Integration of Pre-aligned Liquid Metal Electrodes for Neuronal Stimulation within an easy-to-assemble Microfluidic Platform

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Abstract

NICHOLAS HALLFORS**:** Integration of Pre-aligned Liquid Metal Electrodes for Neuronal Stimulation within an easy-to-assemble Microfluidic Platform
(Under the direction of Dr. Anne Marion Taylor)

Neural stimulation and recording techniques play a critical role in understanding the nervous system. Electrophysiological experiments on neurons may lead to new clinical treatments for chronic neurodegenerative disease, as well as acute neural injury. A major limiting factor in the study of neurons is the cost and complexity of existing suitable research platforms.

In this study, a low cost, easily fabricated research platform was developed for the study of neurons. Using basic photolithographic techniques and readily available materials, a compartmentalized microfluidic chamber with pre-aligned microelectrodes was developed. Neurons were grown in the devices and subjected to electrical stimulation, and fluorescence microscopy data was collected on neural response to stimulation and neural compatibility to the devices.

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Definitions and abbreviations

Soma: Neuron cell body

Dendrite: Neural appendage which receives information from other neurons

Glia: Neuroprotective cells, glia maintain homeostasis around neurons, as well as coat certain neurons in Myelin

Somatodendritic compartment: Region containing neuron cell bodies (soma) and dendrites

µf: Microfluidic

MEA: Microelectrode array

CNS: Central Nervous System

PNS: Peripheral Nervous System

CHAPTER 1

INTRODUCTION

Scientists have long known that electricity played a role in human physiology. Beginning with shamanic medicine using electric fish thousands of years ago, to modern deep brain stimulation and cochlear implants, electricity has provided a unique interface to what we now know as the central nervous system. The vast network of nerves in our body is composed of nerve cells, or neurons, which communicate with other neurons by two types of projections known as axons and dendrites. These projections send and receive chemical messages, carrying important information about our surroundings, our thoughts, actions and sensations. In some cases, these messages can be forced by externally applied electrical stimulation, producing a desired physiological outcome in a controlled manner.

1.1 Neuronal Physiology

Neuronal activity can be described as a combination of chemical and electrical events which result in signal transduction. This activity relies on different concentrations of ions in the cell and in the environment surrounding the cell. Three ions of particular importance for neuronal activity are Sodium (Na+), Potassium (K+), and Calcium (Ca2+). All three ions are present both inside and outside the cell, but at greatly different concentrations. The sodium concentration inside a neuron is much lower than the concentration outside, while the potassium concentration inside the cell is much higher than outside. Since the cell membrane is permeable to water, a diffusion gradient is created, whereby Na+ ions want to get into the neuron and K+ ions want to escape. The diffusion gradient of charged particles creates an electric potential, or a voltage across the cell membrane. Bound along the surface of the cell

membrane are a number of ion pumps, which actively maintain the diffusion gradient at the cost of energy.

As well as ion pumps, the cell membrane contains a number of selective ion channels, designed to only allow certain particles in and out, and only under certain conditions. While open, ions are allowed to flow down the diffusion gradient, and across the cell membrane producing an electric current. For the purpose of this study, voltage gated ion channels are of particular importance. The open state of these channels is determined by the voltage across the cell membrane. At rest, any given neuron will have a membrane voltage of around -90mV, calculated as the sum of the contributions from all ionic gradients.

As the cell membrane depolarizes, more voltage gated ion channels open, allowing positive ions into the cell and further depolarizing the membrane. Once the membrane voltage is raised above threshold, a rapid, binary event known as an Action Potential (AP) occurs. During an AP, a cascade of ion channel state changes rapidly occurs, resulting in a large spike in membrane voltage and current. APs are initiated at the axon hillock, propagating down the length of the axon and to the presynaptic terminal, triggering the release of neurotransmitter to induce an effect at the postsynaptic terminal. An AP can be thought of as a unit of neural activity, and can be induced externally by applying electric current via an electrode system.

1.2 Disease and injury

Neurons are complex, critical to our survival, and compared to other cells in our body, very vulnerable to damage and disease. Neurodegenerative diseases such as Alzheimer's and Parkinson's are progressive, debilitating and largely untreatable. Since the number one contributing factor to

neurodegenerative disease is age (1), understanding the pathology behind these diseases and their potential cures becomes ever more important in an increasingly elderly society.

