eliminate this major confounding factor. Finally, cells to be patch-clamped by the standard technique were allowed to stick to PDL-coated Petri dishes and cells to be patch-clamped by devices were picked up and manoeuvred by large fire-polished pipettes. This was to anchor the the cells more firmly whilst patch-clamping with the respective techniques, and to allow the pipette or nozzle to be slightly pressed into the membrane prior to suction.

7.2 Materials and methods

Pipettes

Pipettes were pulled as described in section 4.2 and then fire-polished. To measure the tip size of pipettes prior to use, a 20ml syringe was fitted with a plastic tube. Newly prepared pipettes were attached to the end of the tube and the tip was immersed in 100% ethanol.

The syringe was depressed until air bubbles came from the tip, and the reading on the syringe was taken – this is the "bubble number" (Standen *et al.* 1992 page 70).

A control experiment was carried out in which pipettes of various sizes were pulled. Their bubble numbers were measured as above and their tip diameters were measured as described in section 4.2. A linear regression of 1 / pipette diameter on bubble number gave a means of establishing which pipettes were of appropriate tip diameter after fire-polishing. This allowed pipettes to be adequately matched by tip diameter to the devices. For standard patch-clamping attempts carried out alongside devices with aperture diameters of 3µm pipettes had diameters of mean 3.3µm, s.d. 0.5µm, and for devices with aperture diameters of 2.5µm pipettes had diameters of mean 2.6µm, s.d. 0.5µm.

Snail dissection

Great Pond Snails (*Lymnaea Stagnalis*) (from Blades Biological, Kent) were kept in a freshwater tank at room temperature and fed *ad libitum* with lettuce. A Petri dish was prepared with a layer of cured Sylgard. A snail was de-shelled and placed in the Petri dish with extracellular solution containing (in mM): 30 NaCl, 10 methyl sulphuric acid sodium salt (NaCH3SO4), 10 HEPES, 5 sodium hydrogen carbonate (NaHCO3), 5 D-glucose, 4 CaCl2, 1.7 KCl, 1.5 MgCl2. The solution was then buffered to pH 7.8 with NaOH. (van Soest *et al.* 1998). The ganglia were dissected out and pinned to the Sylgard, as shown in Figure 24. The extracellular solution was replaced with extracellular solution containing 2mg per ml of trypsin. This was applied for ≃30 minutes while the outer membrane of the ganglia was dissected away. The trypsin was then flushed out by an exchange of solution, and was replaced with extracellular solution containing 2mg per ml of ovomucoid (a trypsin inhibitor). This was then flushed out by several exchanges of solution. The ganglia were removed to an Eppendorf tube with a Gilson pipette with approx 200µl of solution. This was triturated about 40 times until the ganglia had visibly dispersed. In a subsequent

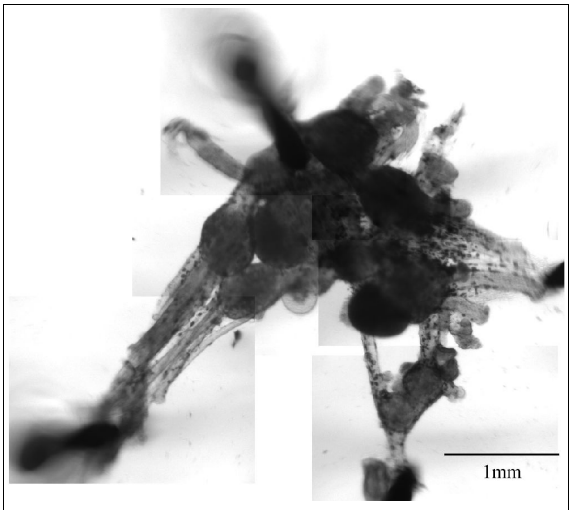

Figure 24 Photo montage from snail dissection, showing central nervous system pinned out in Petri dish.

improvement to this procedure, trituration was carried out in 3 shorter steps with the removal of solution each time, in order to dissociate the largest cells without damaging them.

Cell Tracker Green (CTG) is a fluorescent probe which is taken up through the cell membrane and fluoresces only after reaction with cytoplasmic thiols, indicating the integrity of the cell. This was added to the cells and allowed at least 30 minutes to be taken up.

Patch-clamping

One dissection was carried out per day of testing. Cells dissociated by the above method were used on the same day as dissection, within 8 hours of the start of dissection. Cells in extracellular solution were placed in a Petri dish plated with PDL and allowed to settle. On days where at least one device trial was possible these were followed by standard patch-clamping attempts on cells in the Petri dish.

