AN ACTIVE MICROSCAFFOLD SYSTEM WITH FLUID DELIVERY AND STIMULATION/RECORDING FUNCTIONALITIES FOR CULTURING 3-D IN VITRO NEURONAL NETWORKS

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SUMMARY

The work presented in this dissertation builds upon the conventional concept of passive scaffolds for cell culturing by the addition of active functions (e.g., fluid perfusion, electrodes) integrated directly into the scaffold. Overall, the primary objective of this research was to provide neuroscientists with a better analytical tool for studying 3-D in vitro neuronal networks than was currently available, and was executed through the development of BioMEMS technologies that assisted in the growth, proliferation, and stimulation/recording of 3-D in vitro neuronal networks.

The design, fabrication, and characterization of an active microscaffold system with fluid perfusion and electrical stimulation/recording functionalities for culturing 3-D in vitro neuronal networks is presented. The active microscaffold system consists of an array of microfabricated towers (1-2 mm in height) with integrated fluid perfusion functionality to provide nutrients and aeration to the network of cells growing within the microscaffold, in addition to electrodes for stimulation/recording of the neuronal network. The microtowers provide a structural support system for the neurons to adhere to, enabling branching and network formation with other neurons in the culture. The microtower electrodes, placed at varying heights on the surface of the microtowers, allow for stimulation and recording of the cultured network in a 3-D multielectrode array (MEA) environment.

An active microscaffold system for culturing re-aggregate networks formed from dissociated neurons was designed, fabricated, packaged, and tested, and the biological data gathered from this active microscaffold system demonstrated that the fluid perfusion functionality of the system increased cell viability at 14 and 21 DIV. Additionally, a
separate active microscaffold system for culturing thick (≤ 1 mm) slices of brain tissue was designed, fabricated, and packaged.
CHAPTER 1

INTRODUCTION

1.1 Origin and History of the Problem (Motivation for 3-D Neuronal Cultures)

Most of the neurophysiologic research to date has involved either probing single neurons with single electrodes (single-unit neuronal recordings) [1, 2], or culturing 2-D (monolayer) neuronal cultures on top of planar multielectrode arrays (MEAs) [3, 4, 5, 6]. The problem with single neuron studies is that every task performed by the brain requires the coordinated interaction of multiple neuronal networks, and not the responses of a solitary, independent neuron [1, 3, 7]. Likewise, 2-D neuronal cultures, although useful for investigating planar neuronal network functionality, are not accurate models of the brain, which is a functioning 3-D system. The research in this dissertation involves extending in vitro neuronal cell studies into three dimensions to study the function of 3-D in vitro neuronal networks.

Past studies have theorized that it is the brain’s cytoarchitecture (its geometric and physical layout) that may be the relevant link to the brain’s overall function [8, 9]. Furthermore, it has been shown that 2-D cultures display differing electrical responses from their 3-D counterparts, which implies that 3-D cell cultures allow for more cell-to-cell interactions and therefore, more accurately mimic actual in vivo, cellular networks [10, 11]. In other words, 3-D neuronal cultures can provide neuroscientists with a better model to study the nervous system than 2-D neuronal cultures can provide [12, 13, 14, 15]. However, there are numerous obstacles to performing 3-D neuronal cell culture. Three-dimensional in vitro neuronal cell cultures lack an intact circulatory system. Hence, a lack of nutrient distribution, aeration, and waste removal contribute to rapid
necrosis in the center of these 3-D neuronal cultures [9, 16, 17]. Hence, little knowledge has been gathered on the dynamics of 3-D neuronal networks. As a result, biologists have been unable to obtain an adequate understanding of how the brain functions under normal, healthy conditions and in response to traumatic or degenerative injury [18].

Two-dimensional (monolayer) neuronal culturing has become a fairly standard method for in vitro experiments in cell growth, attachment, proliferation, and electrical stimulation/recording. However, there would be several advantages to 3-D in vitro culturing of neurons. For instance, a 3-D culture provides a more accurate representation of neuronal growth by allowing more cell-to-cell interactions and opportunities for branching and attachment formation to facilitate cell growth and survival [13, 14, 15]. Additionally, studies have shown that 3-D cell cultures exhibit different responses to stimuli than their 2-D counterparts, implying that 3-D cultures display more in vivo like qualities [10, 11].

This dissertation presents the design, fabrication, and characterization of a microscaffold system with fluid and electrical functionality, which is conducive to the culturing of 3-D in vitro neuronal networks. The microscaffold consists of an array of microfabricated towers with integrated fluid perfusion functionality to provide nutrients and aeration to the network of cells growing within the microscaffold, in addition to electrodes for stimulation and recording of the cultured 3-D in vitro neuronal network. The microtowers provide a structural support system for the neurons to branch out and form connections with adjacent neurons, while the fluid perfusion properties of the microscaffold system enable 3-D in vitro culturing of neurons. Finally, the electrodes,
placed at varying heights on the microtower walls, allow for stimulation and recording of the cultured network in a 3-D MEA environment.

1.1.1 In Vivo vs. In Vitro

A large research paradigm is focused specifically on obtaining electrical stimulation/recording data from 3-D in vivo neuronal networks. While in vivo experiments implement the use of live animals and may provide the best real-time response from 3-D neuronal networks, the benefit of in vitro studies lies in the fact that environmental conditions, stimuli, and injury to the cells/network can be controlled with an exact precision that is unattainable in a living animal. Also, numerous challenges arise when trying to image 3-D in vivo neuronal networks, whereas imaging is far more accessible in 3-D in vitro neuronal network studies. Additionally, in vitro culture studies eliminate variables such as immune response and inflammatory factors, which are present within in vivo cultures [9, 17].

Both in vivo and in vitro studies are crucial to further advance the knowledge of functional neuronal networks, but the work in this dissertation focuses specifically on advancing the knowledge of 3-D neuronal networks through in vitro studies. Also, there are times when the knowledge gained from the in vivo research paradigm can be implemented and blended with the in vitro research paradigm and vice versa. For example, much was learned from the 3-D in vivo neuronal probes fabricated by K. Wise at the University of Michigan (see Section 1.1.3) that was applied to the design and fabrication of the active microscaffold system presented in this dissertation.
1.1.2 Multielectrode Arrays (MEAs, 2-D and 3-D)

Neuronal cell culturing on 2-D MEAs has become a standard procedure for studying electrical properties and responses of *in vitro* neuronal networks. Multi Channel Systems provides standard commercial 2-D MEAs for such purposes [11]. However, long-term, 2-D neuronal network studies are difficult to perform on planar MEAs since neurons are not stationary. Hence, repeated electrical measurements from a specific neuron are difficult to acquire. Additionally, to study single neurons within a neuronal network, each neuron must correspond to a particular electrode. To overcome this difficulty, Parylene neuro-cages have been constructed for confining individual neurons to particular electrodes within a network, while channels for neurite outgrowth have been provided to allow these confined neurons to create a functioning network with other neurons in the array. Such neuro-cage devices allow for long-term studies of patterned 2-D neuronal networks [19].

When organotypic brain slices are cultured on MEAs that lack fluidic functionality, it is typically the portions of the slice directly in contact with the electrodes that display the lowest viability. In response, porous MEAs, which are essentially porous membranes with both electrical and diffusion properties, were developed to culture cortical slices with higher viability [16, 17]. However, porous MEAs are only capable of improving the diffusion of nutrients, oxygen, and waste removal at the slice's surface. Porous MEAs still do not enable thick, *in vitro* cortical slice studies. Hence, many obstacles still remain for long-term, thick, *in vitro* organotypic slice studies [8, 9].

Three-dimensional, tip-shaped MEAs have been fabricated by Ayanda Biosystems that are capable of penetrating into an *in vitro* organotypic brain slice to
obtain electrical stimulation/recording data from within the slice. In contrast, traditional 2-D (planar) MEAs are only capable of stimulating and recording on the surface of the slice. Comparisons in electrical recordings of organotypic brain slices cultured on the 3-D tip-shaped MEAs vs. the 2-D (planar) MEAs showed that larger amplitudes (in the millivolt range) were obtained with the 3-D tip-shaped MEAs. These 3-D tip-shaped MEAs are only approximately 60 µm in height, but this study still signifies that 3-D MEAs more accurately represent the functioning of neuronal networks in acute brain slice studies [11].

1.1.3 Michigan Probes

Electrical stimulation/recording of 3-D in vivo neuronal networks has been performed with the advancement of neural probes. Numerous in vivo neural probes have been developed for such studies [7, 20, 21], the most well-known being the neural probes developed by K. Wise at the University of Michigan [2, 10, 22, 23, 24]. The Michigan in vivo probes consist of an array of vertical Si shanks (1.2 mm in height) with integrated 3-D microelectrodes that allow for semichronic and acute stimulation and recording. These Si probes are first fabricated in linear arrays in a 2-D format and are subsequently packaged into an upright, vertical position. The probes contain on-chip complementary metal oxide semiconductor (CMOS) circuitry, which allows for the site selection, amplification, and multiplexing of electrical signals. Initially, the Michigan probes had only electrical functionality, but later fluidic functionality was also added to the probes for drug delivery purposes [2, 24]. Challenges remain for long-term, in vivo implantation, but future goals of such devices are aimed at implantable prosthetics to treat debilitating
disorders of the central nervous system such as paralysis, Parkinson’s disease, epilepsy, stroke, blindness, and deafness [2, 25].

1.1.4 Utah Intracortical Electrode Array (UIEA)

Another, well-recognized, in vivo neural probe is the Utah Intracortical Electrode Array (UIEA), which consists of a $10 \times 10$ array of vertical needles approximately 1.2 mm in height [26, 27]. Each needle contains electrodes for neuronal cell recording, and the corresponding leads are insulated with polyimide. The array of needles protrudes out from an Si substrate, and functional electrical integration of the needle electrodes is performed on the backside of the device. It is also important to note that the UIEAs are purely electrical devices and contain no fluidic functionality.

Several in vivo experiments have been performed with the UIEA to record spatial and temporal neuronal activation patterns, in addition to establishing the potential of the UIEA as a brain-computer interface (BCI) [27]. A BCI, for example, could serve to restore sensory and motor functions in a paralysis victim by transmitting neuronal signals between the brain and external devices. A microelectrode-based BCI would record action potentials and determine whether neuronal activity changes were the result of a person’s intent to execute a particular action [25, 27]. In fact, preliminary BCI studies have enabled paralysis victims to move a cursor on a computer screen in a desired direction by sensing that person’s electrical signals in their brain, and successfully translating those signals as a desire for a specific action [28, 29].

A criticism of both the Michigan and Utah Arrays is that the probes are made of rigid materials that cause damage to the cortex upon insertion. There have been recent
advances in flexible neuronal probes, which were shown to be less invasive than the conventional Si probes, and these flexible probes also can deform to better fit the tissues under analysis [30, 31, 32, 33, 34, 35]. Parylene flexible neuronal probes have been fabricated that contain both electrodes for extracellular recordings, and fluid channels for drug delivery. The fluid channels in the flexible probes are initially filled with polyethylene glycol (PEG), which makes the probes stiff upon insertion into the cortex. However, after insertion, the PEG dissolves when in contact with the biological tissue, giving the Parylene probe flexible freedom, leaving behind a hollow, fluid channel for drug delivery purposes [30].

1.1.5 Traditional Microscaffolds

Traditional microscaffolds are used in tissue engineering applications to culture the cells up to the point that the culture becomes a suitable replacement for an in vivo system [36]. Essentially, these conventional scaffolds serve as temporary structures to support the cultured cells during the process of tissue/network regeneration [37]. The majority of these traditional microscaffolds are composed of organic and inorganic polymer matrices [36]. BD Matrigel™ Matrix is one of the most common of these traditional scaffoldings used in tissue engineering applications; its main components being laminin (a major constituent in basement membranes) and collagen (the primary protein in connective tissue) [38, 39, 40, 41]. Matrigel is a basement membrane complex taken from mice with sarcomas (tumors), which are abundant in extracellular matrix (ECM) proteins [38]. Cell cultures performed on Matrigel tend to mimic the complex cellular architectures and therefore tissue function found in in vivo systems [36], which
otherwise wouldn’t be observed in vitro in the absence of Matrigel. For example, endothelial cells will extend processes and form cellular networks on Matrigel coated surfaces, but endothelial cells will not display this sort of behavior on plastic coated substrates in the absence of Matrigel [39].

Some researchers have used larger volumes of Matrigel to create 3-D cell cultures, with attempts to have the cells navigate from the surface to the center of the gel, thus yielding a culture model that represents tumor or cancer cell metastasis [39]. Also, standard procedures have been developed for creating pores in the Matrigel to promote nutrient distribution and cell infiltration to the center of the gel, followed by seeding of the cells on Matrigel coated surfaces. Matrigel scaffolds are inherently biocompatible and provide the basic ECM proteins required for mimicking certain aspects of cellular behavior in systems in vivo, but Matrigel lacks the mechanical properties required for guiding proper cellular organization in 3-D tissue engineered systems [42]. Researchers are pursuing numerous types of 3-D cell cultures for various reasons. Primarily, 3-D cultures provide a better model of functioning in vivo complexes than can be provided by 2-D cultures. Additionally, tissue engineering has presented itself as a potential technology for tissue repair/replacement [42, 43].

Creating in vitro cultures that have been able to mimic the structural organization of cells found in functioning in vivo systems has been one of the largest challenges in tissue engineering. Furthermore, numerous studies have shown that the cellular organization of in vivo systems has a direct correlation to tissue function. Hence, an in vitro system will not be able to properly function as a tissue replacement if the cellular organization cannot be controlled. In response to this desire to control cellular
organization, polydimethylsiloxane (PDMS) scaffolds have been used in conjunction with collagen matrices to guide the cellular growth of fibroblasts in 3-D. However, these 3-D PDMS microscaffolds were not high-aspect ratio structures since they only extended 70 µm in the z-direction, and could therefore only provide limited 3-D cellular and network organization [36].

The ideal cellular scaffolding would be biocompatible, promote cell adhesion, have appropriate porosity for cell permeation and nutrient distribution, and have suitable mechanical properties to guide cellular organization and maintain tissue structure. Biodegradable synthetic scaffolds composed of polymers like polylactic acid and polyglycolic acid have also been implemented in tissue engineering applications. However, toxicity issues tend to arise with these synthetic polymer scaffolds, and the surface chemistry of such scaffolds does not encourage cell adhesion [42].

Other cellular scaffolds have been created from either collagen or elastin derived from decellularized porcine aorta, with a specific focus placed on creating interconnected pores with favorable dimensions to permit cell infiltration and proliferation. In vitro fibroblast cultures were performed on both the collagen and elastin scaffoldings with excellent viability. Also, these collagen and elastin scaffolds maintained the original structural design of the porcine aorta, and therefore exhibited the mechanical properties required for guiding proper cellular organization. The elastin scaffolds contained larger pores than the collagen scaffolds, and the elastin scaffolds also biodegraded at a lower rate than the collagen scaffolds, implying that the elastin scaffolds may be better for tissue replacement applications, while the collagen scaffolds may be better for controlled tissue regeneration [42].
PEG-fibrinogen hydrogel scaffolds were also developed to manipulate 3-D cellular growth. These scaffolds contained both a synthetic component (PEG) and a biological component (fibrinogen precursor molecules) to establish a biomimetic environment for controlling the morphology and migration of the cells. Experiments involving the culture of smooth muscle cells on the PEG-fibrinogen hydrogel scaffolds displayed a correlation between the morphology of the cells and the molecular organization of the matrix. Furthermore, more control over scaffold properties such as stiffness, density, and the rate and extent to which the scaffold biodegrades are available when synthetic and biological components are merged to form scaffolds for tissue engineering applications than are available with scaffolds composed of only biological components [37].

Specifically for neuronal network cultures, studies have shown that the components of the ECM help direct neurites and nerve cells to the appropriate targets during the growth process, and hence it is theorized that these specific ECM components involved in navigating neurite outgrowth could also promote adult nervous tissue regeneration. Three-dimensional gels containing combinations of collagen, fibronectin, laminin, and agarose were experimented with to study the effect of the extracellular environment on the outgrowth and navigation of neurites. The results showed that neurite outgrowth decreased with increasing collagen concentrations. Also, cultures in pure agarose gels displayed no neurite growth, but small quantities of collagen added to the cultures in these agarose gels displayed significant neurite outgrowth. It is evident that the proper extracellular environment with a precise combination of neurotrophic
factors is essential to guide neurite outgrowth, and could also be useful to guide axonal regeneration and enhance the growth of transplanted neurons in the brain [44].

The research in this dissertation is focused on studying 3-D in vitro neuronal networks, but a significant amount of knowledge from the in vivo probe devices and MEAs was applied to realizing the active microscaffold system for 3-D in vitro neuronal cell culture. For example, the biocompatibility of materials had to be considered in fabricating the active microscaffold system presented in this dissertation. SU-8 photoresist epoxy was a good structural candidate for the scaffold since it could be easily patterned into high-aspect ratio structures [12, 13, 14, 15] with fluidic applications [45, 46], and its cytotoxicity has been under evaluation for implantable BioMEMS devices [47]. Furthermore, the electrode materials that have shown the most promise for long-term studies, extract biologically favorable responses from cells, and form ohmic contacts with biological tissues include platinum (Pt), gold (Au), platinum-iridium, (Pt-Ir), tantalum (Ta), and tungsten (W), while the best insulators have been shown to be either polyimide or glass [48]. In some 3-D probe structures, nickel (Ni) [31] or some other magnetic materials were used to raise 2-D structures into a 3-D position using plastic deformation magnetic assembly (PDMA) [49]. However, Ni or other magnetic materials, though useful in 3-D assembly, will tend to extract a toxic response from any neurons under long-term evaluation.

Furthermore, biocompatible packaging techniques had to be considered for the entire active microscaffold system. Extensive work with polydimethylsiloxane (PDMS) has proven its usefulness in creating 3-D structures, with applications ranging from neuronal cell culture in microfluidic systems, to microcontact printing, to guiding and
directing neuronal cell growth [50, 51, 52]. Additionally, lead (Pb)-free solders (which can often be electroplated) were considered for establishing electrical functionality to the active microscaffold system [53, 54, 55, 56].