During the infancy of neuroscience, neurons were believed to have no intrinsic regenerative capacity whatsoever. Ramon y Cajal's statement, "In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated." (2) is now being called into question, as there seems to be clear evidence that many neurons can in fact regenerate. (2)

Peripheral neurons in general have a greater capacity to generate than do central neurons(3). The sciatic nerve is a popular model in regeneration studies due to its strong regenerative properties, large size, and being relatively easy to access. Sciatic nerve studies have shown rapid hypertrophic responses to axon lesions, resulting in robust regeneration across the lesion site (3). This hypertrophy can be further enhanced by a conditioning lesion prior to total lesion(3). In contrast, central neurons respond to trauma in a manner inhibitory to regeneration, and in some cases, atrophic. Axon lesion results in glial scarring, sealing the damaged axon and preventing regeneration.(2)

One proposed method of enhancing neuronal regeneration is electrical stimulation. Many studies have shown improved regenerative outcomes from electrical stimulation in injury models (3). This improvement has been attributed to increased production of trophic factors such as BDNF, as well as growth factor receptors (3).

The vast majority of neuroregenerative work has been done in a peripheral nerve model because peripheral nerves are easier to work with. Peripheral nerves are much larger, and tend to have a clear origin and terminus. This means a researcher can easily attach an electrode to the sciatic nerve, with a priori knowledge of signal direction and destination. In the CNS, neurons are congregated as homogeneous tissues, like regions of the brain or spinal cord. Within this tissue, a single neuron could be sending projections in every direction. Attempting to penetrate this tissue to interface with target

neurons could cause significantly more damage than you would see from interfacing a peripheral nerve. To avoid the complexity associated with an in-vivo approach to CNS study, new methods have been developed to study CNS neurons in-vitro. Compartmentalized culture chambers, Microelectrode Arrays, and 3D cell culture scaffolds offer new platforms for understanding the nervous system in-vitro.

1.3 Learning and Plasticity

As neurons communicate over time, long lasting changes in signal strength along communication pathways appear. These changes are known as *Synaptic Plasticity,* and are believed to be the fundamental physiology behind learning and memory. Any time an external event is committed to memory, it is manifested as the plastic changes in the connections between many neurons.

1.4 Current methods of Electrical Stimulation

Patch-clamp

A Patch-Clamp electrode consists of a glass pipette with an extremely small open tip. Under a microscope, a researcher can touch the surface of a cell body with the pipette. Gentle suction will attach the pipette to the cell body on a region small enough to contain only a few ion channels. The pipette can be filled with an ionic solution similar to in-vivo extracellular environment, or changed to suit the particular experiment. A microscopic conductor inside the pipette can then pass current across the cell membrane, controlling the membrane voltage and cellular activity.

Impaling electrode

An impaling electrode penetrates through target tissue, or target cells, to bypass the significant electrical impedance of the cell membrane. This way, current can be easily passed into the cell, and

membrane voltage can be well controlled. In myoelectric stimulation for example, this method can evoke a significantly stronger muscular response than a surface electrode. However, impaling tissue has drawbacks which make it impractical for many applications, mostly due to the potential for tissue damage, or discomfort in live subjects.

Cuff/Ring electrode

Cuff electrodes consist of a series of individual conductors embedded in a flexible substrate which can then be wrapped around a target. By having a series of conductors, cuff electrodes can control the direction of propagation by the relative voltage of each conductor. As well as controlling the direction of propagation, cuff electrodes are able to block propagation by hyperpolarizing a specific region. This way, a cuff electrode is capable of evoking a one-way action potential and block unwanted back-propagation.

Microelectrode array (MEA)

Patterned onto glass slides or custom cell imaging platforms by vapor deposition or other metal patterning techniques, MEAs can be designed to suit a wide range of in-vitro research designs. MEAs are a commonly used in dissociated neuron cultures as a means of electrically interfacing with cells. They are able to both stimulate and record neuronal activity of cells adhered directly above their electrodes, and offer excellent spatial and temporal resolution. MEAs can be designed with hundreds or even thousands of electrodes to record from a large number of neurons simultaneously.

CHAPTER 2

DEVICE DESIGN AND EXPERIMENTS

2.1 Introduction

Axons, the long appendages through which neurons communicate, are particularly vulnerable to injury due to their small size and extreme length. The difficulty in separating out axons from the somatodendritic compartment and from glia has limited investigations of axonal biology. Microfluidic devices to isolate axons from somata and dendrites allow unique studies of axonal biology in an easily accessible format for experimentation. Axons have the intrinsic capacity to regenerate after injury, and it may be possible to enhance that regeneration with a proper treatment modality (2).

In microfluidics, we find a powerful tool to study central neurons at the individual level. Using an appropriate microfluidic (μf) device, we are able to isolate small groups of axons and synapses and specifically stimulate regions of interest to monitor behavior. In living systems, it is a challenge to sufficiently untangle, or even to map the network of neuronal connections so as to study a single synapse over a period of time. Studying neuronal polarity and its effect on synapse formation (4), single cell PCR (5), and the interactions of distinct cell types (4) relies on the ability to separate and observe single neurons over time.