For standard patch-clamping attempts, healthy cells were identified with epifluorescence by viewing them under light of the excitation wavelength of CTG of 492nm from a monochromatic source (Polychrome IV, TILL Photonics). Pipettes were back-filled with intracellular solution which consisted of (in mM): 64 KCl, 11 EGTA, 10 HEPES, 2.3$CaCl_2$, 2 NaCl. The solution was buffered to pH 7.4 with KOH (adapted from van Soest *et al.* 1998). In all other respects these patch-clamping attempts used the same equipment and proceeded as described for AtT-20 cells in section 4.2.

For device patch-clamping attempts, type III nozzle devices were used, with diameters of either $\simeq$2.5μm or $\simeq$3μm. These devices had been through an additional thermal oxidation process and therefore the apertures may have been slightly squarer than normal. Devices were pre-mounted onto perspex frames as described in section 5.2. They were then mounted on the rig using the configuration shown in Figure 12 though with the well empty of solution, and positive pressure was applied to test for leaks in the PDMS seal. The devices were then unmounted and filled with the snail intracellular solution described above using the method described in section 5.2. The devices were re-mounted on the rig with the well filled with intracellular solution and were then observed under the microscope to check for air bubbles in the well. Furthermore suction was applied followed by positive pressure, and where this led to a lasting change in the circuit

resistance, this was taken as a sign of air bubbles in the well, in which case the mounting step was repeated. It was also necessary in some cases to vacuum the filled rig (as described for devices in section 5.2) in order to de-gas the intracellular solution to assist mounting. Where devices were mounted with a stable and appropriate circuit resistance, a small amount of cells in solution was applied to the top surface, and the cells were allowed to settle for $\simeq$5 minutes before more solution was carefully applied.

The rig was connected to a headstage as described in section 5.2 and in other respects recording equipment was as described in section 4.2. Additionally a large fire-polished pipette was back-filled with intracellular solution, and mounted to a headstage (unattached to the recording system) on a micromanipulator. This was used to pick up a cell and place it over the patch nozzle, by the following procedure. Firstly a healthy cell was identified by viewing under the excitation wavelength of CTG as described above. Figure 25A shows two cells on the surface of a device. One of these is fluorescing and is therefore considered to have survived dissociation. The pipette tip was brought to the cell membrane and suction was applied (Figure 25B-C). The pipette was lifted up taking the cell with it. The platform on which the device was resting was manoeuvred so that the cell was over the nozzle (Figure 25D-E). The cell was lowered onto the nozzle until a deflection was observed. Thereafter suction was applied and the patch-clamping attempt proceeded as described in section 4.2. Note that the photographs Figure 25B-E show pipettes with relatively small tips, however to achieve the results presented below, larger pipettes were used, for example Figure 25F, so as to firmly anchor the cells during patch-clamping.

Statistical analysis

Maximum seal resistances obtained from the first patch-clamping attempt with each device were compared with maximum seal resistances from the standard patch-clamping controls. Data points from all dissections were pooled to yield two samples, standard versus planar patch-clamping attempts. Seal resistances obtained from patch-clamping do not appear to be normally distributed (see Figure 9, note the log scale). Therefore data points from the two samples were ranked by maximum seal resistance. These two samples were compared with a two-tailed Mann-Whitney Rank Sum test.