In conclusion, the presented active microscaffold system with fluid delivery and stimulation/recording functionalities builds upon past BioMEMS neuronal cell research, providing an analytical tool upon which neuroscientists can create 3-D in vitro neuronal cell cultures. The active microscaffold system contains an array of high-aspect ratio microfabricated towers that were first fabricated in a 2-D (planar) format, and then were subsequently packaged into a 3-D (vertical) position in a manner similar to the packaging of the Michigan probe arrays [2]. The active microscaffold system contains both electrical and fluid functionality, combining the electrical/stimulation recording capabilities of 3-D MEAs with the nutrient and drug delivery capabilities of porous MEAs and fluidic neuronal probes. The overall objective of this research is to provide neuroscientists with a better BioMEMS device for studying 3-D in vitro neuronal networks than is currently available.

1.2 Objective: Design, Fabricate, and Test an Active Microscaffold System with Fluid Delivery and Stimulation Recording Functionalities for Culturing 3-D in Vitro Neuronal Networks

The objective of this research is to further the comprehension of both healthy and damaged 3-D neuronal networks through BioMEMS technologies that assist in the growth, proliferation, and stimulation/recording of 3-D in vitro neuronal networks. This proposal presents an active microscaffold system with fluid delivery and
stimulation/recording functionalities for culturing 3-D neuronal networks created from dissociated hippocampal cells. *In vitro* culturing of 3-D neuronal networks has not been very successful, since such cultures lack intact, functioning circulatory systems. Typically, attempts at 3-D *in vitro* neuronal cultures have failed, since all the cells at the center of the culture rapidly become necrotic due to a lack of nutrients, gas exchange, and waste removal. Because of the lack of an appropriate, investigative, analytical instrument (i.e. an active microscaffold system with fluid delivery and electrical stimulation/recording functionalities), little information has been gathered on the dynamics of 3-D neuronal complexes.

The active microscaffold systems presented in this dissertation consist of arrays of microfabricated towers with integrated microfluidic channels and electrodes. The microfluidic channels allow for the perfusion of nutrients, gas exchange, and biochemical control of the extracellular environment throughout the 3-D culture, while the electrodes allow for active stimulation/recording of the 3-D neuronal network. In essence, the fluidic capabilities of the microscaffold serve as an artificial circulatory system to enable 3-D *in vitro* culturing of dissociated neuronal cells. Additionally, the microtower scaffold is built upon a substrate that can both control and process electrical signals to and from the microtower electrodes through neural processing chips (NPCs).

The overall goal of this research is to provide neuroscientists with a better analytical tool for studying 3-D *in vitro* neuronal networks than is otherwise currently available. The research was organized into four specific aims and one reach aim. Furthermore, each specific aim was divided into a series of subaims. The four specific aims, corresponding subaims, and reach aim are the following:
Specific Aim #1: Design 2-D (planar) microtower structures that are conducive to neuronal growth and allow for integration of both fluidic and electrical components

Subaim 1.1: Design 2-D microtower structures with both fluidic and electrical components that are conducive to culturing 3-D neuronal networks formed from dissociated neurons

Subaim 1.2: Design 2-D microtower structures with both fluidic and electrical components that are conducive to culturing thick slices of brain tissue

Specific Aim #2: Fabricate 2-D (planar) microtower structures

Subaim 2.1: Fabricate 2-D microtower structures with both fluidic and electrical components that are conducive to culturing 3-D neuronal networks formed from dissociated neurons

Subaim 2.2: Design 2-D microtower structures with both fluidic and electrical

Subaim 2.3: Test the biocompatibility of the 2-D microtower structures by culturing primary hippocampal neurons on the unpackaged microtower structures (This was a collaborative effort with the Brewer research lab at the Southern Illinois University School of Medicine and the Wheeler lab at the University of Illinois at Urbana-Champaign)

Specific Aim #3: Package the 2-D (planar) dissociated neuronal cell microtower structures into a 3-D format, yielding the active microscaffold system

Subaim 3.1: Design a packaging and fabrication methodology for assembling the microtower arrays

Subaim 3.2: Package the dissociated neuronal cell microtowers into a 3-D format, yielding the active microscaffold, composed of a vertical array of microtowers
that are conducive to culturing 3-D in vitro neuronal networks formed from dissociated neurons

**Specific Aim #4:** Integrate fluid and electrical functionality of the 3-D packaged microtowers

**Subaim 4.1:** Integrate fluid functionality

**Subaim 4.1.1:** Design and fabricate a fluid manifold that acts as an interface between the dissociated neuronal cell microscaffold and the external fluid delivery system (This was a collaborative effort with the Glezer research lab at the Georgia Institute of Technology)

**Subaim 4.1.2:** Characterize 3-D dissociated neuronal cell culture with nutrient perfusion on the packaged microscaffold/fluid manifold system (This was a collaborative effort with the Brewer research lab at the Southern Illinois University School of Medicine)

**Subaim 4.2:** Integrate electrical functionality of the 3-D, packaged, dissociated neuronal cell microtowers for electrical stimulation/recording of the 3-D neuronal networks

**Subaim 4.2.1:** Form 90° electrical connections between the vertical microtower leads and the planar substrate leads to validate electrical functionality of the dissociated neuronal cell microscaffold

**Subaim 4.2.2:** Enable electrical stimulation/recording from 3-D networks of dissociated neurons that are being cultured on the electrically active dissociated neuronal cell microscaffold, using the commercially available Multi Channel Systems preamplifier setup obtain electrical data
stimulate/record from 59 sites (This was a collaborative effort with the Brewer research lab at the Southern Illinois University School of Medicine and the Wheeler lab at the University of Illinois at Urbana-Champaign)

Reach Aim: Integrate simultaneous fluid and electrical functionality into the dissociated neuronal cell microscaffold

Reach Subaim 5.1: Deliver a fully functioning, integrated, fluidic/electronic, dissociated neuronal cell microscaffold for culturing 3-D neuronal networks from dissociated neurons

Reach Subaim 5.2: Test the fully functioning, integrated, fluidic/electronic, dissociated neuronal cell microscaffold by perfusing 3-D neuronal cultures with nutrients, while simultaneously performing electrical stimulation/recording from the neuronal network cultured on the integrated system (This was a collaborative effort with the Brewer research lab at the Southern Illinois University School of Medicine and the Wheeler research lab at the University of Illinois at Urbana-Champaign)

1.3 Overview of the Thesis and What Each Chapter Entails

Chapter 1 of this dissertation includes the introduction, origin and history of the problem (motivation for 3-D neuronal cell cultures), background, and research objective. The design of the active microscaffold system with fluid delivery and stimulation/recording functionalities for culturing 3-D in vitro neuronal networks is presented in Chapter 2. Additionally, the theoretical analysis and modeling of the fluidics in the active microscaffold system are also presented in Chapter 2.
Chapter 3 goes into the detail of the microfabrication and packaging of the active microscaffold system. Materials selection is also discussed in Chapter 3 and includes discussions on SU-8 structural material for BioMEMS devices, biocompatible metals for electrodes, Si as a well established material in microfabrication, and PDMS as a moldable material for BioMEMS packaging applications. Also, the step-by-step 2-D microfabrication process for the linear microtower arrays, the 3-D vertical packaging of the microtower arrays, and the fluid and electrical packaging are presented in detail in Chapter 3.

Chapter 4 embodies the experimental methods used in the thesis project. Initial biological tests on the unpackaged SU-8 microtower arrays are presented, in addition to biological tests on packaged microscaffolds with perfusion. Device tests on fluid flow and suitable fluid flow rates are also discussed. Additionally, electrode characterizations on the electrically functioning microscaffold system are presented in Chapter 4.

Results and discussion of the fabrication, packaging, and biological testing are addressed in Chapter 5. Material selection, SU-8 lithography, electroplating, and sacrificial layer fabrication are all discussed. Furthermore, packaging results, fluid flow experiments, electrode characterizations, and the results of the biological experimentation are also examined. Chapter 6 concludes the thesis and summarizes the presented active microscaffold system with fluid perfusion and electrical stimulation/recording functionalities for culturing 3-D in vitro neuronal networks. Additionally, possible future paths and alternative applications for the presented microscaffold system are examined. Appendices are also included for convenient access to the microfabrication protocols.
CHAPTER 2

MICROSYSTEM DESIGN AND THEORETICAL ANALYSIS

2.1 Microsystem Design

The original 3-D Nets project [Bioengineering Research Partnership (BRP) National Institutes of Health (NIH) proposal (grant 1 R01 EB007861; Principal Investigator Stephen P. DeWeerth)] proposed a microscaffold consisting of a 16×16 array of hollow microtowers (1 mm in height) with a diameter ≤ 250 µm, as shown in figure 2.1 [57].

Figure 2.1. A schematic of the proposed active microscaffold system, showing a 16×16 array of microtowers with integrated electrodes that connect to neural processing chips (NPCs). Also, the schematic shows fluid channels underneath the substrate that deliver nutrients to the base of each microtower. The nutrients then travel up through the length of each microtower and perfuse out of the fluid side ports to deliver nutrients/aeration to the 3-D neuronal network cultured within the microtower array. (Image from the original NIH BRP proposal, p. 61).

The microtowers have integrated electrodes than run out to neural processing chips (NPCs) to enable electrical stimulation/recording functionality. The BRP project hypothesized that 3-D in vitro neuronal cultures grown on the active microscaffold
system with integrated fluid perfusion and electrical stimulation/recording functionalities would achieve more rapid maturity rates than would be observed by 2-D in vitro neuronal cultures. Additionally, the microtower array was theorized to provide a structural surface upon which the neurons could attach and proliferate and would therefore facilitate in 3-D network formation from dissociated neurons. The fluid perfusion functionality of the microscaffold enables nutrient delivery to the interior of the 3-D culture, thus enabling the formation of a 3-D in vitro neuronal network. Furthermore, the integrated microtower electrodes open up a new paradigm of research by enabling stimulation/recording from 3-D in vitro neuronal networks.

It was desired that the microtowers be fabricated upon a substrate with fluid delivery capabilities that would enable fluid delivery to the base of each hollow microtower so that the fluids could travel up the length of each microtower and perfuse out the top port and side ports of each microtower to nourish the 3-D network of cells being cultured within the microtower array. Also, as shown in figure 2.2, it was desired that each microtower contain several electrodes (for stimulation/recording), and the corresponding leads insulated within the microtower walls would interface with the NPCs. Furthermore, as can be seen in figure 2.2, structural cross-members that ran horizontally to form connections between each of the microtowers in the array were also hypothesized to aid in 3-D neuronal network formation.
The microtowers with electrical and fluidic interconnects. Each tower incorporates four microfabricated wires and one fluidic channel that run vertically through the tower and make contacts via electrodes and ports that project through the side walls (and top) of the tower at selected points. Each tower incorporates four electrodes and two fluidic ports. The resulting 16×16 array of towers includes a total of 1024 electrodes and 512 fluidic ports.

Figure 2.2. A schematic of the proposed microtowers with hollow channels for nutrient delivery and exposed electrodes (for stimulation/recording) with corresponding leads insulated within the microtower walls. Also, structural cross-members that run horizontally serve as bridges to connect each of the microtowers in the array. (Image from the original NIH BRP proposal, p. 62).

2.1.1 Design Versions of the Microsystem

The microtower arrays were initially fabricated in a 2-D (planar) fashion on top of a 4 in Si wafer using a six photomask microfabrication process. The completed 2-D microtower arrays were then released from their original Si substrate and were subsequently packaged into a Si orifice plate in a vertical, upright position. Four separate microscaffold systems were designed for this dissertation. The first set of six photomasks designed contained the original 8×8 and 16×16 microtower array designs for culturing re-aggregate neuronal networks from dissociated neuronal cells. A second set of six photomasks was later designed that contained a revised 12×12 microtower array design for culturing re-aggregate neuronal networks from dissociated neuronal cells and a 12×12 microtower array design for culturing thick slices of brain tissue. In the second
photomask set, the design dimensions of the fluid channels and side ports were calculated to achieve uniform flow rates out of each of the side ports. Additionally, the 12×12 microtower array design for culturing re-aggregate neuronal networks from dissociated neuronal cells had reduced dimensions to further accommodate neuronal culturing and data acquisition from the cultured system. Also, the 12×12 microtower array for culturing thick brain slices was also designed to the closest approximation of the proposed dimensions from the original BRP NIH proposal [57].

2.1.1.1 Original 8×8 and 16×16 Designs

A schematic of the first active microscaffold system designed for this dissertation, consisting of an 8×8 array of vertical microtowers, with integrated cross-connects running parallel to both the x and y-directions is provided in figure 2.3.

Figure 2.3. Schematic showing an 8×8 array of hollow microtowers projecting 1.5 mm from a Si orifice plate. There are functional cross-connects along the x-direction and structural cross-connects along the y-direction.
The microscaffold shown in figure 2.3 consists of 8 separate linear microtower arrays (with 8 microtowers per linear array) vertically packaged in succession one adjacent to the next. Each microtower (extending in the z-direction) and functional cross-member (running along the x-direction) contains a hollow fluid channel and multiple fluid ports to aid in fluid perfusion/nutrient delivery to the 3-D neuronal network cultured on the topside of the microscaffold device. However, the cross-members that run parallel to the y-direction were designed for purely structural purposes to form bridges between the adjacent linear microtower arrays, but contained no fluid or electrical functionality.

The fluid channels in the microtowers and functional cross-members served as a means of fluid/nutrient transport in the system. To facilitate fluid perfusion/nutrient delivery, there were numerous fluid ports (20×20 µm²) situated along the length of each microtower and functional cross-member, which allowed for nutrient perfusion, gas exchange, and biochemical control of the extracellular environment throughout the 3-D culture growing on the topside of the microscaffold system. The total active volume of the microscaffold available for cell culture in the 8×8 microtower arrays was 18.7 µL, 28.0 µL, or 37.3 µL, depending on the height (1, 1.5, or 2.0 mm) of the microtowers in the array. Similarly, the total active volume of the microscaffold available for cell culture in the 16×16 microtower arrays was 83.2 µL, 124.8 µL, or 166.3 µL, depending on the height (1, 1.5, or 2.0 mm) of the microtowers in the array.

The original 8×8 and 16×16 microtower array designs were laid out on a set of six 5×5 in² photomasks that contained linear microtower arrays consisting of either 8 or 16 microtowers per linear array. Using this photomask set, 180 linear microtower arrays (90 linear microtower arrays with 8 microtowers per linear array and 90 linear
microtower arrays with 16 microtowers per linear array) could be produced on a single 4 in Si wafer. Each 4 in Si wafer produced 30 microtower arrays. The microtowers were designed to have a 120 µm width, an 85 µm depth, and a 600 µm center-to-center horizontal spacing. The functional cross-members were designed to have a 110 µm width and a 500 µm center-to-center vertical spacing. Once vertically packaged, the microtowers would to project 1, 1.5, or 2.0 mm above the Si orifice plate (depending on the design), and 1.5 mm out the backside of the Si orifice plate. A fluid channel with a cross-sectional area of 70×23 µm² ran along the entire length of each microtower and in each functional cross-member.

Furthermore, a series of fluid ports (20×20 µm²) were placed at varying heights along the length of each microtower and in the functional cross-members. The fluid side ports were positioned directly along the center of each microtower, but only on the section of each microtower that projected from the topside of the Si orifice plate. No fluid side ports were placed on the sections of the microtowers that projected out the back of the Si orifice plate once packaged, since it was desired for all of the fluid perfusion to occur on the topside of the device where the neurons were being cultured.

Also, each microtower was designed to have two Au electrodes (at varying heights) for stimulation/recording of the cultured neuronal network. It was desired for the dimensions of each electrode to be on the order of the size of a single neuron. Since the cell body of a neuron has a diameter of 15-20 µm, each electrode was designed to have an exposed surface area of 15×15 µm². The Au electrodes were placed at varying heights either along the length of each microtower or in the horizontal cross-arms. Also, each exposed microtower electrode had a corresponding Au lead that was insulated.
within the microtower walls (running along either side of the fluid side ports) and attached to large, thick, electroplated Au tabs at the base of each microtower where the microtower intersected the top surface of the Si orifice plate. The large Au tabs at the base of each microtower were designed for the following two reasons; (1) to serve as a structural stop during the 3-D vertical packaging to prevent the microtower arrays from sliding further through the Si orifice plate, and (2) to later interface the microtower electrodes to electrical traces patterned on the Si orifice plate to provide electrical functionality to the microscaffold system.

The structural cross-members were also designed in linear arrays and were also initially fabricated in a 2-D (planar) format on top of a 4 in Si wafer. The structural cross-members (referred to as “pitchforks”) were solid SU-8 structures, and therefore could be fabricated using only a single photomask. Using a 3-D packaging technique that was similar to that of the SU-8 linear microtower arrays, the completed SU-8 pitchfork structures were released from their original Si wafer and were manually slid in between the vertically packaged SU-8 microtower arrays. The SU-8 pitchforks were sealed into place at the periphery of the packaged microtower array using PDMS.

The linear arrays of 8 and 16 microtowers were first fabricated in a 2-D (planar) fashion on a Si orifice plate, and were subsequently released from the original Si wafer. The linear arrays of 8 or 16 SU-8 microtowers were then manually packaged and sealed into a Si orifice plate, in a 3-D (vertical) format, in succession, one adjacent to the next. The structural SU-8 pitchforks were then inserted into the packaged microtower arrays to provide bridging structures that connected adjacent microtower arrays, thus forming a 3-
D grid for 3-D re-aggregate neuronal cell growth and analysis (see Chapter 3: Microsystem Fabrication and Packaging).

### 2.1.1.2 Revised Microtower Design (12×12 Microtower Array)

Some of the problems with the initial 8×8 and 16×16 microscaffold designs were the following; (1) the center-to-center spacing (600 µm) of the microtowers was too large for the neuron processes to branch from microtower-to-microtower in the absence of and additional ingredient such as Matrigel, (2) the 2.0 mm towers were too tall and presented depth of field imaging problems, and (3) the fluid side ports (all sized at 20×20 µm²) weren’t designed with the appropriate dimensions to achieve uniform fluid volume flow rates throughout the microscaffold system. Therefore, a redesign of the microscaffold system was performed that consisted of a 12×12 array of SU-8 microtowers with a height of 1.0 mm and a 200 µm center-to-center spacing.