Several groups have approached this problem by different avenues. Tooker et al. (6) devised parylene neurocages to trap and isolate single neurons on patterned electrodes. Pan et al. (7) make use of microfluidics in their approach to separate axons and cell bodies, incorporating commercially

available MEAs and microfluidic axon isolation to study unidirectional AP conduction. Here, we take a different approach: guiding synaptic formation into predefined channels stably aligned with stimulating electrodes.

By exploiting a procedure elucidated by Michael Dickey's group (8), we are able to combine microfluidic axonal growth platforms and inherently aligned microelectrodes for highly selective stimulation on easily fabricated devices. Gallium is a low melting point metal alloy with a native oxide layer or "skin" which mechanically stabilizes the metal in its liquid state, preventing it from diffusing into the cell media. These unique properties make Gallium ideal for our electrode material, and allow us to combine our entire test platform into a single PDMS chip.

2.2 Objectives

1 Grow neurons in specially designed µf devices

2 Demonstrate biocompatibility of liquid metal microelectrodes with axons

3 Electrically stimulate axons to induce AP

Justification

The nervous system is monumentally complex and sophisticated. Simplifying it to a level where individual connections can be studied will help with basic understanding of how connections are formed and changed over time. The majority of neuroscience research focuses on average behavior of a large number of neurons (9). To better understand the specific nature of overall activity, it is necessary to understand interactions between individual neurons. Results could be relevant to nervous system injury and disorders and their respective therapies, as well as better understanding the mechanisms behind neural plasticity, how we learn both physically and mentally.

Better methods are needed to investigate single neurons and synapses. Previous research has focused on average behavior for a large population of neurons. Such averaging may overlook important dynamics taking place at the level of individual neurons and synapses (2). Platforms designed to investigate small populations can provide insights not otherwise apparent. Even within a seemingly heterogeneous population of neurons, some cells respond to different cues (temperature vs chemical for example) so single neuron is critical to differentiate the two different behaviors (10). While single neuron studies do exist, they are labor and equipment intensive, and cannot be performed in a highly parallel manner (11).

Background

It has long been known that neural activity could be manipulated by electric current (12). Recent investigation has focused on electrically induced activity for therapeutic purposes (3). Studies have found that external nerve stimulation enhances intrinsic regenerative response of neurons (9), as well as offering a variety of long-term benefits to victims of spinal cord injury or brain damage (3). Deep brain stimulation has been successfully used to reduce tremors associated with Parkinson's, as well as treating severe depression. Cochlear implants have been used to restore hearing in acutely deaf individuals by directly stimulating the cochlear nerve, bypassing the tympanic membrane (11).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Design and fabrication of μf devices

The device consists of two main microchannels, connected by a series of smaller microgrooves wide enough for a few axons to pass through, but narrow enough to keep cell bodies out (Fig. 1). Two fluid electrodes connect to one of the microchannels to selectively stimulate any neurons contained within. Alternate configurations include electrodes which meet at a single microgroove, designed to stimulate only a very small population of axons.

Device design started with the original design described by Taylor et al. (13). From there, devices were modified to include two additional microfluidic channels into which liquid metal is flowed.