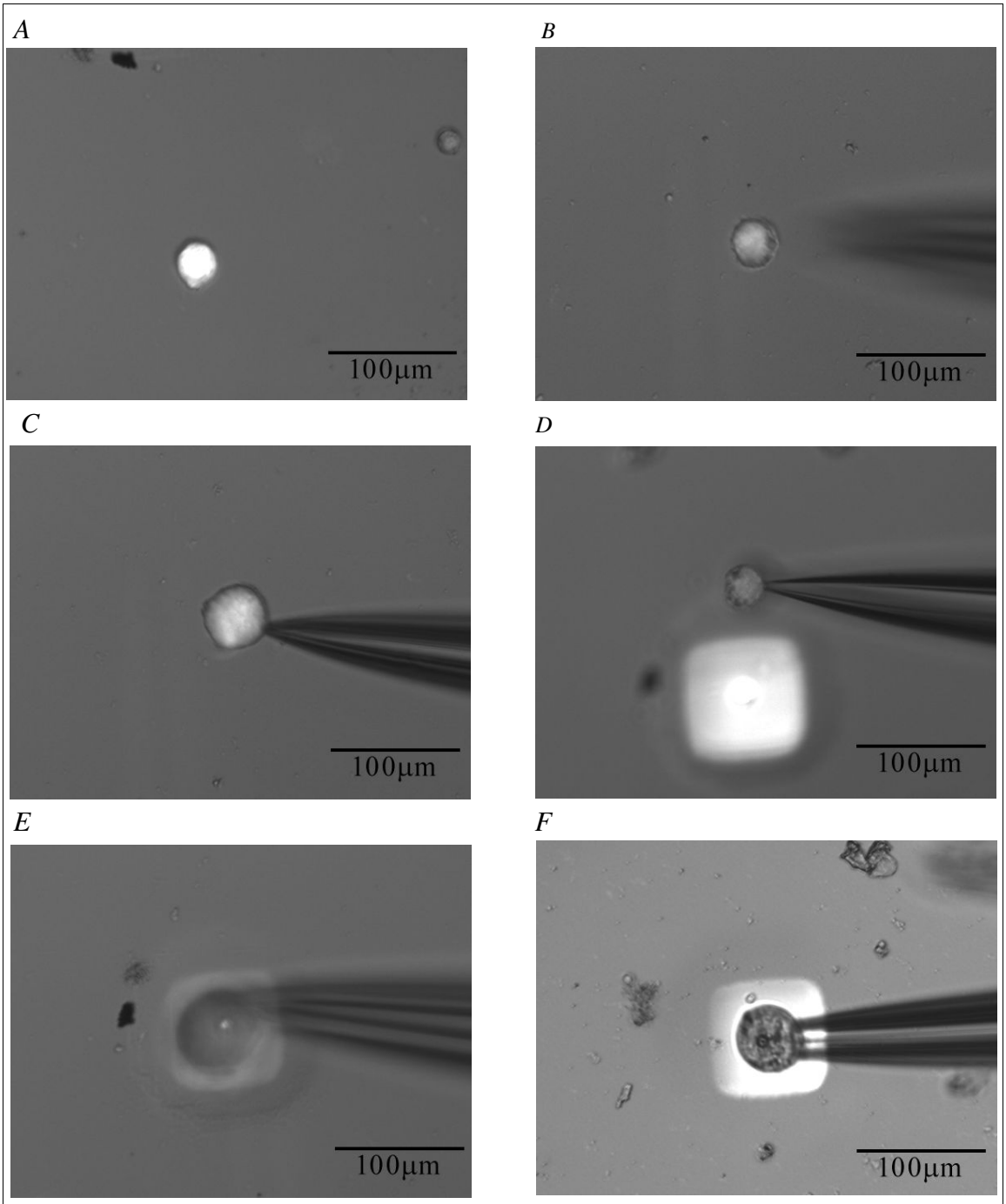

Figure 25 Method for lowering dissociated cells on patch aperture (photos B and D are of the same cell). A: Dissociated cells which have been allowed to settle on the surface of the chip. Some are fluorescent indicating that they are alive (centre), whereas others do not (right). B: A suitable cell is chosen. C: The pipette is brought to the membrane and suction is applied. the cell can then be lifted up. D: The pipette is brought to the patch aperture. Note the loss of fluorescence compared to B. E: The cell is lowered onto the nozzle until a deflection is observed. F: An improvement of technique was to use larger fire-polished pipettes for manoeuvring cells. These cells could be lowered more firmly onto the nozzle.

7.3 Results

A batch of 17 devices were used. Problems with the PDMS seals around the devices reduced the usable yield to 8 devices and a further attempt was rejected due to persistent air bubbles below the device. Results from days where no planar patch-clamping attempts could be completed have been discarded. In order to increase experimental throughput, most devices were used for two patch-clamping attempts on different cells. However for the following quantitative analysis only the result of first attempt for each device is included. The results are summarised in Table 2.

Table 2

Dissection	**Aperture diameter of planar devices (μm)**	**Planar devices Maximum seal resistance (MΩ)**	**Glass pipettes Maximum seal resistance (MΩ)**
1	3	2 0.25	*125 20 15
2	3	2 0.25 0	120 100 30
3	2.5	5	90 40 1
4	2.5	15	*80 20 15 3

** On these attempts sealing was followed by signs of whole-cell breakthrough.*

Data points from all dissections were pooled to yield two samples, standard versus planar patch-clamping attempts. Standard patch-clamping results had mean 51MΩ s.d. 28MΩ, whereas planar patch-clamping results had mean 4MΩ s.d. 5MΩ. Data was ranked

according to maximum seal resistance achieved and a Mann-Whitney Rank Sum test was performed, which confirmed the significance of this difference ($U_{pipette}$=6.5, n_{device}=7, $n_{pipette}$=13, two-tailed, P<0.01).