Each microtower in the new design had an 80 µm width and an 85 µm depth. Similarly, each microtower in the 12×12 design had a hollow, fluid channel (with a cross-sectional area of 40×23 µm²) that ran along the entire length of each microtower. Likewise, the functional horizontal cross-members were designed with a fluid channel of identical dimensions. However, in the redesign, fluid side ports were deemed unnecessary in the functional cross-members to achieve adequate perfusion to the culture, so fluid side ports were only placed along the vertical length of each microtower. Three, square, fluid side ports having dimensions of 8.5×8.5 µm², 9.8×9.8 µm², and 11.6×11.6 µm² were placed along the length of each microtower at vertical heights of 200 µm, 400 µm, and 600 µm, respectively (see Section 2.1.2.1 Fluid Channel and Port Calculations.
for the Revised 12×12 Microtower Array Design). The larger ports were placed at higher elevations, while the smaller ports were placed at lower elevations to optimize uniform fluid flow rates in the system. The total active volume for cell culture in the redesigned microscaffold system for re-aggregate culturing of dissociated neurons was 10.4 µL.

### 2.1.1.3 Brain Slice Design

A separate active microscaffold with fluid perfusion and electrical stimulation/recording functionalities was designed for culturing thick (400 µm – 1.0 mm) slices of brain tissue. The schematics in figures 2.4a and 2.4b provide topside and backside views of the brain slice microscaffold device, consisting of a 12×12 array of hollow, tapered, SU-8 microtowers (1.0 mm in height).

![Schematics showing the 12×12 array of hollow, tapered, microtowers with integrated electrodes for culturing thick slices of brain tissue in vitro. (a) Topside; (b) Backside.](image)

Each brain slice microtower was designed with a 50 µm base width and tapered down to 24 µm at the microtower tip on the topside of the microscaffold system. The
microtowers were designed to be tapered and without cross-members on the topside of the device so that the microtowers could pierce through the brain slice being cultured within the microtower array on the topside of the device. The microtowers extend 1.0 mm out the topside of the Si orifice plate and 2.0 mm out the backside of the Si orifice plate.

Also, in the brain slice microscaffold design, functional cross-members were implemented on the backside of the device to connect adjacent microtowers, thus forming linear microtower arrays with 12 microtowers per linear array, to greatly simplify the 3-D, vertical packaging process. The microtowers in each linear array have a 200 µm center-to-center spacing, but each separate linear microtower array was packaged with a 400 µm center-to-center spacing from adjacent linear microtower arrays. Therefore, the total volume available for cell culture on the topside of the active brain slice microscaffold device was 11.5 µL.

Each brain slice microtower contains a hollow channel with a width of 12 µm on the topside of the device. The hollow channels on the backside of the device are much wider (100 µm) than on the front side of the device (12 µm), and hollow channels were also placed in each of the cross-members on the backside of the device to assist in fluid delivery in case a blockage occurred in one of the other microtowers in the linear array. Six, square, fluid side ports having dimensions of 4.0×4.0 µm², 4.5×4.5 µm², 5.0×5.0 µm², 5.5×5.5 µm², 6.0×6.0 µm², and 7.0×7.0 µm² were placed along the length of each tower at vertical heights of 200 µm, 300 µm, 400 µm, 500 µm, 600 µm, and 700 µm, respectively (see Section 2.1.2.2: Fluid Channel and Port Calculations for the Brain Slice Microtower Array Design). The larger ports were placed at higher elevations, while
the smaller ports were placed at lower elevations to optimize uniform fluid flow rates in
the system.

Additionally, each brain slice microtower was designed with 2 stimulation/recording electrodes placed at varying heights on each of the microtowers. Each microtower electrode was also linked to a corresponding vertical lead that was insulated within the microtower walls and connected to large electroplated Au tabs at the base of each microtower. However, in the brain slice microscaffold design, all of the electrical integration occurred on the backside of the Si orifice plate. Therefore, the microtower leads run from the stimulation/recording microtower electrodes to the thick electroplated Au tabs at the base of each microtower where each microtower intersects the backside of the Si orifice plate (see figure 2.4b). The thick electroplated Au tabs are bonded to the corresponding horizontal electrical leads patterned on the backside of the Si orifice plate, which each have a corresponding mega contact pad at the periphery of the Si orifice plate. The brain slice microscaffold system, like the dissociated neuronal cell microscaffold system, was designed to fit into the commercially available Multi Channel Systems preamplifier setup to obtain stimulation/recording data from the thick slice of brain tissue being cultured on the microscaffold system.

The brain slice microtower array design was incorporated onto the same set of six photomasks that was also used for the revised 12×12 microtower array design for re-aggregate neuronal cultures. The six photomask layers were the following; (1) the first SU-8 layer, (2) the electroplated tabs, (3) the electrodes and leads, (4) the second SU-8 layer, (5) the sacrificial layer, and (6) the third SU-8 layer.
2.1.2 Fluidic Design Analysis

The original 8×8 and 16×16 microtower array designs had fluid ports running along the vertical length of each microtower and along the horizontal length of each cross-member. All of these fluid ports were designed to be 20×20 µm². However, from a microfluidics analysis, uniform port sizes along the length of each tower do not yield uniform fluid volume flow rates. With the fluidic port design of the original 8×8 and 16×16 microtower arrays (where all of the side ports had dimensions of 20×20 µm²), larger fluid flow rates would occur out of the side ports located at lower vertical heights along each microtower than would occur out of the side ports located at greater vertical heights along the length of each microtower. To achieve uniform fluid volume flow rates, the ports that are located at higher vertical heights should be larger than the ports located at lower vertical heights. For the revised 12×12 microtower array design and for the brain slice microtower array design, ideal fluid port dimensions were calculated in collaboration with the Glezer Lab (Mechanical Engineering, Fluidics) at the Georgia Institute of Technology.

2.1.2.1 Fluid Channel and Port Calculations for the Revised 12×12 Microtower Array Design

In the revised 12×12 microtower array design for dissociated neuronal cultures, each microtower was designed to have three fluid side ports along its length, having dimensions of 8.5×8.5 µm², 9.8×9.8 µm², and 11.6×11.6 µm², located at vertical heights of 200 µm, 400 µm, and 600 µm, respectively. The dimensions for each side port were calculated, taking into account the cross-section of the fluid channel (40×23 µm²), the
width of the microtower wall (10 µm) containing the fluid side ports, and the height of each port (200 µm, 400 µm, and 600 µm). Also, the horizontal cross-members in the revised 12×12 microtower array design would still contain a fluid channel to assist in nutrient re-distribution in the case of a blockage in one of the microtower channels. However, since it was assumed and later confirmed by modeling (see Section 2.1.2.3.1: FEMLab Fluidic Model for the 12×12 Dissociated Microtower Design) that little fluid flow would actually occur in the horizontal cross-members, no fluid side ports were placed in the cross-members of the revised microtower arrays.

The one-dimensional (1-D) energy equation from fluid mechanics [58] was used to calculate the ideal dimensions for the fluid side ports that were placed along the length of each microtower. Three, square, fluid side ports were placed along the length of each microtower, centered at vertical heights of \( h_1 = 200 \mu m \), \( h_1 + h_2 = 400 \mu m \), and \( h_1 + h_2 + h_3 = 600 \mu m \), as shown in figure 2.5. Furthermore, each microtower was designed to have a height of 1.0 mm, and a hollow fluid channel with a rectangular cross-section \( (a_0 \times b_0) \) running along the entire length. The width of the fluid channel was designed to be \( b_0 = 40 \mu m \), and the depth of the fluid channel was designed to be \( a_0 = 23 \mu m \).
Since the hollow channel running through the microtower does not have a circular cross-section like a standard pipe, the hydraulic diameter ($d_i$) of the hollow channel had to be calculated from the dimensions of the rectangular cross-section ($a_0 \times b_0$) (see equation 1), yielding $d_0 = 29.2 \, \mu m$.

$$h_1 = 200 \, \mu m$$
$$h_2 = 200 \, \mu m$$
$$h_3 = 200 \, \mu m$$
$$h_4 = 400 \, \mu m$$

$(0), (1), (2), (3), and (4)$ refer to the nodes (inlet and outlet ports) for which the constant volume flow rates were evaluated.

$$Q = \text{volume flow rate} \ (\text{mm}^3/\text{s})$$
$$Q_0 = \sum_{i=1}^{4} Q_i$$
$$Q_0 = Q_1 + Q_2 + Q_3 + Q_4$$
$$Q_1 = Q_2 = Q_3 = Q_4 = Q$$

since the ideal system has equal volume flow rates out of each port.

$$Q_0 = 4Q$$
$$Q_{bd} = 3Q$$
$$Q_{df} = 2Q$$

**Figure 2.5.** Cross-sectional schematic of a microtower for dissociated neuronal cell culture, specifying variables for the microtower and fluid channel dimensions, and the locations of each fluid side port. There are three side ports per tower. This schematic was used to calculate the ideal dimensions for each side port to achieve uniform volume flow rates from the system.
\[ \text{hydraulic diameter} = d_i = \frac{2a_i b_i}{a_i + b_i} \text{, where } i = 0, 1, 2, 3, 4 \tag{1} \]

Also, as can be seen from figure 2.5, \( d_0 = d_4 \), since the hollow channel has identical dimensions at the base (node 0) and top (node 4) of the microtower. The cross-sectional dimensions of the three fluid side ports located at nodes 1, 2, and 3, are represented by the variables \( a_1 \times b_1, a_2 \times b_2, \) and \( a_3 \times b_3 \), respectively. Since all of the side ports are square, \( a_1 = b_1, a_2 = b_2, \) and \( a_3 = b_3 \), yielding \( d_1 = a_1, d_2 = a_2, \) and \( d_3 = a_3 \). Therefore, the 1-D energy equation was used to calculate values for \( d_1, d_2, \) and \( d_3 \), which directly provided the dimensions for each of the square side ports that would provide uniform fluid volume flow rates.

\( \beta_i \) is a ratio of the dimensions defined by equation 2

\[ \beta_i = \frac{a_i}{b_i} \text{, where } i = 0, 1, 2, 3, 4 \tag{2}. \]

Using the values, \( a_0 = 23 \, \mu\text{m} \), and \( b_0 = 40 \, \mu\text{m} \), gives \( \beta_0 = \beta_4 = 0.575 \). Also, since all of the fluid side ports were designed to have a square shape, \( \beta_1 = \beta_2 = \beta_3 = 1 \). The \( c_i \) value is then determined from a table of \( \beta_i \) vs. \( c_i \) values (see Table 2.1). Therefore, for all of the square side port calculations, \( c_1 = c_2 = c_3 = 56.9 \). Additionally, a \( \beta_0 = 0.575 \) value corresponds to a \( c_0 = 60.9 \) value, since there is a linear relationship between \( \beta_0 \) and \( c_i \).
Table 2.1. Specific $c_i$ values corresponding to specific $\beta_i$ values.

<table>
<thead>
<tr>
<th>$\beta_i$</th>
<th>$c_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>0.05</td>
<td>89.9</td>
</tr>
<tr>
<td>0.1</td>
<td>84.7</td>
</tr>
<tr>
<td>0.25</td>
<td>72.9</td>
</tr>
<tr>
<td>0.5</td>
<td>62.2</td>
</tr>
<tr>
<td>0.75</td>
<td>57.9</td>
</tr>
<tr>
<td>1</td>
<td>56.9</td>
</tr>
</tbody>
</table>

$Q$ (mm$^3$/s) refers to the volume flow rate, where $Q_0 = \sum_{i=1}^{4} Q_i$. Also, $K$ is defined to be the loss coefficient, and $\alpha$ is defined to be the kinetic energy coefficient that takes into account the velocity profile. $K_1 = K_2 = K_3 = K_4 = \alpha = 2$ for laminar flow in a pipe [58]. The specific weight is given by $\gamma$ (kN/m$^3$), which is a constant, implying that $\gamma_1 = \gamma_2 = \gamma_3 = \gamma_4 = \gamma$.

The hydrostatic pressure at each exit port is given by $p_i$, and since the system was designed to have equal pressure drops at each port, the relation between the hydrostatic pressures at each node are given by equation 3

$$p_i = p_{i+1} + \gamma h_{i+1} \text{ where } i = 0, 1, 2, 3 \quad (3).$$

Therefore, the relations $p_0 = p_1 + \gamma h_1$, $p_1 = p_2 + \gamma h_2$ and $p_2 = p_3 + \gamma h_3$ hold. The variable $\nu$ (m$^2$/s) represents the kinematic viscosity, $\rho$ (kg/m$^3$) represents the density, and $g=$
9.8 m/s² is the acceleration of gravity, \( v_i \) (m/s) represents the velocity, and \( f \) represents the friction factor given by equation 4

\[
f = \frac{c_i}{v_i d_i} \gamma_i, \quad \text{where } i = 0, 1, 2, 3, 4 \quad (4).
\]

A set of energy equations were developed corresponding to pressure drops at nodes 0-1, 0-2, 0-3, and 0-4. The energy equations corresponding to the microtower design are listed below. As shown in the energy equations for each set of nodes, the minor loss terms can be neglected since each minor loss term is \( (10^{-4}) \) times smaller than the friction term for \( v \approx 100 \mu m/s \).

For nodes (0-1), the energy equation is given by

\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_1}{\gamma} + h_i + f(h_i, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(t, v_i) \frac{t}{d_1} \frac{v_i^2}{2g} + \left( k_a \frac{v_i^2}{2g} + K_1 \frac{v_i^2}{2g} \right) \text{, where the}
\]

\[
\left( k_a \frac{v_i^2}{2g} + K_1 \frac{v_i^2}{2g} \right) \text{ term represents the minor losses and can therefore be neglected, yielding equation 5.}
\]

\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_1}{\gamma} + h_i + f(h_i, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(t, v_i) \frac{t}{d_1} \frac{v_i^2}{2g} \quad (5)
\]

For nodes (0-2), the energy equation is given by

\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_2}{\gamma} + (h_1 + h_2) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} + \left( k_b \frac{v_{bd}^2}{2g} + k_c \frac{v_2^2}{2g} + K_z \frac{v_2^2}{2g} \right) \text{, where the}
\]

\[
\left( k_b \frac{v_{bd}^2}{2g} + k_c \frac{v_2^2}{2g} + K_z \frac{v_2^2}{2g} \right) \text{ term represents the minor losses and can therefore be neglected, yielding equation 6.}
\]
\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_2}{\gamma} + (h_1 + h_2) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} \tag{6}
\]

For nodes (0-3), the energy equation is given by

\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_3}{\gamma} + (h_1 + h_2 + h_3) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} + \left( k_b \frac{v_{bd}^2}{2g} + k_c \frac{v_2^2}{2g} + k_e \frac{v_3^2}{2g} + K_3 \frac{v_3^2}{2g} \right),
\]

where the \( \left( k_b \frac{v_{bd}^2}{2g} + k_c \frac{v_2^2}{2g} + k_e \frac{v_3^2}{2g} + K_3 \frac{v_3^2}{2g} \right) \) term represents the minor losses and can therefore be neglected, yielding equation 7.

\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_3}{\gamma} + (h_1 + h_2 + h_3) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} \tag{7}
\]

For nodes (0-4),

\[
\frac{p_0}{\gamma} + \alpha \frac{V_{i}^2}{2g} = \frac{p_4}{\gamma} + (h_1 + h_2 + h_3 + h_4) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_4) \frac{h_4}{d_4} \frac{v_4^2}{2g} + \left( k_b \frac{v_{bd}^2}{2g} + k_d \frac{v_{df}^2}{2g} + k_f \frac{v_4^2}{2g} + K_4 \frac{v_4^2}{2g} \right),
\]

where the \( \left( k_b \frac{v_{bd}^2}{2g} + k_d \frac{v_{df}^2}{2g} + k_f \frac{v_4^2}{2g} + K_4 \frac{v_4^2}{2g} \right) \) term represents the minor losses and can therefore be neglected yielding equation 8.
\[ p_0 + \frac{\alpha V_0^2}{\gamma} = p_4 + (h_1 + h_2 + h_3 + h_4) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{\text{bd}}) \frac{h_2}{d_0} \frac{v_{\text{bd}}^2}{2g} + f(h_3, v_{\text{df}}) \frac{h_3}{d_0} \frac{v_{\text{df}}^2}{2g} \]

\[ f(h_3, v_{\text{df}}) \frac{h_3}{d_0} \frac{v_{\text{df}}^2}{2g} + f(h_4, v_4) \frac{h_4}{d_0} \frac{v_4^2}{2g} \]  \hspace{1cm} (8)

Setting (5) = (6) gives

\[ \frac{p_1}{\gamma} + f(t, v_1) \frac{t}{d_1} \frac{v_1^2}{2g} = \frac{p_2}{\gamma} + h_2 + f(h_2, v_{\text{bd}}) \frac{h_2}{d_0} \frac{v_{\text{bd}}^2}{2g} + f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} \].

Since \( p_1 = p_2 + \gamma h_2 \), the above equation simplifies to yield equation 9.

\[ f(t, v_1) \frac{t}{d_1} \frac{v_1^2}{2g} = f(h_2, v_{\text{bd}}) \frac{h_2}{d_0} \frac{v_{\text{bd}}^2}{2g} + f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} \]  \hspace{1cm} (9)

Setting (6) = (7) gives

\[ \frac{p_2}{\gamma} + f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} = \frac{p_3}{\gamma} + h_3 + f(h_3, v_{\text{df}}) \frac{h_3}{d_0} \frac{v_{\text{df}}^2}{2g} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} \].

Since \( p_2 = p_3 + \gamma h_3 \), the above equation simplifies to yield equation 10.

\[ f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} = f(h_3, v_{\text{df}}) \frac{h_3}{d_0} \frac{v_{\text{df}}^2}{2g} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} \]  \hspace{1cm} (10)

Setting (7) = (8) gives

\[ \frac{p_3}{\gamma} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} = \frac{p_4}{\gamma} + h_4 + f(h_4, v_4) \frac{h_4}{d_0} \frac{v_4^2}{2g} \].

Since \( p_3 = p_4 + \gamma h_4 \), the above equation simplifies to yield equation 11.

\[ f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} = f(h_4, v_4) \frac{h_4}{d_0} \frac{v_4^2}{2g} \]  \hspace{1cm} (11)
Now, it is necessary to use equation 4 for the friction factor \( f = \frac{C_f}{v_i d_i} \), and the general relation for the velocity out of each port given by equation 12.

\[
v_i = \frac{Q_i}{a_i b_i} = \frac{\beta_i Q_i}{a_i^2} = \frac{4\beta_i Q_i}{(1 + \beta_i)^2 d_i^2}
\] (12)

Therefore, the left side of equation (11) becomes

\[
f(t, v_3) = \frac{t v_3^2}{d_3^2 g} = c_3 \gamma t \frac{v_3}{2g} = c_3 \gamma t \frac{4\beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)}
\] (13),

and the right side of equation (11) becomes

\[
f(h_4, v_4) = \frac{h_4 v_4^2}{d_0^2 g} = c_4 \gamma h_4 \frac{v_4}{2g} = c_4 \gamma h_4 \frac{4\beta_4 Q_4}{(1 + \beta_4)^2 d_4^4 (2g)}
\] (14).