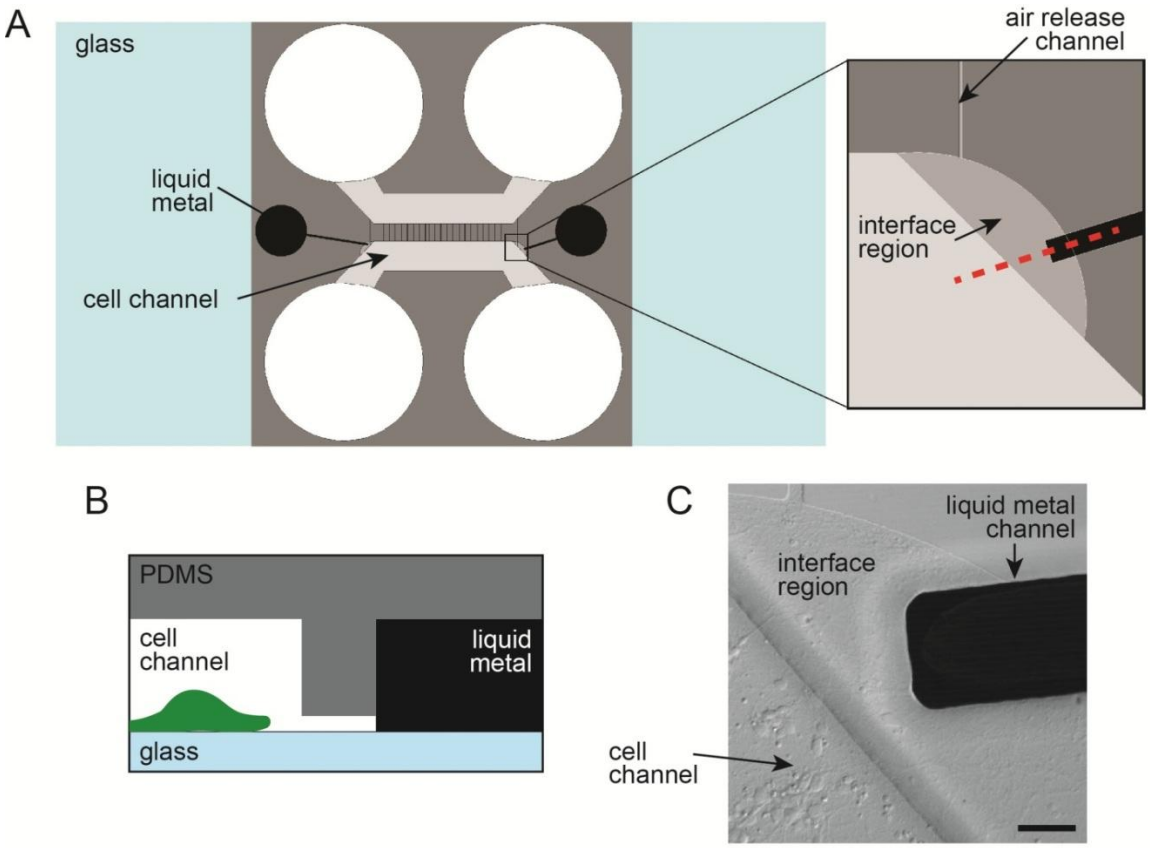

Fig.1 (a) Top-down illustration of PDMS device on glass coverslip. (b) Scaled side view illustration electrode terminal (rotated vertically 90 degrees away from the viewer). Electrode buffer region surface area is only 3% of the electrode surface area. Also visible is Cell body compartment, which is same height as electrode. (c) Wide field micrograph of electrode with neurons visible. (Scale bar= 50µm)

3.2 Electrode preparation and testing

The use of Gallium as a microfluidic electrode relies on its inherent ability to spontaneously form a stable oxide layer at its surface (8). This oxide layer mechanically stabilizes the metal, even in its liquid form, making it a non-Newtonian fluid. In order to make the liquid metal flow through a channel, enough pressure must be applied to break the oxide layer. The pressure required to break the oxide is inversely proportional to the cross sectional area of the channel. In this device, the cross sectional area of the electrode channel is 10^4 µm2, whereas that of the axonal microgroove is only $3x10^2$ µm2. As a result, it is possible to flow Gallium right up to the interface between the two channels without leaking

into the second channel (8). The pressure required to flow Gallium into the microgroove is orders of magnitude higher than to flow into the electrode channel.

Gallium is a conductive metal, and the electrodes have a resistance of approximately 40 ohms, calculated by the dimensions of the electrode and the resistivity of Gallium provided by Sigma Aldrich. The majority of resistance in the circuit comes from the region between the two electrode terminals consisting of cell growth media and axonal biomaterial. The total resistance of the circuit is around 2 megaohms. Electrodes were tested under a stimulating AC voltage from a function generator and they remained stable through a relevant range of voltages and frequencies (0-5V, 0-100kHz). At very high voltages, reductive current flow caused the stabilizing layer to break, and surface tension pulled the electrodes back into their wells. In some device configurations, high voltages would also lead to bubble formation due to hydrolysis of cell media.

3.3 Neuronal growth

Neuron growth in the devices was comparable to growth in similar compartmentalized culture devices without Gallium (Fig. 2). In small regions near electrodes, some abnormal growth was observed, however this did not affect the overall prosperity of the cells.

The devices were designed such that only axons would pass through the microgrooves. The microgrooves are 10 µm wide, and the average diameter of a hippocampal neuron is about 20 µm. Because of this, very few cell bodies drifted into the microgrooves, while axons grew readily through. As expected, the devices had one channel of cell bodies, and another of all axons.