Electrical recordings of sealing attempts (sealing traces) are presented for discussion. Figure 26 is an example of sealing with a glass pipette. This example is from an attempt on a day where no results with devices could be achieved. It went on to yield the best whole-cell recordings, an example of which is shown in Figure 27. Figure 28 is a typical sealing trace for a device (from the second attempt with the device used in dissection 3). Figure 29 is the most successful attempt at patch-clamping with a device in this batch of experiments, from the first attempt with the device in dissection 4.

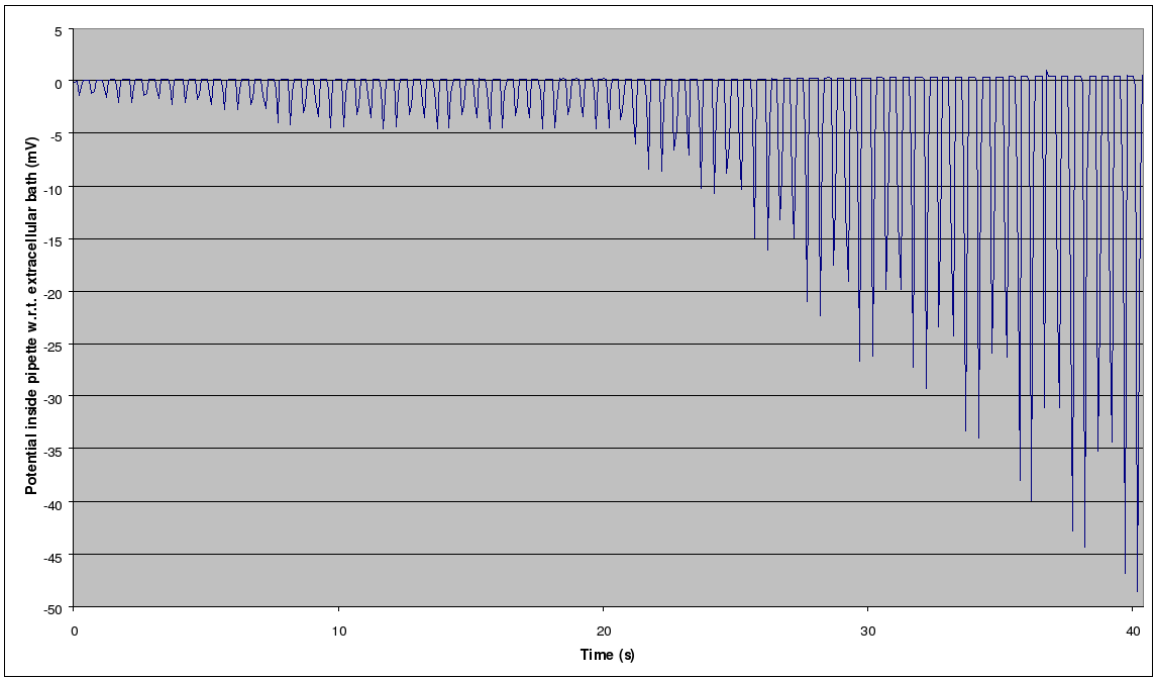

Figure 26 Example sealing trace with glass pipette. There is an intermittent square pulse of 5nA every 500ms for 100ms which was not varied for the duration of this trace. Data initially sampled at 62.5kHz then resampled at 12.5Hz, yielding voltage responses which appear spiked. The pipette has been touched to the membrane prior to the start of the trace and is advanced further at ≃8s. Suction is applied at ≃20s and resistance starts to rise. By the end of this trace there is a voltage response of ≃50mV which indicates a seal resistance of ≃10MΩ. On this occasion, the resistance continued to rise steadily to reach ≃200MΩ. (This patch-clamping attempt was performed by Dr John Curtis).