So, setting the right sides of (13) = (14) gives

\[
c_3 \gamma t \frac{4\beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)} = c_4 \gamma h_4 \frac{4\beta_4 Q_4}{(1 + \beta_4)^2 d_4^4 (2g)}
\]

which simplifies to

\[
c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4} = c_0 h_4 \frac{\beta_0}{(1 + \beta_0)^2 d_0^4}, \text{ yielding}
\]

\[
\frac{d_3}{d_0} = \left[ \frac{\beta_3 (1 + \beta_0)^2}{\beta_0 (1 + \beta_3)^2} \frac{c_3}{c_0} \frac{t}{h_4} \right]^{0.25}
\] (15).

Likewise, the left side of equation (10) becomes

\[
f(t, v_2) = \frac{t v_2^2}{d_2^2 g} = c_2 \gamma t \frac{v_2}{2g} = c_2 \gamma t \frac{4\beta_2 Q_2}{(1 + \beta_2)^2 d_2^4 (2g)}
\] (16)

and the right side of equation (10) becomes
\[ f(h_3, v_{df}) \frac{h_3}{d_0} v_{df}^2 + f(t, v_3) \frac{t}{d_3} v_3^2 = c_0 \gamma h_3 \frac{v_{df}}{2g} + c_3 \gamma t \frac{v_3}{2g} \]

\[ = c_0 \gamma h_3 \frac{4 \beta_0 Q_{df}}{(1 + \beta_0)^2 d_0^4 (2g)} + c_3 \gamma t \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)} \] \quad (17)

So, setting the right sides of (16) = (17) gives

\[ c_3 \gamma t \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)} = c_0 \gamma h_3 \frac{4 \beta_0 Q_{df}}{(1 + \beta_0)^2 d_0^4 (2g)} + c_3 \gamma t \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)}, \]

which simplifies to

\[ c_3 \gamma t \frac{\beta_3 Q}{(1 + \beta_3)^2 d_3^4} = c_0 \gamma h_3 \frac{\beta_0 (2Q)}{(1 + \beta_0)^2 d_0^4} + c_3 \gamma t \frac{\beta_3 Q}{(1 + \beta_3)^2 d_3^4}, \]

\[ c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4} = c_0 \gamma h_3 \frac{2 \beta_0}{(1 + \beta_0)^2 d_0^4} + c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4}. \]

Then, substituting from (15) the relation \( d_3 = d_0 \left[ \frac{\beta_3 (1 + \beta_0)^2 c_3 t}{\beta_0 (1 + \beta_3)^2 c_0 h_4} \right]^{0.25} \) yields

\[ c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4} = c_0 \gamma h_3 \frac{2 \beta_0}{(1 + \beta_0)^2 d_0^4} + c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4} \frac{\beta_0 (1 + \beta_3)^2 c_0 h_4}{\beta_3 (1 + \beta_0)^2 c_3 t}, \]

which simplifies to

\[ c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4} = c_0 \gamma h_3 \frac{2 \beta_0}{(1 + \beta_0)^2 d_0^4} + \frac{\beta_0 c_0 h_4}{(1 + \beta_3)^2 d_0^4}, \text{ and} \]

\[ c_3 \gamma t \frac{\beta_3}{(1 + \beta_3)^2 d_3^4} = \frac{c_0 \beta_0}{(1 + \beta_0)^2 d_0^4} (2h_3 + h_4), \text{ yielding} \]

\[ \frac{d_2}{d_0} = \left[ \frac{c_3 \beta_3 (1 + \beta_0)^2 t}{c_0 \beta_0 (1 + \beta_2)^2 (2h_3 + h_4)} \right]^{0.25} \] \quad (18)

Likewise, the left side of equation (9) becomes
\[ f(t,v) \cdot \frac{v_1^2}{d_1^4 \cdot 2g} = c_1 \cdot \frac{4\beta_1 Q_1}{(1 + \beta_1)^2 d_1^4 (2g)} \]  \tag{19},

and the right side of equation (9) becomes
\[ f(h_2, v_{bd}) \cdot \frac{h_2^2}{d_0^4 \cdot 2g} + f(t,v) \cdot \frac{v_2^2}{d_2^4 \cdot 2g} = c_0 \cdot \frac{4\beta_0 Q_{bd}}{(1 + \beta_0)^2 2g} + c_1 \cdot \frac{4\beta_2 Q_2}{(1 + \beta_2)^2 2g} \]  \tag{20}.

So, setting (19) = (20) yields
\[ c_1 \cdot \frac{4\beta_1 Q_1}{(1 + \beta_1)^2 d_1^4 (2g)} = c_0 \cdot \frac{4\beta_0 Q_{bd}}{(1 + \beta_0)^2 2g} + c_1 \cdot \frac{4\beta_2 Q_2}{(1 + \beta_2)^2 2g} \]

which simplifies to
\[ c_1 \cdot \frac{\beta_1 Q}{(1 + \beta_1)^2 d_1^4} = c_0 \cdot \frac{\beta_0 (3Q)}{(1 + \beta_0)^2} + c_1 \cdot \frac{\beta_2 Q}{(1 + \beta_2)^2} \]
\[ c_1 \cdot \frac{\beta_1}{(1 + \beta_1)^2 d_1^4} = c_0 \cdot \frac{3\beta_0}{(1 + \beta_0)^2} + c_1 \cdot \frac{\beta_2}{(1 + \beta_2)^2} \]

Then, substituting from (18) the relation \( d_2 = d_0 \left[ \frac{c_2 \beta_2 (1 + \beta_2)^2 t}{c_0 \beta_0 (1 + \beta_2)^2 (2h_3 + h_4)} \right]^{0.25} \) yields
\[ c_1 \cdot \frac{\beta_1}{(1 + \beta_1)^2 d_1^4} = c_0 \cdot \frac{3\beta_0}{(1 + \beta_0)^2} + c_1 \cdot \frac{\beta_2}{(1 + \beta_2)^2} \cdot \frac{c_0 \beta_0 (1 + \beta_2)^2 (2h_3 + h_4)}{d_0^4 c_2 \beta_2 (1 + \beta_0)^2 t} \]
\[ \frac{c_0 \beta_0}{d_0^4 (1 + \beta_0)^2} \cdot (3h_2 + 2h_3 + h_4) \]

yielding
\[ \frac{d_1}{d_0} = \left[ \frac{c_1 \beta_1 (1 + \beta_1)^2 t}{c_0 \beta_0 (1 + \beta_1)^2 (3h_2 + 2h_3 + h_4)} \right]^{0.25} \]  \tag{21}.

To solve for \( d_1 = a_1 = b_1 \) and therefore obtain dimensions for the lowermost side port, the known values were plugged into equation (21).
\[ d_1 = d_0 \left[ \frac{c_1 \beta_1 (1 + \beta_0)^2 t}{c_0 \beta_0 (1 + \beta_1)^2 (3h_2 + 2h_3 + h_4)} \right]^{0.25} \]

\[ = 29.2 \mu m \left[ \frac{(56.9)(1)(1 + 0.575)^2 (10 \mu m)}{(60.9)(0.575)(1+1)^2 [3(200 \mu m) + 2(200 \mu m) + 400 \mu m]} \right]^{0.25} = 8.5 \mu m \]

Hence, the lowermost side port centered at a vertical height of 200 \mu m was designed to be 8.5 \times 8.5 \mu m^2.

To solve for \( d_2 = a_2 = b_2 \) and therefore obtain dimensions for the side port centered at a height of 400 \mu m, the known values were plugged into equation (18).

\[ d_2 = d_0 \left[ \frac{c_2 \beta_2 (1 + \beta_0)^2 t}{c_0 \beta_0 (1 + \beta_2)^2 (2h_3 + h_4)} \right]^{0.25} \]

\[ = 29.2 \mu m \left[ \frac{(56.9)(1)(1 + 0.575)^2 (10 \mu m)}{(60.9)(0.575)(1+1)^2 [2(200 \mu m) + 400 \mu m]} \right]^{0.25} = 9.8 \mu m \]

Hence, the side port centered at a vertical height of 400 \mu m was designed to be 9.8 \times 9.8 \mu m^2.

To solve for \( d_3 = a_3 = b_3 \) and therefore obtain dimensions for the side port centered at a height of 600 \mu m, the known values were plugged into equation (15).

\[ d_3 = d_0 \left[ \frac{\beta_3 (1 + \beta_0)^2 c_3}{\beta_0 (1 + \beta_3)^2 c_0 h_4} \right]^{0.25} \]

\[ = 29.2 \mu m \left[ \frac{(1) (1 + 0.575)^2 (56.9) (10 \mu m)}{(0.575) (1+1)^2 (60.9) (400 \mu m)} \right]^{0.25} = 11.6 \mu m \]

Hence, the topmost side port centered at a vertical height of 600 \mu m was designed to be 11.6 \times 11.6 \mu m^2.
2.1.2.2 Fluid Channel and Port Calculations for the Brain Slice Microtower Array

Design

For the 12×12 brain slice microtower array design, each microtower was designed to have six fluid side ports along its length, having sizes of 4.0×4.0 µm², 4.5×4.5 µm², 5.0×5.0 µm², 5.5×5.5 µm², 6.0×6.0 µm², and 7.0×7.0 µm², located at vertical heights of 200 µm, 300 µm, 400 µm, 500 µm, 600 µm, and 700 µm, respectively. The sizes of each side port were calculated, taking into account the cross-section of the fluid channel (12×23 µm²), the width of the microtower wall (10 µm) containing the fluid side ports, and the height of each port.

The 1-D energy equation from fluid mechanics [58] was used to calculate the ideal dimensions for the fluid side ports that were placed along the length of each brain slice microtower. Six, square, fluid side ports were placed along the length of each microtower, centered at vertical heights of \( h_1 = 200 \, \mu m \), \( h_1 + h_2 = 300 \, \mu m \), and \( h_1 + h_2 + h_3 = 400 \, \mu m \), \( h_1 + h_2 + h_3 + h_4 = 500 \, \mu m \), \( h_1 + h_2 + h_3 + h_4 + h_5 = 600 \, \mu m \), and \( h_1 + h_2 + h_3 + h_4 + h_5 + h_6 = 700 \, \mu m \), as shown in figure 2.6. Furthermore, each brain slice microtower was designed to have a height of 1.0 mm, and a hollow fluid channel with a rectangular cross-section \((a_0 \times b_0)\) running along the entire length. The width of the fluid channel was designed to be \( a_0 = 12 \, \mu m \), and the depth of the fluid channel was designed to be \( b_0 = 23 \, \mu m \).
Figure 2.6. Cross-sectional schematic of a microtower for brain slice cultures, specifying variables for the microtower and fluid channel dimensions, and the locations of each fluid side port. There are six side ports per tower. This schematic was used to calculate the ideal dimensions for each side port to achieve uniform volume flow rates from the system.
Since the hollow channel running through the microtower does not have a circular cross-section like a standard pipe, the hydraulic diameter \( d_i \) of the hollow channel had to be calculated from the dimensions of the rectangular cross-section \( (a_0 \times b_0) \) (see equation 22), yielding \( d_0 = 15.78 \, \mu\text{m} \).

\[
\text{hydraulic diameter} = d_i = \frac{2a_ib_i}{a_i + b_i}, \quad \text{where} \quad i = 0, 1, 2, 3, 4, 5, 6, 7 \quad (22)
\]

Also, as can be seen from figure 2.6, \( d_0 = d_7 \), since the hollow channel has identical dimensions at the base (node 0) and top (node 7) of the microtower. The cross-sectional dimensions of the six fluid side ports located at nodes 1, 2, 3, 4, 5, and 6 are represented by the variables \( a_1 \times b_1, a_2 \times b_2, a_3 \times b_3, a_4 \times b_4, a_5 \times b_5, \) and \( a_6 \times b_6 \), respectively. Since all of the side ports are square, \( a_1 = b_1, a_2 = b_2, \ldots, a_6 = b_6, \) yielding \( d_1 = a_1, d_2 = a_2, \ldots, d_6 = a_6. \) Therefore, the 1-D energy equation was used to calculate values for \( d_1, d_2, d_3, d_4, d_5, \) and \( d_6, \) which directly provided the dimensions for each of the square side ports that would provide uniform volume flow rates.

\[ \beta_i \] is a ratio of the dimensions defined by equation 23.

\[
\beta_i = \frac{a_i}{b_i}, \quad \text{where} \quad i = 0, 1, 2, 3, 4, 5, 6, 7 \quad (23)
\]

Using the values, \( a_0 = 12 \, \mu\text{m} \), and \( b_0 = 23 \, \mu\text{m} \), gives \( \beta_0 = \beta_7 = 0.522 \). Also, since all of the fluid side ports have a square shape, \( \beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5 = \beta_6 = 1 \). The \( c_i \) value is then determined from a table of \( \beta_i \) vs. \( c_i \) values (see Table 2.1). Therefore, for all of the square side port calculations, \( c_1 = c_2 = c_3 = c_4 = c_5 = c_6 = c = 56.9 \). Additionally, a \( \beta_0 = 0.522 \) value corresponds to a \( c_0 = 61.8 \) value.
Q (mm³/s) refers to the volume flow rate, where \( Q_0 = \sum_{i=1}^{7} Q_i \). Also, K is defined to be the loss coefficient, and \( \alpha \) is defined to be the kinetic energy coefficient that takes into account the velocity profile. \( K_1 = K_2 = K_3 = K_4 = K_5 = K_6 = K_7 = \alpha = 2 \) for laminar flow in a pipe [58]. The specific weight is given by \( \gamma \) (kN/m³), which is a constant, implying that \( \gamma_1 = \gamma_2 = \gamma_3 = \gamma_4 = \gamma_5 = \gamma_6 = \gamma_7 = \gamma \).

The hydrostatic pressure at each exit port is given by \( p_i \), and since the system was designed to have equal pressure drops at each port, the relation between the hydrostatic pressures at each node are given by equation 24.

\[
p_i = p_{i+1} + \gamma h_{i+1}, \text{ where } i = 0, 1, 2, 3, 4, 5, 6 \quad (24)
\]

Therefore, the relations \( p_0 = p_1 + \gamma h_1 \), \( p_1 = p_2 + \gamma h_2 \) and \( p_2 = p_3 + \gamma h_3 \), ..., \( p_5 = p_6 + \gamma h_6 \) hold. The variable \( \nu \) (m²/s) represents the kinematic viscosity, \( \rho \) (kg/m³) represents the density, \( g = 9.8 \text{ m/s}^2 \) is the acceleration of gravity, \( \nu_1 \) (m/s) represents the velocity, and \( f \) represents the friction factor given by equation 25.

\[
f = \frac{c_i}{\nu_i d_i} \gamma_i, \text{ where } i = 0, 1, 2, 3, 4, 5, 6, 7 \quad (25)
\]

A set of energy equations were developed corresponding to pressure drops at nodes 0-1, 0-2, 0-3, 0-4, 0-5, 0-6, and 0-7. The energy equations corresponding to the brain slice microtower design are listed below. As shown in the energy equations for each set of nodes, the minor loss terms can be neglected since each minor loss term is \((10^{-4})\) times smaller than the friction term for \( \nu \approx 100 \text{ µm/s} \).
For nodes (0-1), the energy equation is given by

\[
\frac{P_0}{\gamma} + \alpha \frac{v_0^2}{2g} = P_{1} + h_1 + f(h_1, v_0) \frac{h_1 v_0^2}{d_0 2g} + f(t, v_1) \frac{t v_1^2}{d_1 2g} + \left( k_a + K_1 \right) \frac{v_1^2}{2g},
\]

where the \( \left( k_a + K_1 \right) \frac{v_1^2}{2g} \) term represents the minor losses and can therefore be neglected yielding equation 26.

\[
\frac{P_0}{\gamma} + \alpha \frac{v_0^2}{2g} = P_{1} + h_1 + f(h_1, v_0) \frac{h_1 v_0^2}{d_0 2g} + f(t, v_1) \frac{t v_1^2}{d_1 2g}
\]

For nodes (0-2), the energy equation is given by

\[
\frac{P_0}{\gamma} + \alpha \frac{v_0^2}{2g} = P_{2} + (h_1 + h_2) + f(h_1, v_0) \frac{h_1 v_0^2}{d_0 2g} + f(h_2, v_{bd}) \frac{h_2 v_{bd}^2}{d_0 2g} + f(t, v_2) \frac{t v_2^2}{d_2 2g} + \left( k_b + k_c + K_2 \right) \frac{v_2^2}{2g},
\]

where the \( \left( k_b + k_c + K_2 \right) \frac{v_2^2}{2g} \) term represents the minor losses and can therefore be neglected, yielding equation 27.

\[
\frac{P_0}{\gamma} + \alpha \frac{v_0^2}{2g} = P_{2} + (h_1 + h_2) + f(h_1, v_0) \frac{h_1 v_0^2}{d_0 2g} + f(h_2, v_{bd}) \frac{h_2 v_{bd}^2}{d_0 2g} + f(t, v_2) \frac{t v_2^2}{d_2 2g}
\]

For nodes (0-3), the energy equation is given by

\[
\frac{P_0}{\gamma} + \alpha \frac{v_0^2}{2g} = P_{3} + (h_1 + h_2 + h_3) + f(h_1, v_0) \frac{h_1 v_0^2}{d_0 2g} + f(h_2, v_{bd}) \frac{h_2 v_{bd}^2}{d_0 2g} + f(h_3, v_{d}) \frac{h_3 v_{d}^2}{d_0 2g} + f(t, v_3) \frac{t v_3^2}{d_3 2g} + \left( k_b + k_d + k_e + K_3 \right) \frac{v_3^2}{2g},
\]

where the \( \left( k_b + k_d + k_e + K_3 \right) \frac{v_3^2}{2g} \) term represents the minor losses and can therefore be neglected, yielding equation 28.
\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_2}{\gamma} + (h_1 + h_2 + h_3) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} \quad (28)
\]

**For nodes (0-4)**, the energy equation is given by

\[
\frac{p_0}{\gamma} + \alpha \frac{V_4^2}{2g} = \frac{p_4}{\gamma} + (h_1 + h_2 + h_3 + h_4) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_{jh}) \frac{h_4}{d_0} \frac{v_{jh}^2}{2g} + f(t, v_4) \frac{t}{d_4} \frac{v_4^2}{2g} + \left( k_b + k_d + k_f + k_i + K_4 \right) \frac{v_4^2}{2g},
\]

where the \( \left( k_b + k_d + k_f + k_i + K_4 \right) \frac{v_4^2}{2g} \) term represents the minor losses and can therefore be neglected yielding equation (29).