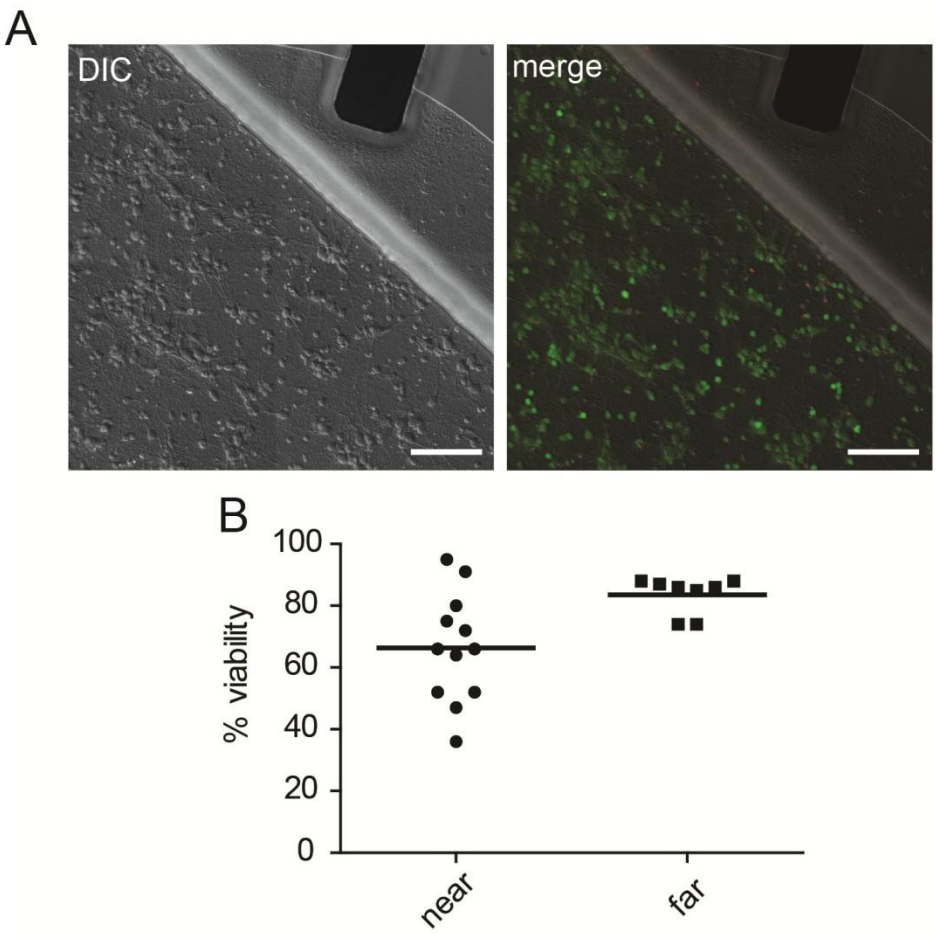

Fig 2 (a) Widefield micrograph of device with electrode visible (top right). (b) Same field of view, imaged with celltracker green 488 live-cell dye and propidium iodide dead cell dye, overlayed on (a) (scale bar = 100μm). (b) % viability for cells near the electrode vs. the center of the cell chamber.

3.4 Calcium Imaging

A fluorescent calcium marker was used to image neural activity (Fluo-4 NW Calcium Assay, Invitrogen, Inc.). Details on the assay are described in Taylor et al., Neuron, 2010 (14). Using this assay, many neurons show a low brightness, baseline fluorescence which is magnified many times during synaptic activity (Fig 3 b-c).

Using Calcium fluorescence, we were able to determine when the neurons were activated, and what electrical stimulation was required to induce activity. Figure 3 shows the fluorescence response to three separate stimulation periods at .2mA, .6mA and 1mA. .6mA was found to be the ideal stimulus amplitude to elicit repeated responses without damaging neurons.

Under a stimulus amplitude of .6mA, optimal neuronal response was observed. A stimulus of .6mA could also be repeatedly applied for an equally powerful response, unlike 1mA which appeared to be harmful to neurons and evoked less dramatic subsequent responses.