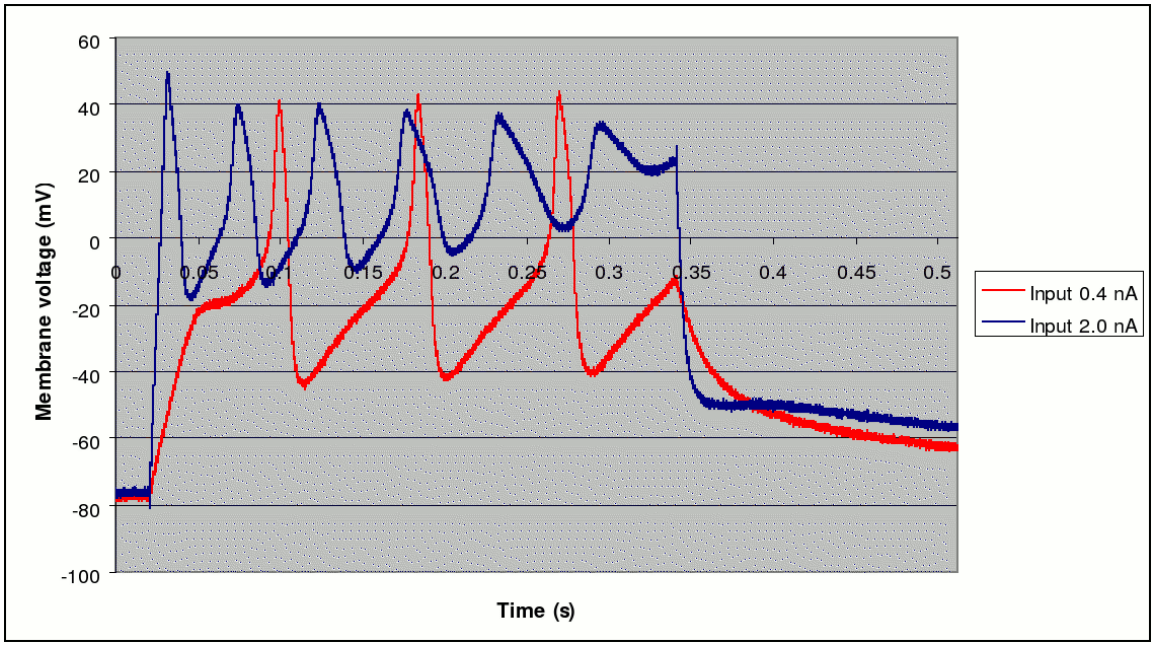

Figure 27 Recordings obtained from a snail neuron of spike bursts in response to current pulses. The pulses were applied from 0.02s to 0.34s. Note the faster spike rate in response to a higher current input.