\[
\frac{p_0}{\gamma} + \alpha \frac{V_4^2}{2g} = \frac{p_4}{\gamma} + (h_1 + h_2 + h_3 + h_4) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_{jh}) \frac{h_4}{d_0} \frac{v_{jh}^2}{2g} + f(t, v_4) \frac{t}{d_4} \frac{v_4^2}{2g} \quad (29)
\]

**For nodes (0-5)**, the energy equation is given by

\[
\frac{p_0}{\gamma} + \alpha \frac{V_5^2}{2g} = \frac{p_5}{\gamma} + (h_1 + h_2 + h_3 + h_4 + h_5) + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_{jh}) \frac{h_4}{d_0} \frac{v_{jh}^2}{2g} + f(h_5, v_{hj}) \frac{h_5}{d_0} \frac{v_{hj}^2}{2g} + f(t, v_5) \frac{t}{d_5} \frac{v_5^2}{2g} + \left( k_b + k_d + k_f + k_i + k_h + K_5 \right) \frac{v_5^2}{2g},
\]

where the \( \left( k_b + k_d + k_f + k_h + K_5 \right) \frac{v_5^2}{2g} \) term represents the minor losses and can therefore be neglected yielding equation (30).
For nodes (0-6), the energy equation is given by

\[
p_0 + \alpha \frac{v_0^2}{2g} = \frac{p_s}{\gamma} + \sum_{i=1}^{5} h_i + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{id}) \frac{h_2}{d_0} \frac{v_{id}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_{fh}) \frac{h_4}{d_0} \frac{v_{fh}^2}{2g} +
\]

\[
f(h_5, v_{hf}) \frac{h_5}{d_0} \frac{v_{hf}^2}{2g} + f(h_6, v_{hf}) \frac{h_6}{d_0} \frac{v_{hf}^2}{2g} + f(t, v_6) \frac{t}{d_0} \frac{v_6^2}{2g} + \left( k_h + k_d + k_f + k_h + k_j + k_1 + \frac{v_6^2}{2g} \right)
\]

where the \[ \left( k_h + k_d + k_f + k_h + k_j + k_1 + \frac{v_6^2}{2g} \right) \] term represents the minor losses and can therefore be neglected, yielding equation (31).

\[
p_0 + \alpha \frac{v_0^2}{2g} = \frac{p_s}{\gamma} + \sum_{i=1}^{5} h_i + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{id}) \frac{h_2}{d_0} \frac{v_{id}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_{fh}) \frac{h_4}{d_0} \frac{v_{fh}^2}{2g} +
\]

\[
f(h_5, v_{hf}) \frac{h_5}{d_0} \frac{v_{hf}^2}{2g} + f(h_6, v_{hf}) \frac{h_6}{d_0} \frac{v_{hf}^2}{2g} + f(t, v_6) \frac{t}{d_0} \frac{v_6^2}{2g} \quad (31)
\]

For nodes (0-7), the energy equation is given by

\[
p_0 + \alpha \frac{v_0^2}{2g} = \frac{p_s}{\gamma} + \sum_{i=1}^{6} h_i + f(h_1, v_0) \frac{h_1}{d_0} \frac{v_0^2}{2g} + f(h_2, v_{id}) \frac{h_2}{d_0} \frac{v_{id}^2}{2g} + f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(h_4, v_{fh}) \frac{h_4}{d_0} \frac{v_{fh}^2}{2g} +
\]

\[
f(h_5, v_{hf}) \frac{h_5}{d_0} \frac{v_{hf}^2}{2g} + f(h_6, v_{hf}) \frac{h_6}{d_0} \frac{v_{hf}^2}{2g} + f(h_7, v_{hf}) \frac{h_7}{d_0} \frac{v_{hf}^2}{2g} + \left( k_b + k_d + k_f + k_h + k_j + k_1 + \frac{v_7^2}{2g} \right)
\]

where the \[ \left( k_b + k_d + k_f + k_h + k_j + k_1 + \frac{v_7^2}{2g} \right) \] term represents the minor losses and can therefore be neglected, yielding equation (32).
\[
\frac{p_0}{\gamma} + \alpha \frac{v_0^2}{2g} = \frac{p_2}{\gamma} + \sum_{i=1}^{2} h_i + f(h_1,v_i)\frac{h_1 v_0^2}{d_0 2g} + f(h_2,v_{zd})\frac{h_2 v_{zd}^2}{d_0 2g} + f(h_3,v_{zd'})\frac{h_3 v_{zd'}^2}{d_0 2g} + f(h_4,v_{zd''})\frac{h_4 v_{zd''}^2}{d_0 2g} + f(h_5,v_{zd''})\frac{h_5 v_{zd''}^2}{d_0 2g} + f(h_6,v_{zd'})\frac{h_6 v_{zd'}^2}{d_0 2g} + f(h_7,v_{zd''})\frac{h_7 v_{zd''}^2}{d_0 2g} + f(h_8,v_{zd'})\frac{h_8 v_{zd'}^2}{d_0 2g} + f(h_9,v_{zd''})\frac{h_9 v_{zd''}^2}{d_0 2g} + f(h_{10},v_{zd''})\frac{h_{10} v_{zd''}^2}{d_0 2g} \]

\[ (32) \]

**Setting (26)=(27) [for nodes (0-1)↔(0-2)] gives**

\[
\frac{p_1}{\gamma} + f(t,v_1)\frac{t v_1^2}{d_1 2g} = \frac{p_2}{\gamma} + h_2 + f(h_2,v_{zd})\frac{h_2 v_{zd}^2}{d_0 2g} + f(t,v_2)\frac{t v_2^2}{d_2 2g}
\]

Since \( p_1 = p_2 + \gamma h_2 \), the above equation simplifies to yield equation (33).

\[
f(t,v_1)\frac{t v_1^2}{d_1 2g} = f(h_2,v_{zd})\frac{h_2 v_{zd}^2}{d_0 2g} + f(t,v_2)\frac{t v_2^2}{d_2 2g} \]

\[ (33) \]

**Setting (27)=(28) [for nodes (0-2)↔(0-3)] gives**

\[
\frac{p_2}{\gamma} + f(t,v_2)\frac{t v_2^2}{d_2 2g} = \frac{p_3}{\gamma} + h_3 + f(h_3,v_{zd'})\frac{h_3 v_{zd'}^2}{d_0 2g} + f(t,v_3)\frac{t v_3^2}{d_3 2g}
\]

Since \( p_2 = p_3 + \gamma h_3 \), the above equation simplifies to yield equation (34).

\[
f(t,v_2)\frac{t v_2^2}{d_2 2g} = f(h_3,v_{zd'})\frac{h_3 v_{zd'}^2}{d_0 2g} + f(t,v_3)\frac{t v_3^2}{d_3 2g} \]

\[ (34) \]

**Setting (28)=(29) [for nodes (0-3)↔(0-4)] gives**

\[
\frac{p_3}{\gamma} + f(t,v_3)\frac{t v_3^2}{d_3 2g} = \frac{p_4}{\gamma} + h_4 + f(h_4,v_{zd''})\frac{h_4 v_{zd''}^2}{d_0 2g} + f(t,v_4)\frac{t v_4^2}{d_4 2g}
\]

Since \( p_3 = p_4 + \gamma h_4 \), the above equation simplifies to yield equation (35).

\[
f(t,v_3)\frac{t v_3^2}{d_3 2g} = f(h_4,v_{zd''})\frac{h_4 v_{zd''}^2}{d_0 2g} + f(t,v_4)\frac{t v_4^2}{d_4 2g} \]

\[ (35) \]

**Setting (29)=(30) [for nodes (0-4)↔(0-5)] gives**

\[
\frac{p_4}{\gamma} + f(t,v_4)\frac{t v_4^2}{d_4 2g} = \frac{p_5}{\gamma} + h_5 + f(h_5,v_{zd''})\frac{h_5 v_{zd''}^2}{d_0 2g} + f(t,v_5)\frac{t v_5^2}{d_5 2g}
\]
Since \( p_5 = p_6 + \gamma h_5 \), the above equation simplifies to yield equation (36).

\[
f(t,v_4) \frac{t_{v_4}^2}{d_4} = f(h_5,v_{hi}) \frac{h_5 v_{hi}^2}{d_0} + f(t,v_5) \frac{t_{v_5}^2}{d_5} \tag{36}
\]

Setting (30)=(31) [for nodes (0-5)↔(0-6)] gives

\[
p_5 + f(t,v_5) \frac{t_{v_5}^2}{d_5} 2g = p_6 + f(h_6,v_{hi}) \frac{h_6 v_{hi}^2}{d_0} + f(t,v_6) \frac{t_{v_6}^2}{d_6} 2g
\]

Since \( p_5 = p_6 + \gamma h_6 \), the above equation simplifies to yield equation (37).

\[
f(t,v_5) \frac{t_{v_5}^2}{d_5} \frac{2g}{2} = f(h_6,v_{hi}) \frac{h_6 v_{hi}^2}{d_0} 2g + f(t,v_6) \frac{t_{v_6}^2}{d_6} 2g \tag{37}
\]

Setting (31)=(32) [for nodes (0-6)↔(0-7)] gives

\[
p_6 + f(t,v_6) \frac{t_{v_6}^2}{d_6} \frac{2g}{2} = p_7 + f(h_7,v_{hi}) \frac{h_7 v_{hi}^2}{d_0} 2g + f(t,v_7) \frac{t_{v_7}^2}{d_7} 2g
\]

Since \( p_6 = p_7 + \gamma h_7 \), the above equation simplifies to yield equation (38).

\[
f(t,v_6) \frac{t_{v_6}^2}{d_6} \frac{2g}{2} = f(h_7,v_{i}) \frac{h_7 v_{i}^2}{d_0} 2g \tag{38}
\]

Now, it is necessary to use equation 25 for the friction factor \( \left( f = \frac{c_i}{v_i d_i^2} \gamma_i \right) \), and

the general relation for the velocity out of each port given by equation 12.

\[
v_i = \frac{Q_i}{a_i b_i} = \frac{\beta_i Q_i}{a_i^2} = \frac{4 \beta_i Q_i}{(1 + \beta_i)^2 d_i^2} \tag{12}
\]

Therefore, the left side of equation (38) becomes

\[
f(t,v_6) \frac{t_{v_6}^2}{d_6} \frac{2g}{2} = \frac{c_6}{d_6^2} \frac{\gamma_t}{2g} \frac{v_6}{d_6} = c_6 \gamma_t \frac{4 \beta_6 Q_6}{(1 + \beta_6)^2 d_6^2 (2g)} \tag{39},
\]

and the right side of equation (38) becomes

\[
\frac{c_6}{d_6^2} \frac{\gamma_t}{2g} \frac{v_6}{d_6} = c_6 \gamma_t \frac{4 \beta_6 Q_6}{(1 + \beta_6)^2 d_6^2 (2g)}
\]

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\[ f(h_7, v_7) \frac{h_7}{d_0} \frac{v_7^2}{2g} = \frac{c_0 \gamma h_7}{d_0} \frac{v_7}{2g} = c_0 \gamma h_7 \frac{4\beta_0 Q_7}{(1 + \beta_0)^2 d_0^4(2g)} \]  
(40).

So, setting the right sides of (39) = (40) gives

\[ c_5 \gamma t \frac{4\beta_0 Q_6}{(1 + \beta_0)^2 d_6^4(2g)} = c_0 \gamma h_7 \frac{4\beta_0 Q_7}{(1 + \beta_0)^2 d_0^4(2g)} \]

\[ c_6 t \frac{\beta_6}{(1 + \beta_0)^2 d_6^4} = c_0 h_7 \frac{\beta_0}{(1 + \beta_0)^2 d_0^4}, \text{ yielding} \]

\[ d_6 = d_0 \left[ \frac{ct}{4c_0 \beta_0^2} \frac{(1 + \beta_0)^2}{h_7} \right]^{0.25} \]  
(41).

Likewise, the left side of equation (37) becomes

\[ f(t, v_5) \frac{t}{d_5} \frac{v_5^2}{2g} = \frac{c_5 \gamma t}{d_5} \frac{v_5}{2g} = c_5 \gamma t \frac{4\beta_5 Q_5}{(1 + \beta_5)^2 d_5^4(2g)} \]  
(42),

and the right side of equation (37) becomes

\[ f(h_6, v_{jl}) \frac{h_6}{d_0} \frac{v_{jl}^2}{2g} + f(t, v_6) \frac{t}{d_6} \frac{v_6^2}{d_6} \frac{v_6}{2g} = c_0 \gamma h_6 \frac{4\beta_6 Q_{jl}}{(1 + \beta_6)^2 d_6^4(2g)} + c_6 \gamma t \frac{4\beta_6 Q_6}{(1 + \beta_6)^2 d_6^4(2g)} \]

(43).

So, setting the right sides of (41) = (43) gives

\[ c_5 \gamma t \frac{4\beta_5 Q}{(1 + \beta_5)^2 d_5^4(2g)} = c_0 \gamma h_6 \frac{4\beta_0(2Q)}{(1 + \beta_0)^2 d_0^4(2g)} + c_6 \gamma t \frac{4\beta_6 Q}{(1 + \beta_6)^2 d_6^4(2g)} \], yielding

\[ d_5 = d_0 \left[ \frac{ct(1 + \beta_0)^2}{4c_0 \beta_0^2(2h_6 + h_7)} \right]^{0.25} \]  
(44).

Likewise, the left side of equation (36) becomes
\[ f(t, v_t) \frac{t}{d_4} \frac{v_t}{2g} = \frac{c_4}{d_4^2} \gamma t \frac{v_t}{2g} = \frac{4 \beta_4 Q_4}{(1 + \beta_4)^2 d_4^4 (2g)} \]  
(45)

and the right side of equation (36) becomes

\[
f(h_5, v_{h_5}) \frac{h_5}{d_0} \frac{v_{h_5}}{2g} + f(t, v_t) \frac{t}{d_0^2} \frac{v_t}{2g} = \frac{c_0}{d_0^2} \gamma h_5 \frac{v_{h_5}}{2g} + \frac{c_5}{d_5^2} \gamma t \frac{v_t}{2g},
\]

\[
= c_0 \gamma h_5 \frac{4 \beta_0 O_{h_5}}{(1 + \beta_0)^2 d_0^4 (2g)} + c_5 \gamma t \frac{4 \beta_5 Q_5}{(1 + \beta_5)^2 d_5^4 (2g)} \]  
(46).

So, setting the right sides of (45) = (46) gives

\[
c_4 \gamma t \frac{4 \beta_4 Q_4}{(1 + \beta_4)^2 d_4^4 (2g)} = c_0 \gamma h_5 \frac{4 \beta_0 (3Q)}{(1 + \beta_0)^2 d_0^4 (2g)} + c_5 \gamma t \frac{4 \beta_5 Q_5}{(1 + \beta_5)^2 d_5^4 (2g)},
\]

yielding

\[ d_4 = d_0 \left( \frac{ct(1 + \beta_0)^2}{4c_0 \beta_0 (3h_5 + 2h_6 + h_7)} \right)^{0.25} \]  
(47).

Likewise, the left side of equation (35) becomes

\[ f(t, v_3) \frac{t}{d_3} \frac{v_3}{2g} = \frac{c_3}{d_3^2} \gamma t \frac{v_3}{2g} = \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)} \]  
(48)

and the right side of equation (35) becomes

\[
f(h_4, v_{h_4}) \frac{h_4}{d_0} \frac{v_{h_4}}{2g} + f(t, v_t) \frac{t}{d_0^2} \frac{v_t}{2g} = \frac{c_0}{d_0^2} \gamma h_4 \frac{v_{h_4}}{2g} + \frac{c_4}{d_4^2} \gamma t \frac{v_t}{2g},
\]

\[
= c_0 \gamma h_4 \frac{4 \beta_0 (4Q)}{(1 + \beta_0)^2 d_0^4 (2g)} + c_4 \gamma t \frac{4 \beta_4 Q_4}{(1 + \beta_4)^2 d_4^4 (2g)} \]  
(49).

So, setting the right sides of (48) = (49) gives

\[
c_3 \gamma t \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)} = c_0 \gamma h_4 \frac{4 \beta_0 (4Q)}{(1 + \beta_0)^2 d_0^4 (2g)} + c_4 \gamma t \frac{4 \beta_4 Q_4}{(1 + \beta_4)^2 d_4^4 (2g)},
\]

yielding

\[ d_3 = d_0 \left( \frac{ct(1 + \beta_0)^2}{4c_0 \beta_0 (4h_4 + 3h_5 + 2h_6 + h_7)} \right)^{0.25} \]  
(50).
Likewise, the left side of equation (34) becomes

$$f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} = c_2 \gamma t \frac{v_2}{2g} = c_2 \gamma t \frac{4 \beta_2 Q_2}{(1 + \beta_2)^3 d_2^4 (2g)}$$  \hspace{1cm} (51)$$

and the right side of equation (34) becomes

$$f(h_3, v_{df}) \frac{h_3}{d_0} \frac{v_{df}^2}{2g} + f(t, v_3) \frac{t}{d_3} \frac{v_3^2}{2g} = c_0 \gamma h_3 \frac{v_{df}}{2g} + c_3 \gamma t \frac{v_3}{2g}$$

$$= c_0 \gamma h_3 \frac{4 \beta_0 Q_{df}}{(1 + \beta_0)^2 d_0^4 2g} + c_3 \gamma t \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 2g}$$  \hspace{1cm} (52).$$

So, setting the right sides of (51) = (52) gives

$$c_2 \gamma t \frac{4 \beta_2 Q}{(1 + \beta_2)^3 d_2^4 (2g)} = c_0 \gamma h_3 \frac{4 \beta_0 (5Q)}{(1 + \beta_0)^2 d_0^4 2g} + c_3 \gamma t \frac{4 \beta_3 Q}{(1 + \beta_3)^2 d_3^4 2g}$$

$$d_2 = d_0 \left( \frac{ct (1 + \beta_0)^2}{4 c_0 \beta_0 (5h_3 + 4h_4 + 3h_5 + 2h_6 + h_7)} \right)^{0.25}$$  \hspace{1cm} (53).$$

Likewise, the left side of equation (33) becomes

$$f(t, v_1) \frac{t}{d_1} \frac{v_1^2}{2g} = c_1 \gamma t \frac{v_1}{2g} = c_1 \gamma t \frac{4 \beta_1 Q_1}{(1 + \beta_1)^3 d_1^4 (2g)}$$  \hspace{1cm} (54)$$

and the right side of equation (33) becomes

$$f(h_2, v_{bd}) \frac{h_2}{d_0} \frac{v_{bd}^2}{2g} + f(t, v_2) \frac{t}{d_2} \frac{v_2^2}{2g} = c_0 \gamma v_{bd} t \frac{v_{bd}}{2g} + c_2 \gamma t \frac{v_2}{2g}$$

$$= c_0 \gamma h_2 \frac{4 \beta_0 Q_{bd}}{(1 + \beta_0)^2 d_0^4 (2g)} + c_2 \gamma t \frac{4 \beta_3 Q_3}{(1 + \beta_3)^2 d_3^4 (2g)}$$  \hspace{1cm} (55).$$

So, setting the right sides of (54) = (55) gives

$$c_1 \gamma t \frac{4 \beta_1 Q}{(1 + \beta_1)^3 d_1^4 (2g)} = c_0 \gamma h_2 \frac{4 \beta_0 (6Q)}{(1 + \beta_0)^2 d_0^4 (2g)} + c_2 \gamma t \frac{4 \beta_3 Q}{(1 + \beta_3)^2 d_3^4 (2g)}$$

yielding
\[ d_1 = d_0 \left( \frac{c1 + \beta_0}{4c_0 \beta_0 \left( 6h_2 + 5h_3 + 4h_4 + 3h_5 + 2h_6 + h_7 \right)} \right)^{0.25} \]  \hspace{1cm} (56).