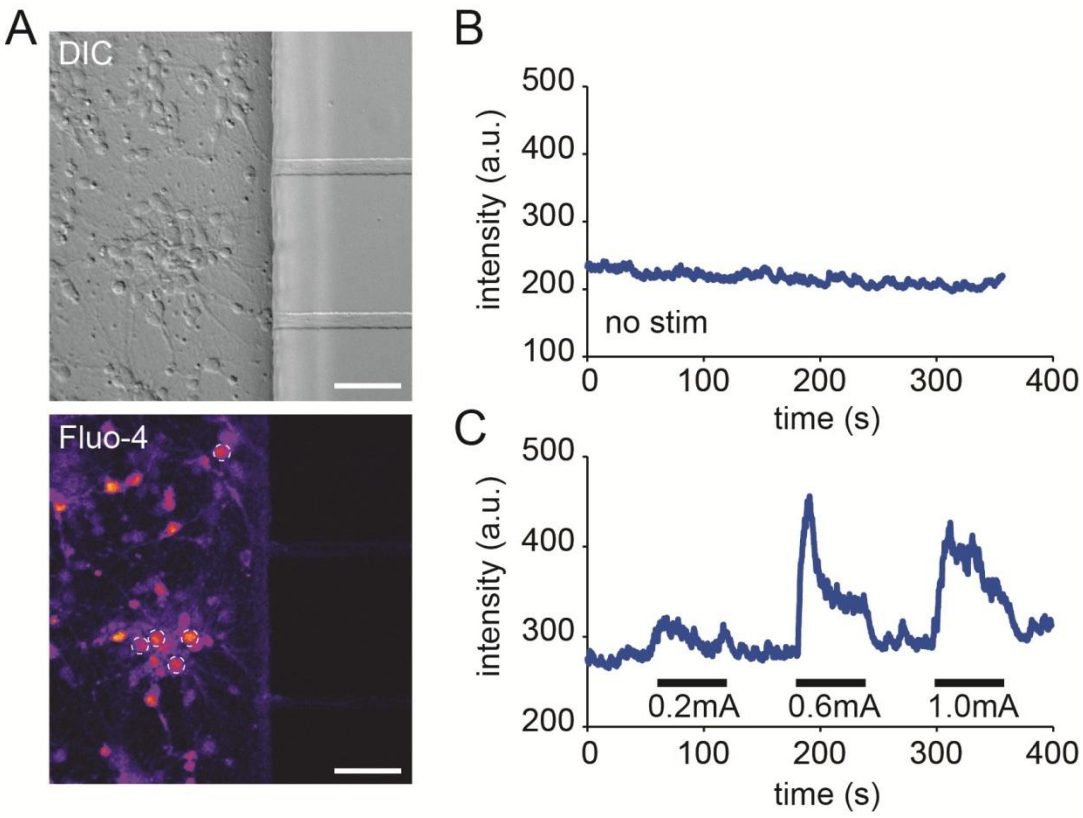

Fig 3: (A) Widefield (top) and fluorescent (bottom) images of target neurons with ROIs circled in white. (B) Baseline fluorescence without stimulatiion. (C) Evoked activity fluorescence during stimulation (underlined) of .2mA, .6mA and 1mA.

Stimulating an axon directly yields the same result. Using the microgroove stimulation chambers (Fig. 4 A,B), we stimulated a single axon and imaged the calcium fluorescence response (Fig. 4 C-E). Immediately following electrical stimulation, the axon exhibits a rapid rise in fluorescence followed by a slower decay, characteristic of evoked neuronal calcium activity (Fig. 4 D).