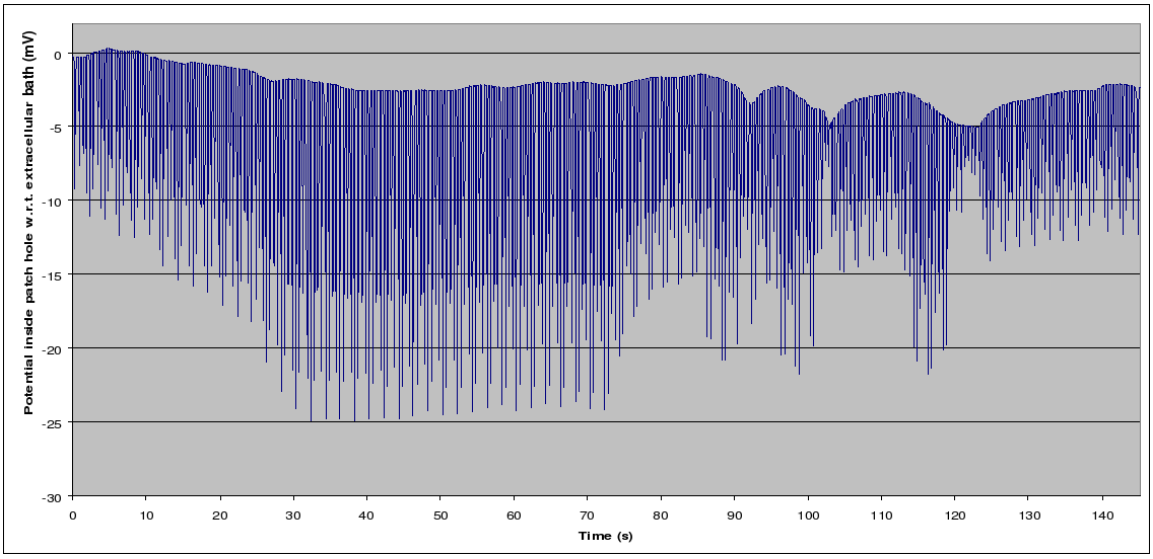

Figure 28 Example sealing trace for device. There is an intermittent square pulse of 5nA every 500ms for 100ms which was not varied for the duration of this trace. Data initially sampled at 62.5kHz then resampled at 12.5Hz. Around the beginning of the trace, suction is applied and resistance rises. After an initial rise, no further growth occurs and when suction is released at ≃75s resistance reduces to where it started. Further attempts to apply suction, at ≃85s, ≃95s and ≃115s also fail to initiate sealing. Note that with devices the baseline potential tends to "wander"more than in standard patch-clamping.

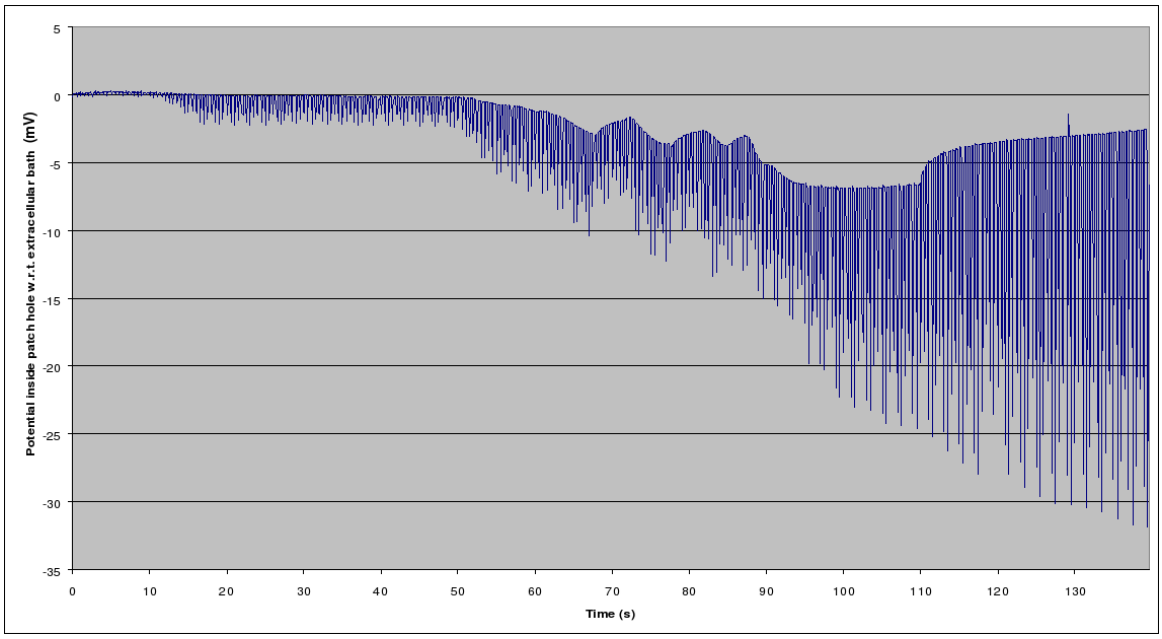

Figure 29 A solitary example of a sealing trace for a device where a phase of seal formation occurred. There is an intermittent square pulse of 5nA every 500ms for 100ms which was not varied for the duration of this trace. Data initially sampled at 62.5kHz then resampled at 12.5Hz. Cell is touched to nozzle at ≃10s. Suction is applied at ≃50s, initiating a rise of resistance. When suction is released at ≃110s seal formation continues. By the end of this trace the voltage response is ≃30mV, indicating a resistance of ≃6MΩ. On this occasion seal formation continued to a maximum of ≃15MΩ. (This patch-clamping attempt was performed by Dr John Curtis).

7.4 Discussion

The experimental yield was reduced by frequent failure of the device mounting technique described in 5.2. The method for creating a seal around the edge of the device needs to be improved in order to raise experimental throughput. Possibilities are successive layers of PDMS or an alternative bonding agent.

Gigaseals were not achieved by either planar devices or glass pipettes. However there is an obvious difference in the quality of sealing achieved between planar devices and glass pipettes and this difference is statistically significant, although the number of trials remains low.