To solve for \( d_1 = a_1 = b_1 \) and therefore obtain dimensions for the lowermost side port, the known values were plugged into equation (56).

\[ \frac{d_1}{15.78 \mu m} = \left( \frac{(56.9)(10 \mu m)(1 + 0.522)^2}{4(61.8)(0.522)(6(100 \mu m) + 5(100 \mu m) + 4(100 \mu m) + 3(100 \mu m) + 2(100 \mu m) + (300 \mu m))} \right)^{0.25} \]

\[ d_1 = 4.07 \mu m \]

Hence, the lowermost side port centered at a vertical height of 200 µm was originally designed to be 4.0×4.0 µm² for the brain slice microtowers.

To solve for \( d_2 = a_2 = b_2 \) and therefore obtain dimensions for the side port centered at a vertical height of 300 µm, the known values were plugged into equation (53).

\[ d_2 = d_0 \left( \frac{c1 + \beta_0}{4c_0 \beta_0 \left( 6h_3 + 4h_4 + 3h_5 + 2h_6 + h_7 \right)} \right)^{0.25} \]

\[ d_2 = (15.78 \mu m) \left( \frac{(56.9)(10 \mu m)(1 + 0.522)^2}{4(61.8)(0.522)(5(100 \mu m) + 4(100 \mu m) + 3(100 \mu m) + 2(100 \mu m) + (300 \mu m))} \right)^{0.25} \]

\[ d_2 = 4.39 \mu m \]

Hence, the side port centered at a vertical height of 300 µm was originally designed to be 4.5×4.5 µm² for the brain slice microtowers.

To solve for \( d_3 = a_3 = b_3 \) and therefore obtain dimensions for the side port centered at a vertical height of 400 µm, the known values were plugged into equation (50).
\[ d_3 = d_0 \left( \frac{ct(1 + \beta_0)^2}{4c_0\beta_0(4h + 3h_5 + 2h_6 + h_7)} \right)^{0.25} \]
\[ d_3 = (15.78 \mu\text{m}) \left( \frac{(56.9)(10 \mu\text{m})(1 + 0.522)^2}{4(61.8)(0.522)[4(100 \mu\text{m}) + 3(100 \mu\text{m}) + 2(100 \mu\text{m}) + (300 \mu\text{m})]} \right)^{0.25} \]
\[ d_3 = 4.79 \mu\text{m} \]

Hence, the side port centered at a vertical height of 400 \( \mu\text{m} \) was originally designed to be 5.0\( \times \)5.0 \( \mu\text{m}^2 \) for the brain slice microtowers.

To solve for \( d_4 = a_4 = b_4 \) and therefore obtain dimensions for the side port centered at a vertical height of 500 \( \mu\text{m} \), the known values were plugged into equation (47).

\[ d_4 = d_0 \left( \frac{ct(1 + \beta_0)^2}{4c_0\beta_0(3h_5 + 2h_6 + h_7)} \right)^{0.25} \]
\[ d_4 = (15.78 \mu\text{m}) \left( \frac{(56.9)(10 \mu\text{m})(1 + 0.522)^2}{4(61.8)(0.522)[3(100 \mu\text{m}) + 2(100 \mu\text{m}) + (300 \mu\text{m})]} \right)^{0.25} \]
\[ d_4 = 5.30 \mu\text{m} \]

Hence, the side port centered at a vertical height of 500 \( \mu\text{m} \) was originally designed to be 5.5\( \times \)5.5 \( \mu\text{m}^2 \) for the brain slice microtowers.

To solve for \( d_5 = a_5 = b_5 \) and therefore obtain dimensions for the side port centered at a vertical height of 600 \( \mu\text{m} \), the known values were plugged into equation (44).

\[ d_5 = d_0 \left( \frac{ct(1 + \beta_0)^2}{4c_0\beta_0(2h_6 + h_7)} \right)^{0.25} \]
\[ d_5 = (15.78 \mu\text{m}) \left( \frac{(56.9)(10 \mu\text{m})(1 + 0.522)^2}{4(61.8)(0.522)[2(100 \mu\text{m}) + (300 \mu\text{m})]} \right)^{0.25} \]
\[ d_5 = 5.97 \mu\text{m} \]
Hence, the side port centered at a vertical height of 600 µm was originally designed to be 6.0×6.0 µm² for the brain slice microtowers.

To solve for \( d_6 = a_6 = b_6 \) and therefore obtain dimensions for the side port centered at a vertical height of 700 µm, the known values were plugged into equation (41).

\[
d_6 = d_0 \left[ \frac{ct \ (1 + \beta_0)}{h_7} \right]^{0.25}
\]

\[
d_6 = (15.78\mu m) \left[ \frac{(56.9) \ (10\mu m) (1 + 0.522)^2}{4(61.8) \ (0.522) \ (300\mu m)} \right]^{0.25}
\]

\[d_6 = 6.78\mu m\]

Hence, the side port centered at a vertical height of 700 µm was originally designed to be 7.0×7.0 µm² for the brain slice microtowers. To simplify the brain slice microtower design and to accommodate the accuracy of the photomask resolution, the dimensions of the side ports in the brain slice microtower design were rounded to the nearest 0.5 µm.

### 2.1.2.3 FEMLab Fluidic Model

FEMLab software was used to model the functioning fluidics in the 12×12 microtower array design for re-aggregate cultures and the microtower array design for brain slice cultures.

#### 2.1.2.3.1 FEMLab Fluidic Model for the 12x12 Dissociated Microtower Array Design

A 2-D FEMLab fluidic model was performed on a single linear microtower array for dissociated neuronal cultures, containing 12 microtowers per linear array. The calculated fluidic design parameters were used in the modeling, with a 40 µm wide fluid
channel, and the fluid side ports were placed at vertical heights of 200 µm, 400 µm, and 600 µm, having dimensions of 8.5×8.5 µm², 9.8×9.8 µm², and 11.6×11.6 µm², respectively. The fluid entered the microtower array through the base of each microtower. The fluid experiments provided fluid to the microscaffold system via a syringe pump, and the biological experiments confirmed an optimal flow rate of 0.05 µL/hr for the dissociated neuronal cultures [see Section 4.3.1: Biological Tests on the Packaged Microscaffolds with Perfusion (Compared to Cultures on the Packaged Microscaffolds without Perfusion) Performed by the Brewer Lab at the Southern Illinois University School of Medicine], so a flow rate of 0.05 µL/hr was used in these FEMLab simulations.

Also, the calculated side port dimensions were performed using a hollow channel of cross-section 40×23 µm², however the microtowers that were fabricated actually had hollow channels with cross-sections of 40×17 µm². The FEMLab model uses the dimensions of the microtower arrays that were actually fabricated. Also, each of the four horizontal cross-members contained a hollow channel of identical dimensions that connected to the vertical hollow channel in each microtower. Figure 2.7 shows the arrow velocity field in the microtower array, and confirms that minimal fluid flow is actually observed in the horizontal cross-members.
However, it was thought that the horizontal fluid channels could assist in fluid delivery if a blockage occurred somewhere in the microtower array. For instance, if the inlet port at the base of one of the microtowers becomes blocked, then the horizontal fluid channels allow for fluid to be redistributed to that blocked microtower and permit nutrient perfusion from the side ports in that blocked microtower.

The general behavior of the fluid velocity in the microtower array is provided in figure 2.8, where it can be seen that the velocity of the fluid decreases significantly when the level of the fluid reaches the first set of side ports, which also align with the
lowermost horizontal fluid channel. Also, it can be seen in figure 2.8 that the velocity of the fluid decreases the higher the fluid travels up each microtower.

Figure 2.8. FEMLab model of the microtower design with 12 microtowers per linear array, showing the general fluid velocity field at different locations in the linear microtower array. The velocity of the fluid drops significantly once the fluid reaches the first set of side ports (centered at a vertical height of 200 µm for a packaged microtower array).

A magnified view of the fluid velocity field in the microtower array is provided in figure 2.9, specifying fluid flow rates out of the side ports and in the horizontal cross-members. In general, the linear velocity decreases as the fluid travels farther up the length of each microtower. However, the volume flow rates out of each side port are obtained by multiplying the linear fluid velocity by the side port area, yielding approximately 0.126 µm³/s, 0.145 µm³/s, and 0.200 µm³/s, out of the lowest (8.5×8.5
µm²), middle (9.8×9.8 µm²), and highest (11.6×11.6 µm²) side ports, respectively. This shows that the fluid volume flow rate slightly increases as the height of the side ports increases in this design, but overall, the flow is fairly uniform. Additionally, it can be seen in figure 2.9 that minimal fluid flow is seen in the horizontal channels. Also, the velocity of the fluid decreases significantly once the fluid reaches the lowermost side port. Furthermore, before the fluid reaches the lowest most side port, it can be seen that the fluid velocity is greater at the center of each vertical channel than it is at the edge of each vertical channel, which is in agreement with theoretical velocity profiles for laminar flows [58].

Figure 2.9. FEMLab model of the microtower design, showing the fluid velocities out of the side ports and at different locations in the linear microtower array.
A simulation was also performed when the inlet port at the base of the second microtower from the left was blocked. Sometimes, due to anomalies in the microtower fabrication or packaging process, the inlet ports at the base of some of the microtowers would become blocked, so a simulation was performed to see how such a blockage at an inlet port would affect the overall velocity field. Figure 2.10 provides the general velocity field in a microtower array where the inlet port at the base of the second microtower was blocked. The dark blue color along the lower portion of the second microtower in the array shows that no fluid was able to enter at the base of that second microtower.

Figure 2.10. FEMLab model of the microtower design with 12 microtowers per linear array, showing the general fluid velocities at different locations in the linear microtower array when the inlet port is blocked on the second microtower from the left.
A magnified view of the fluid velocity field in the microtower array with the inlet port blocked on the second microtower is provided in figure 2.11, specifying fluid flow rates out of the side ports and at varying locations in the microtower array. Even though there is minimal fluid flow in the horizontal fluid channels, fluid still flows out of the bottom side port in the microtower with the blocked inlet port, and the velocity of the lowermost side port in the microtower with the blocked inlet port is only one order of magnitude less than the velocities out of the lowermost side ports in the other microtowers that do not have blocked inlet ports. In fact, an increase in the fluid velocity can be observed in the lowest horizontal fluid channel on either side of the lowest side port in the microtower with the blocked inlet port.

**Figure 2.11.** FEMLab model showing a magnified view of the microtower array, where the inlet port at the base of the second microtower is blocked. An increase in the fluid velocity can be observed in the lowest horizontal fluid channel on either side of the lowest side port in the microtower with the blocked inlet port.
2.1.2.3.2 FEMLab Fluidic Model for the Brain Slice Microtower Array Design

A 2-D FEMLab fluidic model was performed on a single linear brain slice microtower array containing 12 microtowers. The calculated design parameters (see Section 2.1.2.2: Fluid Channel and Port Calculations for the Brain Slice Microtower Array Design) suggested ports sizes of $4.0 \times 4.0 \, \mu m^2$, $4.5 \times 4.5 \, \mu m^2$, $5.0 \times 5.0 \, \mu m^2$, $5.5 \times 5.5 \, \mu m^2$, $6.0 \times 6.0 \, \mu m^2$, and $7.0 \times 7.0 \, \mu m^2$ placed at vertical heights of 200 µm, 300 µm, 400 µm, 500 µm, 600 µm, and 700 µm, respectively, to achieve uniform fluid volume flow rates. These calculated side port dimensions for uniform fluid volume flow rates were implemented into the initial photomask design for the brain slice microtower arrays. However, during the microfabrication process the side ports having dimensions $< 6 \times 6 \, \mu m^2$ were difficult to define (see Section 5.1.2: SU-8 Lithography), resulting in a significant number of unwanted clogged fluid side ports in the microtower arrays. Therefore, a new photomask was designed that enlarged each fluid side port in the brain slice microtower array design by $4.0 \times 4.0 \, \mu m^2$. Hence, the new photomask contained side ports with dimensions of $8.0 \times 8.0 \, \mu m^2$, $8.5 \times 8.5 \, \mu m^2$, $9.0 \times 9.0 \, \mu m^2$, $9.5 \times 9.5 \, \mu m^2$, $10.0 \times 10.0 \, \mu m^2$, and $11.0 \times 11.0 \, \mu m^2$ placed at vertical heights of 200 µm, 300 µm, 400 µm, 500 µm, 600 µm, and 700 µm, respectively. The revised, enlarged fluid side port dimensions were successfully reproducible during the microfabrication process, unlike the smaller, calculated fluid side port dimensions.

The fluidic experiments delivered fluid to the microscaffold system via a syringe pump, where the fluid entered the microtower array through the base of each microtower. The FEMLab simulations were performed using a flow rate of 0.05 µL/hr. Each microtower contained a hollow channel running along its entire length. On the upper
portion of the linear brain slice microtower array where the microtower width is \( \leq 50 \mu m \), the hollow channel has a width of 12 \( \mu m \). On the lower portion of the linear brain slice microtower array where the microtower width is 150 \( \mu m \), the hollow channel has a width of 100 \( \mu m \). Therefore, on the upper portion of the linear brain slice microtower array, the hollow channel has a cross-section of 12×17 \( \mu m^2 \), and on the lower portion of the linear brain slice microtower array the hollow channel has a cross-section of 100×17 \( \mu m^2 \). Also, each of the three horizontal cross-members on the lower portion of the brain slice microtower array contained a hollow channel having a cross-section of 100×17 \( \mu m^2 \) that connected to the large vertical hollow channel in each microtower.

The dimensions of the side ports were calculated using a hollow channel with a cross-section having a depth of 23 \( \mu m \), but the microtower arrays that were actually fabricated contained hollow channels with depths of 17 \( \mu m \). The following FEMLab simulations implemented the parameters of the brain slice microtower arrays that were actually fabricated. Figure 2.12 shows the arrow velocity field in the linear brain slice microtower array, and confirms that minimal fluid flow occurs in the horizontal cross-members. However, the fluid channels in the horizontal cross-members were designed to potentially assist in fluid redistribution in the case of a clogged inlet port at the base of one of the microtowers.
The general behavior of the fluid velocity in the microtower array is provided in figures 2.13 and 2.14, respectively showing the velocity field through the lower and upper portions of the brain slice microtower array. It can be seen that the velocity of the fluid increases significantly in the upper portion of the brain slice microtower array. There is a significant increase in velocity where the vertical fluid channel narrows down from a 100 µm width to a 12 µm width. The velocity where the channel narrows down to a 12 µm width is a little more than 8 times the velocity where the channel has a 100 µm width, which is linearly proportional to the ratio of the channel widths (100 µm/12 µm ≈ 8.33).
Also, figure 2.14 provides numerical values for the fluid velocity out of each side port. All of the side port fluid velocities are on the order of $9 \times 10^{-10}$ m/s, so the flow rates are fairly uniform. To determine the volume flow rates out of each side port, the 2-D velocity field value was multiplied by the side port area, yielding volume flow rates of 0.0618 $\mu$m$^3$/s, 0.0687 $\mu$m$^3$/s, 0.0767 $\mu$m$^3$/s, 0.0838 $\mu$m$^3$/s, 0.0924 $\mu$m$^3$/s, and 0.1110 $\mu$m$^3$/s out of the side ports positioned at vertical heights of 200 $\mu$m, 300 $\mu$m, 400 $\mu$m, 500 $\mu$m, 600 $\mu$m, and 700 $\mu$m, respectively. The volume flow rate is fairly uniform, but the brain slice microtower design actually displays an increase in volume flow out of the side ports the higher the fluid travels up the channel. Due to problems with fabricating the side ports with the originally designed dimensions, the size of each of the side ports was

Figure 2.13. FEMLab model of the microtower design with 12 microtowers per linear array, showing the fluid velocities profiles at different locations in the linear microtower array.
increased by 4×4 µm². Furthermore, the original port size calculations were performed with a hollow channel having a depth of 23 µm. However, the brain slice microtower arrays that were actually fabricated had hollow channels with depths of only 17 µm.

![Surface: Velocity field [m/s]](image)

**Figure 2.14.** FEMLab model of the microtower design with 12 microtowers per linear array, showing the fluid velocity at different locations in the linear microtower array. The velocities at the level of each fluid side port are provided.

A simulation was performed where the inlet port at the base of the second microtower from the left was blocked to see how the blockage would affect the overall velocity field. Such a blockage could arise from anomalies in the microfabrication or the packaging and sealing process. Figure 2.15 provides the general velocity field in the lower portion of a brain slice microtower array when the inlet port at the base of the
second microtower was blocked. The darker blue color signifies that there is no fluid flow at the inlet (base) of the second microtower.

When the inlet at the base of the second microtower is blocked, the velocity of the fluid entering into the 11 microtowers shows an increase to $9.71 \times 10^{-11}$ m/s, which is a minimal increase from the original $9.64 \times 10^{-11}$ m/s into the 12 microtowers in the unblocked system. Also, the system with the blocked inlet port displays a slight increase ($0.03 \times 10^{-10}$ m/s) of velocity fluid flow into horizontal cross-member adjacent to the blockage. Otherwise, the blockage at the inlet port has little effect on the overall fluid dynamics within the brain slice microtower array.