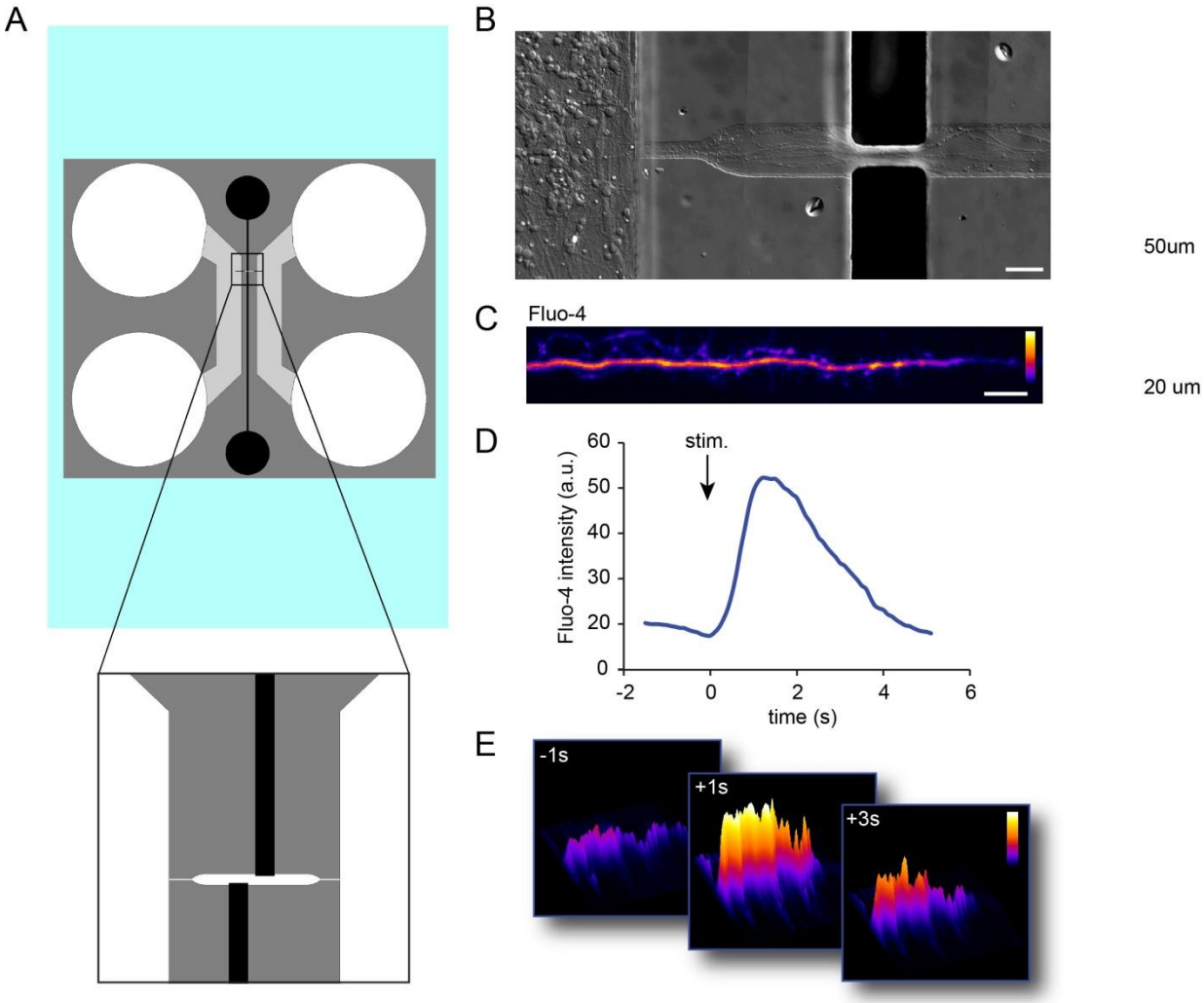

Fig.4. (A) Top-down schematic of microgroove stimulation device. Liquid metal electrodes stimulate a single microgroove across a 60 µm gap. (B) Wide field micrograph of axons growing in device. Biocompatibility is sufficient to sustain cultures for up to two weeks. Scale, 50 µm. (C) Calcium fluorescence image of an electrically stimulated axon. Color added in software. Scale bar, 20 µm. (D) Calcium fluorescence intensity curve over a stimulation period in an axon. (E) Surface intensity maps for an ROI from figure D. Stimulus occurs at t = 0.

3.5 Conclusions

A microfluidic compartmentalized cell culture platform incorporating liquid metal microelectrodes was developed. The electrode material did not adversely affect the growth of neurons within the culture platform, and was able to effectively stimulate target neurons.

This device is easily assembled, using readily available materials and without significant set up time. Liquid metal µf electrodes effectively stimulate target axons with high accuracy and reliability. The device provides a simple electrical interface to hippocampal neurons, as well as a neatly organized array of small axon populations. By organizing the growth of axons and separating axons from cell bodies in CNS neurons, unique research of CNS neuron development, synaptogenesis and axon regeneration is made possible.

The low cost and simple fabrication of these devices means that many samples can be processed in parallel with little startup time.

CHAPTER 4

EXPERIMENTAL

4.1 Fabrication of µf devices

Device plans were drafted in Autocad 2012 (Autodesk Inc.), and printed on transparency films to produce photomasks (CAD/Art Services Inc., Bandon OR). Standard photolithographic procedures were then used to produce device master moulds. In short, SU-8 photoresist was spun onto a silicon wafer at a thickness of 3 um, pre-baked, and exposed to UV light in a mask aligner under the first layer photomask. Next, wafers were developed, cleaned and dried, and a second layer of SU-8 was spun on at a thickness of 100um. Wafers were pre-baked, exposed to UV light in a mask aligner with a second photomask, then post-baked and developed.

Devices were made of PDMS moulded onto an SU-8 master. After being peeled off the master mould, devices were separated with a razor tool and wells were punched out with cutting tools. Devices were then cleaned of debris, sterilized in 70% EtOH and placed onto poly D lysine coated glass coverslip substrates, where they bonded by Van der Waals forces.

4.2 Electrode preparation

Once PDMS devices were bonded to glass coverslips, a drop of liquid Gallium was placed in the electrode wells. Electrodes were then manually injected by applying positive pressure to filled wells with a 1mL BD syringe. Because the pressure required to cause Gallium to flow is inversely proportional

to the cross sectional area of the channel, filling the electrode channel completely, but without causing it to spill out, is a relatively easy task (Dickey et al.). In fact, the difference in surface areas of the axon compartment and electrode channel are so great, the PDMS would separate from the substrate long before any Gallium would leak into the axon compartment (see figure 1 B).

4.3 Neuron transfection and plating

E17 rat hippocampal neurons were used for these experiments. Pregnant rats were euthanized via CO2 chamber and cervical dislocation, then rat embryos were removed and the hippocampi dissected. Cells were disassociated by tripsin and trituration, and plated on poly d lysine coated glass coverslips.

4.4 Incubation and observation

Cells were incubated at 37C and 5% CO2, and periodically observed under a microscope. Once axons and dendrites had sufficiently grown through the channels, usually by 7-14 DIV, cells were subjected to selective stimulation and imaged.

4.5 Electrical Stimulation and Calcium Imaging

Neural activity was monitored via Fluo-4 calcium imaging assay as described previously (Taylor et al., Neuron, 2010). Image acquisition was performed on an Olympus (XYZ) confocal, using a 488nm wavelength laser and ANDOR Iq2 image acquisition software.

Electrical stimulation was supplied by an AD Instruements PowerLab 15T (LTS) (AD Instruments, Inc.). The isolated stimulus unit was used, providing a constant current ranging from .2mA to 1mA.