A qualitative analysis is more informative. Although the actual seal resistance achieved by the nozzle devices varies, all the seal resistances from nozzle devices in Table 2 are loose-cell patches. In standard patch-clamping, the application of suction immediately

invokes a phase of seal formation which can be immediate or can take in the region of seconds or minutes in which the seal resistance rises rapidly. This can be seen in the example trace in Figure 26. During this phase a bleb is forming, as described in section 2.1. In most attempts to patch-clamp with a nozzle device it was possible to observe a "deflection" as the cell contacted the nozzle. However this deflection was usually notably smaller than for a glass pipette and it was often not possible to observe a "bounce" when briefly applying positive pressure. Furthermore suction would mediate a temporary increase in resistance but when suction was released the resistance would decrease again, indicating either that bleb formation had not been initiated or that the bleb had started to form but did not start to seal against the inside surface of the aperture.

One possible explanation is that force due to pressure is not being properly transmitted through the patch aperture. Movement of free-floating debris in the extracellular solution towards or away from the patch aperture has been observed but it is unclear whether the force being transmitted due to pressure is of the same strength as is the case with glass pipettes. Other possibilities are surface roughness or debris and unfavourable surface charge. It should also be noted that these devices had apertures with a certain inherent squareness and that the squareness was enhanced in this case due to an additional thermal oxidation to reduce their size. Such a problem might be avoided in a future round of manufacturing.

It was noticed that snail neurons would stick very firmly to the surface of the device, to such an extent that it was often difficult to then pick it up. This could mean that the cell membrane is being torn in the attempt to pick up the cell. Notice that the cell in Figure 25D is fluorescing less brightly than before it was picked up (Figure 25B). This may indicate that the contents of the cell have been partially dilated out through a rip in the membrane.

It should be noted that in one case, a phase of sealing did occur. This probably indicates that the inability to form seals is not fundamental. However it is possible that the increased resistance observed was not due to bleb formation but rather to an improved contact of the cell membrane with the nozzle.

8 Conclusions

Each of the methods tried for patch-clamping with devices has practical problems which may be to blame for the respective failures to achieve gigaseals. Furthermore the actual number of device trials achieved by any method remains low. However by considering the entire track record of testing it can be seen that there is an apparent difference in the ability of the devices to form seals compared to that of standard glass-pipettes. The results presented in section 7.3 are the clearest indication so far of this difference, and the inability to achieve gigaseals by any method so far tried supports this conclusion. It may be that there is a fundamental problem with the design of the devices which will prevent them from forming seals; the most likely problem is insufficiently smooth surfaces. However it may be that the experimental procedures all allow for debris to contact the patch aperture and prevent seal formation. This may be improved upon. In the case of positioning dissociated neurons, an alternative method for dissociating cells which removes extracellular debris may show an improvement. It is also still possible that all patch-clamping attempts failed due to confounding factors in the experimental design other than problems at the patch aperture. For example, in the method of lowering dissociated neurons the possibility of cells being damaged during lifting has not been eliminated.

Project directions

This project may proceed by one of three means.

1) The devices thus far manufactured are used again with methods having been improved by the work presented in this report. In this case:

    a) The question of the effect of cleaning on surface roughness and biocompatibility needs to be investigated first (this is in fact underway). The question of whether the current method of evaluating surface roughness with an AFM is sufficient to judge the level of smoothness necessary for successful patch-clamping should also be addressed.

    b) Only the type III devices with diameters of $\simeq 1.5\mu m$ should be used and the rest should be rejected. This is because of the finding that AtT-20 cells (the cell type more similar in size and probably in membrane composition to the types

of cells likely to be used with the final product) seal more easily with pipettes of this size and below.

2) New devices are manufactured based on the knowledge now available. In this case:

    a) Aperture diameters of $\simeq 1\mu m$ should be created, the squaring effect of the TO surface treatment notwithstanding.

    b) Devices both with and without nozzles may be created. If however one method is to be prioritised, it is the opinion of this researcher that the paradigm of lowering cultured cells onto devices holds more promise and therefore nozzle devices should be created (this is discussed below).

    c) This work has not advanced understanding of surface treatment to be used, however it is the current opinion that the TO+B surface treatment holds the most promise and nothing herein contradicts this.

    d) The method of mounting devices on perspex frames with Sylgard needs to be improved. Possibilities might be a two-stage application of Sylgard, use of an alternative bonding agent, or manufacture of larger chips.