Figure 2.15. FEMLab model of the microtower design with 12 microtowers per linear array, showing the fluid velocity on the lower portion of the linear microtower array when the second microtower from the left has a blocked inlet port.
CHAPTER 3
MICROSYSTEM FABRICATION AND PACKAGING

3.1 Materials

MEMS fabrication techniques have tended to move away from the traditional Si processing used in integrated circuit (IC) fabrication [59]. The need for alternative materials and processes has become apparent specifically in the fabrication of MEMS devices, since the fabrication of MEMS devices does not necessarily follow the same rules as those of IC fabrication. Additionally, the fabrication of BioMEMS devices has posed ever greater challenges since the finalized BioMEMS devices must be biocompatible. In other words, the materials from which the BioMEMS devices are fabricated should not elicit a biologically unfavorable response from the surrounding cells or tissue. Furthermore, the BioMEMS device must function such that the presence of biological material on the device doesn’t impede its desired function (biofouling) [60]. Proper materials selection was imperative for realizing the desired architecture, functioning, and biological application of the active microscaffold systems presented in this dissertation.

3.1.1 SU-8 as a Structural Material for BioMEMS Devices

SU-8 was chosen as the structural material for the microtower arrays since it can be photolithographically patterned into high-aspect ratio structures [12, 13, 14, 15] with fluidic applications [45, 46], is transparent to visible light, has a low Young’s modulus [59], and its biocompatibility (although still under investigation) is cited in numerous sources [13, 14, 59, 60, 61, 62]. Additionally, SU-8 is an insulator, and since the microtowers were designed to contain multiple electrodes for extracellular stimulation
and recording applications, an insulator was an appropriate choice for the structural material of the microtowers. Also, since completed SU-8 structures can be released from the wafer upon which they were fabricated [61, 62], SU-8 was a good choice for the 2-D fabrication, release, and 3-D packaging process developed in this dissertation.

3.1.1.1 Biocompatibility of SU-8

Photolithographic materials are inherently toxic due to their high solvent content. SU-8 is a negative-tone photoresist epoxy, which raises concern for the biocompatibility of devices fabricated from SU-8. There are a significant number of papers that claim that SU-8 is a biocompatible material [13, 14, 59, 60, 61, 62]. However, attempts at neuronal culturing on 3-D microscaffolds composed of thick layers of SU-8 have displayed a concerning toxicity [13, 14]. Additionally, the cytotoxicity of SU-8 has been under evaluation for implantable BioMEMS devices [47, 63]. However, biocompatibility requirements differ in an in vivo vs. an in vitro application. Biocompatibility protocols for in vivo applications tend to be more complex than biocompatibility protocols for in vitro applications since in vivo devices must endure chronic exposure to the physiological surroundings where systemic immune responses become contributing factors and often the surrounding tissue could impede the overall function of the device [60].

Extensive collaborative experiments were performed by the Allen and LaPlaca labs at the Georgia Institute of Technology and the Brewer lab at the Southern Illinois University (SIU) School of Medicine to determine a biocompatibility protocol that could be performed on the completed SU-8 microscaffolds to improve neuronal cell adhesion and survival on the SU-8 structures. Numerous experimental processing steps including liquid CO₂ treatments, ultra-violet (UV) light exposures, vacuum oven bakes, O₂ plasma
treatments, and glow discharge treatments were evaluated to improve the biocompatibility of the SU-8 in response to neuronal cell culturing [13, 14, 15].

The biocompatibility processing protocol performed on the completed SU-8 microscaffolds presented in this dissertation was obtained via the experimental results obtained from the Allen, LaPlaca, and Brewer labs collaborative biocompatibility experiments. The biocompatibility treatments that performed the best for culturing neurons on the SU-8 microscaffolds presented in this dissertation were a 90,000 mJ/cm² UV light exposure, followed by a 150 °C vacuum oven bake for three days, followed by a 60 sec O₂ plasma treatment, and a 60 sec IPA sonication. The UV light exposure and the vacuum oven bake helped to fully cure and hard bake the SU-8, while the O₂ plasma treatment made the SU-8 surface hydrophilic to aid in cell adhesion to the microtower structures. Finally, the 60 sec IPA sonication removed any remaining photoresist residues in the microtowers, and significantly improved cell survival on the SU-8 microtowers. These biocompatibility treatments mentioned above were all performed after the SU-8 microtowers were vertically packaged and sealed into the Si orifice plate, and after the sacrificial photoresist had been removed from the channels in the microtowers, but prior to packaging the device into the PDMS fluid manifold. Additionally, other treatments such as liquid CO₂, glow discharge, a piranha etch, and poly-D-lysine (PDL) coatings were also experimented with to enhance the biocompatibility of the SU-8, but the extent that each of these treatment aids in enhancing the biocompatibility of the SU-8 is still debatable [64].
3.1.2 Metals

Proper choice of metals was particularly significant in regards to biocompatibility of the completed microscaffold device since it is well known that many metals commonly used in microfabrication applications, such as Cr, copper (Cu), nickel (Ni), aluminum (Al), and iron (Fe) are not biocompatible and therefore would induce necrosis in neuronal cell culturing applications. However, metals such as Au, Pt, and iridium (Ir) tend to generate a more favorable cellular response in biological applications and were therefore considered to be appropriate metals for forming the electrical components of the active microscaffold system.

3.1.2.1 Chromium (Cr)

Cr was chosen as the sacrificial release layer upon which the 2-D, linear SU-8 microtower arrays were fabricated. The use of Cr as a sacrificial release layer for micro-scale devices has been well-established [61, 62, 64, 65], suggesting that Cr was an appropriate candidate for releasing the linear SU-8 microtower arrays. Also, even though Cr is not considered to be a biocompatible metal, the Cr served only as a sacrificial layer to release the completed 2-D microtower arrays, so no Cr was present on the completed and released microtower arrays.

Also, Cr is often used as an adhesion layer underneath thin Au films to resolve any Au/Si adhesion problems. SU-8 also tends to have adhesion problems with Si, but SU-8 patterned on Cr displayed good adhesion. Therefore, the use of Cr as a release layer resolved any SU-8 on Si delamination problems. Delamination of the first SU-8 layer that was patterned on top of the Cr was only observed when the SU-8 wasn’t properly cross-linked, which was a behavior related to undercured SU-8 and not a
property of the Cr layer upon which the SU-8 layer was patterned [66] (see Section 3.2.4: Planar Fabrication with Multiple SU-8 Layers).

Cr can be etched in several ways. CR-7S (Cyantek Corp.) was used to etch the Cr release layer and release the microtower arrays. CR-7S was an appropriate etchant for the desired application since the CR-7S only attacked the Cr release layer, and caused no damage to the completed microtower arrays unless the microtower arrays were left in the CR-7S for an excessive amount of time (over 5 days). A Cr etch consisting of a 1:1 ratio of hydrochloric acid (HCl) and DI water, using a piece of Al foil as a Cr etch catalyst, was also experimented with to etch the Cr release layer, but this method didn’t work as well as using the CR-7S. The piece of Al foil must be briefly dipped into the diluted HCl solution and touch the Cr surface on the wafer to catalyze the Cr etch reaction, which can be visualized in the form of rapid bubble formation. However, once the piece of Al foil is removed from the acid solution, the bubble formation ceases, along with the Cr etch. Also, if the Al foil is left in the diluted HCl solution for too long, then the Al foil will begin to etch and could contaminate the microtower arrays with the Al, which is a metal that is toxic to the cells. Hence, the HCl/DI water Cr etch wasn’t of long enough duration to etch the Cr underneath the planar microtowers to induce the desired release of the planar microtowers from the Si substrate.

The Cr release layer was initially deposited using the CVC DC sputtering system, which functioned well as release layer for the initial microtower designs that had either 8 or 16 microtowers per array, a microtower width of 120 µm, and a microtower center-to-center spacing of 600 µm. Also, the sputtered 1600 Å Cr layer functioned well to release the revised dissociated microtower designs that had 12 microtowers per array, a
microtower width of 80 µm, and a 200 µm center-to-center microtower spacing. However, to release the brain slice microtower arrays, it was found that the 1600 Å Cr release layer had to be evaporated onto the substrate by an e-beam or PVD-75 Filament evaporator system. The evaporated Cr provided a higher quality film than the sputtered Cr film, and greatly improved the release process. The microtowers in the brain slice arrays were only 50 µm wide (with a 200 µm center-to-center spacing) on the top portion of the array that would project above the Si orifice plate upon vertical packaging.

However, the portions of the microtowers on the brain slice arrays that would project out the backside of the Si orifice plate upon vertical packaging were much wider (150 µm, with a 200 µm center-to-center spacing). Therefore, the section of exposed Cr between adjacent towers on the backside of the brain slice microtower arrays was only 50 µm wide, which limited flow of the Cr etchant underneath the wide microtowers on the backside of the brain slice microtower arrays. Basically, the backside of the brain slice microtower arrays had a much larger SU-8 surface area than the other microtower array designs, which complicated the release process (see Section 3.2.7: Release Process) and required the use of an evaporated Cr release layer as opposed to a sputtered Cr release layer.

3.1.2.2 Gold (Au)

Au was chosen as the material for the microtower electrodes and leads since Au is biocompatible, has a low electrical resistance, and the deposition and etching of Au in microfabrication technology has been well-established. Also, Au is the standard lead material used in 2-D MEAs [11] for extracellular stimulation and recording of neuronal networks, so Au was a logical choice for the electrodes and leads used in the microtower
arrays. However, the impedance of the Au electrodes is too high for most neuronal stimulation/recording applications, so usually the electrodes in an MEA will be electroplated with Pt black to lower the electrode impedance (see Section 3.1.2.4: Platinum (Pt) Black).

Electroplated Au (Techni Gold 25 ES, Technic Inc.) was chosen to form the thick Au tabs at the base of each microtower. Techni Gold 25 ES is a neutral, non-cyanide plating bath that was able to electroplate biocompatible Au tabs at the base of each microtower to the desired thickness (between 13-25 µm) for each given microtower design. Also, the sputtered Au seed layer (1800 Å thick) used for the electroplating was the same Au layer from which the microtower electrodes and leads would be patterned. The Au microtower electrodes and leads were photolithographically patterned using Shipley 1827 photoresist, MF-319 developer (Shipley), and were wet etched using a potassium iodide (100 g/L) and iodine (25 g/L) solution (KI/I₂) etchant. The KI/I₂ was able to selectively etch the Au microtower electrodes and leads without causing any damage to the Cr, first SU-8 layer, or thick electroplated Au tabs already patterned onto the wafer.

3.1.2.3 Tin-silver (Sn-Ag) Solder

Flip-chip bonding was originally introduced by IBM in the 1960s, and has recently received a large amount of interest in microelectronic packaging and MEMS integration applications. Flip-chip bonding involved integrating ICs with an electrical packaging via tin-lead (Sn-Pb) solder bumps, and in many circumstances is a superior alternative to time-intensive wire bonding. Sn-Pb eutectic solders, due to their material properties and low cost, have been widely used in microelectronic packaging
technologies [54]. However, Pb based solders are inherently toxic and are therefore unsuitable for enabling electrical functionality to the active microscaffold system presented in this dissertation.

Tin-Silver (Sn-Ag) eutectic solder is a nontoxic alternative to traditional lead based solder. Sn-Ag solders exhibit good mechanical properties and have similar wetting characteristics to lead based solders, but are less compliant than the traditional lead based solders [67]. Sn-Ag solder can be deposited via an electroplating setup using a Sn-Ag alloy bath and a Pt mesh electrode using either DC plating or a pulse plating setup. Also, the reflow temperature of the Sn-Ag solder is between 220-250 °C [54], which is a low enough temperature that will not damage the SU-8 microtowers during the electrical integration/functionalization process. The SU-8 microtower array structures would maintain their structural integrity at temperatures up to ~250 °C but would either begin to warp or char at temperatures above 250 °C.

3.1.2.4 Platinum (Pt) Black

Most MEAs for extracellular stimulation/recording of neuronal networks use platinized electrodes to lower the impedance of the stimulation/recording electrodes. Electroplated Pt black has a rough, textured topography that yields a high surface area, and therefore produces electrodes with a significantly lower impedance than that of Au electrodes [68]. Therefore, to achieve optimum performance from the electrically functional microscaffold system, Pt black should be electroplated onto the Au microtower electrodes to lower the impedance of the stimulation/recording electrodes.
3.1.3 Silicon (Si)

Silicon is the most widely used material in the semiconductor/IC industry and therefore its use in bulk and surface micromachining has been well-established. Silicon is a semiconductor that can be selectively doped to form p-n junction diodes and transistors, which serve as the basis for today’s modern ICs. Also, Si wafers are the primary substrates used in most microfabrication processes. For this reason, Si wafers were chosen as the substrate upon which the 2-D (planar) microtower arrays were fabricated. Additionally, due to the ease with which Si can undergo bulk and surface micromachining processes, Si was also chosen as the material for the orifice packaging plate upon which the microtower electrodes would also be functionalized. The biocompatibility of Si is still under investigation [60, 69], but numerous in vivo probes for extracellular stimulation and recording of neuronal networks have been fabricated [2, 10, 69] from Si due to its extensive use and applications in microfabrication technology.

Deep reactive ion etching (DRIE) of Si, specifically the Bosch process, introduced in the 1990’s, revolutionized MEMS fabrication technologies. The Bosch process enabled anisotropic plasma etching of Si through alternating cycles of polymer deposition (using C₄F₈) and etching of the deposited polymer and Si substrate (using SF₆). Prior to the development of the Bosch process, Si was mainly etched anisotropically using a potassium hydroxide (KOH) wet etch solution that rapidly etched the (100) planes of the Si wafer and slowly etched the (111) planes of the Si wafer, resulting in diamond-shaped cavities in the Si wafer [70]. The Bosch process enabled the fabrication high-aspect ratio through wafer vias in Si wafers that are especially of interest for through wafer interconnect applications [71, 72, 73].
For the purposes of this dissertation, 4 in Si wafers were utilized as the substrates upon which the 2-D (planar) microtower arrays were fabricated and subsequently released. In addition, the Bosch process was used to fabricate Si orifice plates into which the microtower arrays were packaged into a 3-D, vertical format. The Si orifice plates were also patterned with appropriate electrical circuitry to functionalize the microtower electrodes and electrically integrate the active microscaffold system with the Multi Channel Systems preamplifier setup.

3.1.4 Polydimethylsiloxane (PDMS)

Polydimethylsiloxane (PDMS, Dow Corning) is a biocompatible polymer that is often micromolded into devices for microfluidic applications [74]. Pouring a highly viscous solution of PDMS and curing agent into an SU-8 master mold that contains the negative pattern of the desired 3-D PDMS structure has become a standard approach for fabricating 3-D PDMS structures with microfluidic channels for cell culture applications [50, 51, 52, 75]. The PDMS typically cures over a 24 hr period at room temperature, after which the PDMS structure is simply peeled away from the SU-8 master mold. In our application, 184 PMDS base was mixed with the PDMS curing agent (10:1), and was poured into 3-D wax molds (Thermojet) to create the top and bottom pieces of the PDMS fluid manifolds (see Section 3.4: Fluidic Packaging).

Additionally, in this dissertation 184 PDMS diluted with hexane (3:1) was used to form a fluidic seal between the microtower arrays and the Si orifice plate once the microtower arrays were packaged into a 3-D vertical format (see Section 3.3: Vertical Packaging and Sealing Process). Since PDMS is inexpensive, biocompatible, and can be micromolded into detailed 3-D structures, PDMS was a good choice for creating the fluid
manifold pieces and for creating a fluid seal between the microtower arrays and the Si orifice plate.

### 3.2 Microfabrication Process

The 2-D microtower arrays are fabricated from a set of six photomasks; (1) the first SU-8 layer, (2) the electroplated Au tabs, (3) the Au electrodes and leads, (4) the second SU-8 layer, (5) the sacrificial layer, and (6) the third SU-8 layer. The original microtower array designs that had either 8 or 16 microtowers in a single linear microtower array were fabricated from one set of six photomasks. In a similar manner, the revised microtower array design having 12 microtowers in a single linear microtower array and the brain slice microtower array design were fabricated from a separate set of six photomasks.

#### 3.2.1 2-D Planar Fabrication Process for the Original Microtower Array Designs

Top views of the fabrication process for the original microtower design (with 8 microtowers per linear array), including inserts that depict the microtower detail are shown in Figure 3.1. Cross-sections of the planar fabrication process are provided in Figure 3.2. The 5 in Cr on glass photomasks were created by Sine Patterns LLC (Rochester, NY). In the original 8×8 and 16×16 microtower array designs, each photomask contained a total of 180 linear microtower arrays (90 linear microtower arrays containing 8 microtowers each, and 90 linear microtower arrays containing 16 microtowers each), thus having a potential yield of 180 microtower arrays per 4 in Si wafer.
Figure 3.1. Top views of the fabrication process for each photomask layout for the dissociated neuronal cell microtower arrays are shown. A 1.5 mm tall microtower array is shown, with enlarged inserts in each picture to depict the microtower detail. (a) The first SU-8 layer is defined, which contains fluid side ports, openings for electrodes, and structural tabs for packaging. (b) Thick, structural, Au tabs are electroplated that will later be used to provide electrical functionality to the tower electrodes. (c) Two Au electrodes and corresponding leads are patterned per tower. (d) The second SU-8 layer is defined, which insulates the electrodes and leads. The second SU-8 layer also contains fluidic ports. (e) The sacrificial layer, which defines the hollow fluid channels, is patterned. (f) The third SU-8 layer is patterned to complete the microtower structure.
Figure 3.2. Cross-sections of the planar fabrication process for the SU-8 microtower arrays are shown. (a) A 1600 Å Cr release layer is sputtered, followed by the definition of the 5 µm first SU-8 layer. (b) A 1900 Å Au layer is sputtered on the wafer. (c) A 23 µm thick electroplating mold is patterned from AZ 4620 photoresist. (d) Thick (23 µm) Au solder tabs are electroplated. (e) The AZ 4620 electroplating mold is removed and the Au electrodes and leads are patterned. (f) The second SU-8 layer (5 µm thick) is patterned, and insulates the Au electrodes. (g) The hollow channel is defined by a 23 µm sacrificial layer of AZ 4620 photoresist. (h) A conformal Parylene C layer (2 µm) is deposited. (i) The 70 µm thick, third SU-8 layer is patterned. (j) Exposed Parylene C is etched away.
First, a 1600 Å chromium (Cr) release layer is deposited on the wafer by DC sputtering at 5.0 mTorr in an Argon (Ar) atmosphere. Next, the outer wall of the tower arrays is photolithographically patterned using a 5 μm thick SU-8 layer, and is hardbaked at 150 °C for 30 min to improve adhesion and durability. The outer SU-8 wall contains 20×20 μm² fluidic side ports and 15×15 μm² openings to expose the electrodes that will be later defined. Next, a 1900 Å Au layer is DC sputtered, and a thick (23 μm) AZ 4620 (Clariant) photoresist layer is spun onto the wafer and patterned to form an electroplating mold. Au tabs (40×100 μm²) are then electroplated inside the photoresist mold on both sides of each SU-8 microtower to a thickness of 23 μm. The wafer is then cleaned with acetone and Isopropanol (IPA) and dried with N₂. The Au electrodes and corresponding leads (two per microtower) are patterned and wet etched using a KI/I₂ solution. The Au leads are then insulated with a second SU-8 layer having a thickness of 5 μm.