Frequency was 10hz and pulse width .2ms. Leads from the stimulator were clipped to copper wires, which were inserted into the gallium wells.

CHAPTER 5

ADDITIONAL RESULTS

An alternate design featured electrodes that met at a single microgroove in order to stimulate only a very small number of neurons, directly at the axon. In many cases, the direct proximity of the axon to the electrodes caused some irregular growth, and resulted in axons not proceeding through the length of the microgroove.

Steps taken to improve axonal growth:

Polyvinyl Alcohol

In order to avoid metal poisoning of target neurons, a passivating layer of Polyvinyl Alcohol (PVA) was applied by flowing a 1% weight solution of PVA in sterile water into the device and through the microgroove for periods ranging from 10 minutes to 1 hour. The goal was to create a stable, thin layer of PVA which would block the diffusion of metal ions, but still allow current to flow.

Some problems arose from the use of PVA:

1) The extremely small volume of the microgrooves means very little crossflow occurs. Applying enough PVA to the electrode terminals is a challenge. It is possible to increase the flow by applying pressure to the liquid, however too much pressure will separate the device from the substrate, ruining it.
2) After applying PVA, the excess solution must be washed away prior to plating neurons. The same problem of low flow rate makes it difficult to flush the channel with Neurobasal media without damaging the device.

3) Since Neurobasal media is water-based, and PVA is water soluble, the barrier does not last for the entire lifespan of the cell culture. After a few days, the same problems will likely be encountered as without the PVA barrier.

Chloroauric Acid

Chloroauric acid (CAA) is a gold salt which can be easily reduced to pure gold. Gallium's natural tendency to oxidize makes it a particularly interesting partner to Chloroauric acid. It was thought that by applying an aqueous solution of CAA to the electrodes, Gallium would reduce CAA into a layer of gold on the surface of the electrode.

The Gallium-CAA redox process ended up being so vigorous that rather than making a stable gold layer at the electrode surface, the electrode was destabilized and moved out of the channel, up the flow gradient of the applied solution (Fig 5).

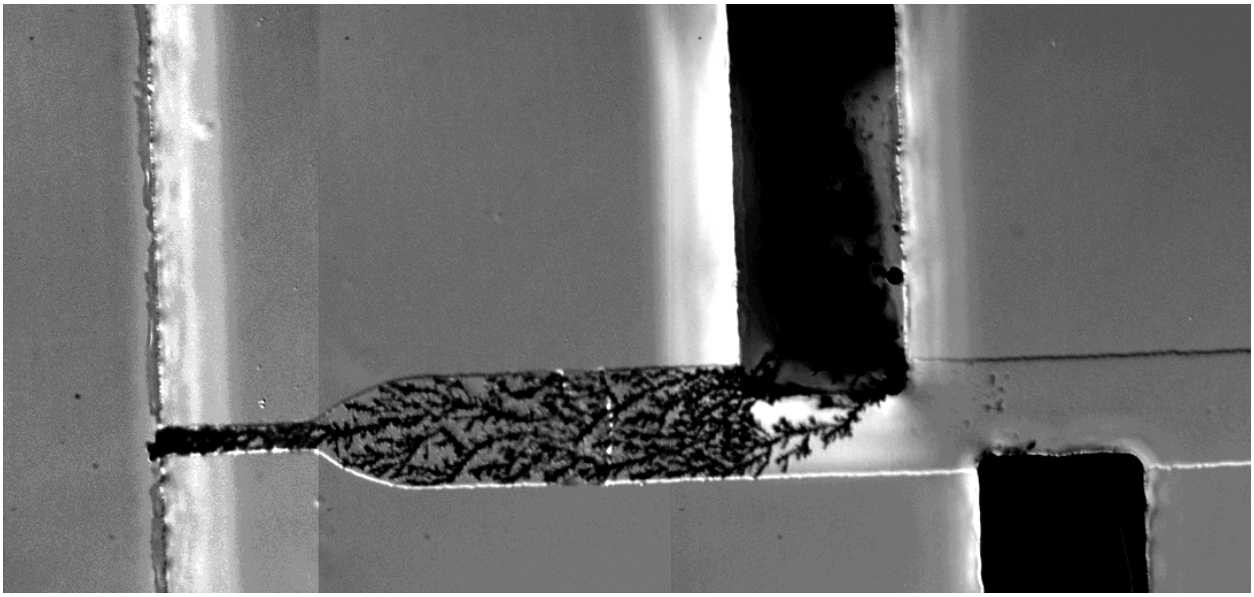

Figure 5: Micrograph of microchannel electrode design. Passing an aqueous solution of Chloroauric acid through the groove causes gallium to grow out of its channel, reducing gold in the process.

Future Direction

Future work with these devices will require a reliable, stable passivating barrier layer at the electrode terminals. Gold is an attractive candidate for barrier material due to its excellent electrical characteristics and proven biocompatibility.

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