3) All aspects of device design are re-considered. Many of the technological aims which this project set out to achieve have been achieved by other research teams within the timespan of the project. However the commercial patch-clamping systems now existing will not necessarily lead to the development of multichannel recording systems because there may not be sufficient financial incentive. Given this, as much knowledge as possible should be obtained about the successes of alternative approaches. It may be for example that a hybrid approach with an oxygen-plasma-treated air-moulded PDMS array of patch apertures (developed from the work presented in Klemic *et al.* 2005) aligned over a silicon MEMS substrate for fluidic control and electrical recording could ultimately be more successful.

Preferred paradigm for use of final devices, and methods for testing devices

Regarding the supplementary hypothesis stated in section 3.2, it is the opinion of this researcher that the paradigm of lowering cultured networks of neurons onto devices,

especially those with nozzles, holds more promise than the alternative of culturing cells directly on devices. This however is a subjective judgement; both paradigms remain possibilities, as will be seen in the following discussion of the methods used for testing devices. All the methods tried have practical problems, however none can yet be rejected. The furtherance of each method will now be considered in turn.

Culturing cells directly over planar devices

To continue this line of testing there are two possible approaches. One is to bring together work on cell patterning to create devices with surfaces that guide the growth of cells towards the patch aperture, whereas the alternative is to continue using a dividing cell line and to increase the density of cultures in subsequent experiments. The second approach is the simpler and should be continued. The dislodging of cells from the mouth of the patch aperture prior to patch-clamping proved problematic. This problem might be eased by use of pre-mounted devices with self-contained solution baths, as described in section 5.2. More importantly however is the issue of adhesion of cells to devices. It should be noted that all the experiments described in section 5 were on type II devices with PECVD surfaces. Devices with TOB surfaces should be used in subsequent experiments, based on the current understanding presented in section 3.4. Notwithstanding this, the adhesion of cells to the surface of devices may be an issue. One possibility is to reconsider the cell line used. If AtT-20 cells are to be used it would be better to acquire a new batch of cells directly from a cell bank and to achieve good whole-cell recordings by the standard method before proceeding with this approach. Devices with smaller aperture sizes need to be used. The work presented here has led to improved procedures for culturing cells on devices, so further trials should be more successful at yielding cells growing over patch-apertures.

The possibility of solution not compatible with cytosol in the patch aperture preventing seal formation or whole-cell breakthrough remains a problem to be researched. It is also possible that the pre-filling of devices with intracellular solution and the gradual diffusion of this may inhibit the growth of cells over the aperture. Furthermore it is possible that as cells grow toward the edge of the aperture, the curvature of the surface could inhibit further growth in that direction. It is known that cells migrating along a surface can detect and react to surface features (Curtis *et al*. 2001). Different cell types may behave differently in these respects. Each of these possibilities may prove problematic for this approach and should be researched. The possibility of deposited

proteins or other cellular debris preventing seal formation is the problem which in the opinion of this researcher is most likely to cause this approach to fail.

Lowering cultured cells onto nozzle devices

Both of these techniques had some promise. It is the opinion of this researcher that using cultured acetate strips is a more promising technique than using cultured coverslips for the reasons stated in section 6.4 and should be prioritised. Investigation into the composition of acetate and the physiological effects of slight distortion on the cells should be carried out. Coating acetate with PDL prior to use is a strong possibility. Plating cells over a layer of glial cells should be considered.

Lowering dissociated cells onto nozzle devices

It is possible that cells are being damaged whilst being picked up by the pipette, due to sticking to the surface of the device. This possibility could be eliminated by an alternative approach where cells are allowed to settle on an alternative surface to which they do not adhere near the device in the same extracellular bath. In other respects this remains a promising technique. It should be noted however that this technique is only useful at this stage of testing. Ultimately a technique involving cells cultured on a surface must be made to work if this project is to succeed.

An alternative method

Some commercial patch-clamping systems work by having dissociated cells in suspension and applying suction through the patch aperture until a cell is sucked onto the patch aperture. It may be worth attempting this approach. Whilst this would create the problem of extracellular solution in the patch aperture it may still be a valuable approach simply for testing the ability of the devices to form seals. This would not work with dissociated snail cells achieved by the method described in section 7.2 due to the amount of non-cellular debris that is included in the suspension and the possibility of this debris blocking the patch aperture (Klemic *et al*. 2005 note comparable difficulties). However it may be made to work by using an alternative procedure for dissociation using centrifugation. This would also necessitate an investigation of the ability of the devices to transmit force through the patch apertures due to pressure, as discussed in section 7.4.

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