The hollow channel that runs along the length of the towers and through the cross-members is defined using a 23 μm thick AZ 4620 photoresist, which is hardbaked at 95°C for 15 min. This hardbake helps maintain the integrity of the sacrificial photoresist layer during subsequent processing (see Section 5.1.4: Sacrificial Layer Fabrication). Next, a uniform, 2 μm thick Parylene C coating is deposited by chemical vapor deposition (CVD), which prevents mixing of the sacrificial resist with the subsequent (third) SU-8 layer. The third SU-8 layer is spin coated to a thickness of 70 μm and patterned to form the opposing wall of the SU-8 tower array. The exposed Parylene C that is not sandwiched between the second and third SU-8 layers is then etched away in an O₂ plasma using a reactive ion etch (Plasma Therm RIE). Finally, the Cr release layer is wet etched in CR-7S Cr etchant (Cyantek) to release the completed SU-8 structures.
from the Si wafer. The sacrificial photoresist is typically not removed from the channels until after the microtower arrays are packaged in a 3-D vertical position, but it is possible to remove the photoresist from the channels with a 1 hr acetone soak, followed by an IPA rinse if hollow structures are desired prior to vertical packaging of the microtower arrays. A detailed protocol for the fabrication of the original microtower array design is provided in Appendix A. Figure 3.3 shows a microscope image of completed a 2-D microtower array (2.0 mm in height) after it has been released from the original Si substrate upon which it was fabricated. The sacrificial AZ 4620 photoresist had been removed from this microtower array, thus yielding the 80 µm wide fluid channels that run along the entire length of each microtower and through each horizontal cross-member.

Figure 3.3. Microscope image of a completed 2-D (planar) microtower array with two electrodes and leads per microtower. The center-to-center microtower spacing is 600 µm, and the center-to-center cross-member spacing is 500 µm.

The microtower array in figure 3.3 is shown from the side that was in contact with the Si substrate during the planar fabrication process. The SU-8 is transparent, which was convenient for viewing the integrity of the Au leads and hollow fluid channels in the
microtower array. Two Au electrodes and corresponding leads can be seen per microtower. In the microtower array shown in figure 3.3, the 15×15 µm$^2$ Au electrodes are exposed in the horizontal cross-members at varying heights. Each Au electrode connects to an Au lead (20 µm in width) that is insulated within the microtower walls between the first and second SU-8 layers. Each insulated Au lead travels from the microtower electrode, through the horizontal cross-member, down the length of each microtower, and connects to a large Au tab (40×120 µm$^2$) at the base of each microtower where the packaged microtower array will intersect the Si orifice plate. Also, the 20×20 µm$^2$ fluid side ports that were positioned along the center of each microtower and functional cross-member can be seen.

Microscope images of the intersection of an SU-8 microtower and functional cross-member are provided in figure 3.4. These images are from a planar microtower array from which the sacrificial AZ 4620 photoresist has been removed, thus yielding the hollow fluid channel positioned in each microtower and cross-member.

![Microscope images showing the intersection of a microtower and cross-member](image)

**Figure 3.4.** Microscope images showing the intersection of a microtower and cross-member; (a) the focus is on the top (third) SU-8 layer; (b) the focus is on the 70 µm wide hollow microchannels and the 20×20 µm$^2$ fluid ports.
Figure 3.4a is focused on the top (third) SU-8 layer, which is purely structural and contains no fluid ports or electrodes. However, since the SU-8 is transparent, the fluid channels lying underneath the top SU-8 layer, running through the entire length of each microtower and through each cross-member can still be observed in figure 3.4a. Figure 3.4b is focused on the hollow fluid channel buried within the microtower array and also displays the side ports and that were defined in the first and second SU-8 layers. Additionally, figure 3.4b depicts the keg wheel pattern that was defined during the photolithography of the first and second SU-8 layers. As can be observed in figure 3.4a, the top (third) SU-8 layer has smooth edges and does not contain the keg wheel pattern that the first and second SU-8 layers have.

The microtower has the keg wheel pattern on either side of the fluid channel to aid in adhesion of the multiple SU-8 layers, while the cross-member has the keg wheel pattern only on one side of the fluid channel. The SU-8 microtower is 120 µm wide and the SU-8 cross-member is 110 µm wide. Since the hollow fluid channels are 70 µm wide, the microtower has a 25 µm side structural SU-8 component on either side of the fluid channel. The cross-member has a 25 µm structural SU-8 component on the side of the fluid channel containing the keg wheel pattern, and a 15 µm structural SU-8 component on the side of the fluid channel that doesn’t contain the keg wheel pattern.

3.2.2 2-D Planar Fabrication Process for the Revised Dissociated Microtower Design

An additional six photomask set was created that included both the revised 12×12 microtower array design and the brain slice microtower array design. This 5×5 in² Cr on
glass photomask set was also created by Sine Patterns LLC. Each photomask contained 180 linear microtower arrays for dissociated cultures (containing 12 microtowers per linear array) and 180 brain slice microtower arrays, thus having a potential yield of 180 linear microtower arrays for dissociated neuronal cultures and 180 brain slice microtower arrays per 4 in Si wafer.

Top views of the fabrication process for the original microtower design (with 12 microtowers per linear array); including inserts that depict the microtower detail are shown in figure 3.5. Also, a stereoscope image of a revised linear microtower array for dissociated neuronal cultures is provided in figure 3.6. The revised linear microtower arrays for dissociated neuronal cultures were fabricated in a similar manner to the original microtower array design (see figure 3.2), with some slight changes in the processing that were necessary to accommodate the smaller features on the revised design. A detailed protocol for the fabrication of the revised microtower array design for dissociated neuronal cultures and the brain slice microtower array design is provided in Appendix B.
Figure 3.5. Top views of the fabrication process for each photomask layout for the revised dissociated neuronal cell microtowers. A 1.0 mm tall microtower array is shown, with enlarged inserts in each picture to depict the microtower detail. (a) The first SU-8 layer is defined, which contains fluid ports, openings for electrodes, and structural tabs for packaging. (b) Thick, structural, Au tabs are electroplated that will later be used to provide electrical functionality to the microtower electrodes. (c) Two Au electrodes and corresponding leads are patterned per microtower. (d) The second SU-8 layer is defined, which insulates the electrodes and leads. The second SU-8 layer also contains fluid ports. (e) The sacrificial layer, which defines the hollow fluid channels, is patterned. (f) The third SU-8 layer is patterned to complete the microtower structure.
3.2.3 2-D Planar Fabrication Process for the Brain Slice Microtower Arrays

Top views of the fabrication process for the brain slice microtower array design (with 12 microtowers per linear array); including inserts that depict the microtower detail are shown in figure 3.7. An additional six photomask set was created that included both the revised 12×12 microtower array design and the brain slice microtower array design. This 5×5 in² Cr on glass photomask set was also created by Sine Patterns LLC. Each
photomask contained 180 linear microtower arrays (containing 12 microtowers per linear array) and 180 brain slice microtower arrays, thus having a potential yield of 180 linear microtower arrays for dissociated neuronal cultures and 180 brain slice microtower arrays per 4 in Si wafer.

Also, a stereoscope image of a fabricated linear microtower array for brain slice cultures is provided in figure 3.8. The brain slice microtower arrays were fabricated in a similar manner to the original microtower array design (see figure 3.2), with some changes in the processing that were necessary to accommodate the smaller features on the brain slice microtower array design. A detailed protocol for the fabrication of the revised microtower array design for dissociated neuronal cultures and the brain slice microtower array design is provided in Appendix B.
Figure 3.7. Top views of the fabrication process for each photomask layout for the brain slice microtower arrays. A single brain slice microtower array is shown (with 12 microtowers per array), with enlarged inserts in each picture to depict the microtower detail. (a) The first SU-8 layer is defined, which contains fluid ports, openings for electrodes, and structural tabs for packaging. (b) Thick, structural, Au tabs are electroplated that will later be used to provide electrical functionality to the tower electrodes. (c) Two Au electrodes and corresponding leads are patterned per tower. (d) The second SU-8 layer is defined, which insulates the electrodes and leads, and also contains fluid ports. (e) The sacrificial layer, which defines the hollow fluid channels, is patterned. (f) The third SU-8 layer is patterned to complete the brain slice microtower array structure.
3.2.4 Planar Fabrication with Multiple SU-8 Layers

The microtowers were initially fabricated in 2-D linear arrays with 8, 12, or 16 microtowers per linear array, depending on the design. The 2-D fabrication of the microtowers allowed for multiple photolithographic techniques to be implemented and therefore precise dimensions and placement of all the fluid channels, ports, electrodes, and structural layers in each microtower could be obtained. Additionally, fabricating the microtowers in linear arrays of 8, 12, or 16 simplified subsequent packaging, which would have been complicated had the microtowers not been fabricated in these arrays.

Figure 3.8. Microscope image of a 2-D, linear brain slice microtower array before it is released from its original Si substrate. There are 12 microtowers per linear array.
SU-8 was chosen as the structural material for the microtowers, and each 2-D linear microtower array was composed of three separate SU-8 layers. The first SU-8 layer defined the basic shape of the 2-D linear microtower array. Additionally, the first SU-8 layer contained square-shaped openings centered along the length of each tower which later served as fluid side ports in the microtower walls. Furthermore, the first SU-8 layer also contained two openings per microtower at varying heights that were later filled by exposed Au electrodes for applications in extracellular recording and stimulation. The first SU-8 layer was spun on top of the Cr layer to a thickness of 5 µm and was subsequently patterned by photolithography.

Making the first SU-8 layer thin (5 µm) had the following advantages: (1) small features such as the fluid ports that ranged in size from 6×6 µm² to 20×20 µm² were more likely to be defined with acceptable resolution than if their realization had been attempted using a think SU-8 layer, (2) toxicity is less of a factor in thin SU-8 films than it is in thick SU-8 films, which is important in neuronal cell culture applications, and (3) adhesion problems tend to be less apparent with thinner layers of SU-8 than with thicker layers of SU-8. Also, the outer defining walls of the linear microtower arrays were not designed to be smooth in the first and second SU-8 layers, but rather were designed to have a keg wheel pattern to aid in adhesion of subsequent SU-8 layers. Previous studies have shown that one SU-8 layer patterned on top of another SU-8 layer will tend to have issues with delamination, especially if the borders of each pattern are smooth. The roughened, keg wheel pattern of the first SU-8 layer was intended to minimize the delamination of subsequent SU-8 layers [76].
The second SU-8 layer has the same basic shape as the first SU-8 layer, and likewise, the second SU-8 layer has square openings centered along the length of each microtower that aligned to the square openings centered along the length of each microtower in the first SU-8 layer. The square openings in the first SU-8 layer aligned with the square openings in the second SU-8 layer formed the fluid side ports centered along the length of each microtower. Since the square openings that form the fluid side ports in the second SU-8 layer are more or less the same size as the square openings that define the fluid side ports in the first SU-8 layer, and since there are numerous advantages to having a thin SU-8 layer for the desired application, the second SU-8 layer was also spun on and patterned to a thickness of 5 µm. However, the main purpose of the second SU-8 layer was to insulate the Au leads along the length of each microtower, so the second SU-8 layer does not contain additional square ports to expose the Au electrodes. The second SU-8 layer covers the patterned Au leads, therefore insulating the leads from any fluids that will travel up through the length of the completed, hollow microtowers.

The third SU-8 layer comprises the main structural component of each microtower array, and is therefore the thickest of all three SU-8 layers. The third SU-8 layer aligns to have the same basic shape as the first and second SU-8 layers, and is also responsible for encapsulating the sacrificial photoresist running along the length of each microtower and functional cross-member which will later define the large hollow channels in each microtower array. The third SU-8 layer does not contain any features such as square openings for side ports or electrodes, but merely forms the smooth outer wall of each microtower array. Additionally, the periphery of the microtower arrays, as
defined by the third SU-8 layer, is smooth (without a keg wheel pattern). During processing, the smooth edges in the third SU-8 layer interlock with the keg wheel pattern in the first and second SU-8 layers, therefore promoting better adhesion amongst the three SU-8 layers comprising each microtower array.

Typically, in microfabrication, which is a form of layered processing, it is best to initially pattern the layers with intricate features, and later pattern the layers without intricate features. This is because as more and more layers are added to the wafer, subsequent layers become more difficult to align and pattern. Therefore, it is best to first define the layers in the processing that have the photomasks with the most intricate features, and it is best to finish up with the layers in the microprocessing that have the photomasks with the least detail. The multiple SU-8 layer fabrication process implemented in the fabrication of the microtower arrays followed the standard microprocessing protocol of patterning the SU-8 layers in order of the most detailed to the least detailed to ensure acceptable definition of all pertinent features (fluid side ports and electrodes) in each microtower array.

SU-8 tends to have problems adhering to Si and various other surfaces [60]. It was necessary to hardbake both the first and second SU-8 layers at 150 °C for 30 min to avoid delamination of the SU-8 layers during subsequent processing. Hardbaking the first two SU-8 layers eliminated any delamination problems that arose when hardbakes weren’t performed. However, the third SU-8 layer wasn’t hardbaked since this would have further cured the sacrificial photoresist and would have created additional problems clearing out the sacrificial resist to form the hollow fluid channels. Although, delamination issues were observed between the Parylene C layer and the third SU-8
layer. The delamination between the Parylene C layer and the third SU-8 layer was remedied by performing a 15 sec O\textsubscript{2} plasma treatment on the Parylene C layer prior to spinning on the third SU-8 layer. This 15 sec O\textsubscript{2} plasma treatment made the surface of the Parylene C layer hydrophilic and therefore improved adhesion between the Parylene C and the third SU-8 layer [77].

3.2.5 Au Electroplating

Two Au tabs were electroplated at the base of each microtower. The Au tabs initially functioned as a structural component that limited how far the microtower arrays could be slid into the Si orifice plate. Hence, upon packaging the microtower arrays into the Si orifice plate, each microtower projected a predicted distance out the front (1.0-2.0 mm) and back (1.5-2.5 mm) of the Si orifice plate, with the Au tabs on each microtower making contact with the Si orifice plate. Also, the Au tabs at the base of each microtower connected to corresponding vertical Au traces that ran along the length of each microtower, which connected to one of the microtower electrodes. Additionally, since the Au tabs at the base of each microtower made contact with both the microtower electrodes and the horizontal Au traces on the Si orifice plate that ran out to the mega contact pads at the periphery of the Si orifice plate, the Au tabs played a critical role in the electrical integration of the microtower arrays.

After the first SU-8 layer was patterned and hardbaked, an 1800 Å Au seed layer was sputtered onto the wafer and an electroplating mold was patterned photolithographically using AZ 4620 photoresist. The Au tabs were electroplated into
the mold to a thickness of 23 µm in the 8×8 and 16×16 microtower array designs, and to a thickness of 17 µm in the 12×12 microtower array designs.

The Au tabs were electroplated using Techni Gold 25 ES; a commercially available, non-cyanide electroplating bath (Technic Inc., Caston, RI) [78]. The electroplating was performed at 55 °C, 300 rpm, 1.5 mA, for 4.5 hr in the 8×8 and 16×16 microtower designs, using a Pt mesh counter electrode to obtain Au tabs electroplated to a thickness of ~ 23 µm. The total Au area to be plated in the 8×8 and 16×16 microtower designs was 1.243 cm² per wafer. Therefore, this translated into a current density of 1.2 mA/cm². Likewise, the electroplating was performed at 55 °C, 300 rpm, 1.5 mA, for 2.5 hr in the 12×12 microtower and brain slice designs, using a Pt mesh counter electrode to obtain Au tabs electroplated to a thickness of ~ 17 µm. The total area to be plated in the 12×12 microtower and brain slice designs was 0.75672 cm² per wafer. Therefore, this translated into a current density of 1.98 mA/cm².

3.2.6 Fluid Channel Fabrication

Various photodefinable polymers were experimented with to create the fluid channel in the microtower arrays. For the most part, a thick photoresist layer could be patterned nicely in the shape of the fluid channel. However, once the third SU-8 layer was spun on top of the patterned sacrificial material, the spun SU-8 would mix with the patterned sacrificial material, creating an unidentifiable mess of the formally defined sacrificial layer and the spun on third SU-8 layer. This mixing problem would still occur when the sacrificial material was hardbaked prior to spinning on the third SU-8 layer. Furthermore, caution had to be taken to not hardbake the sacrificial layer to the point that it would be difficult to remove later.
In the microtower array fabrication process, the mixing of the third SU-8 layer with the patterned sacrificial photoresist layer was in agreement with the observations of S. Psoma et al. [61] who claimed that photoresist sacrificial layers failed when used in conjunction with SU-8 since the SU-8 tended to attack the sacrificial photoresist layers. However, S. Psoma et al. were able to reduce the mixing effect of the SU-8 with the sacrificial photoresist when the softbake temperature of the sacrificial photoresist layer was increased. In this dissertation, experiments that included increasing the softbake temperature during the processing of the sacrificial photoresist were not performed. Experiments in increasing hardbake times and temperatures of the sacrificial photoresist layer were performed, but still resulted in mixing of the spun-on SU-8 with the patterned sacrificial photoresist layer.

PiRL®III (Brewer Sciences, Rolla, MO), which is a polyimide that is not photodefinable [79], was the only spun-on sacrificial material experimented with in this dissertation that once patterned, didn’t mix with the third SU-8 layer when the third SU-8 layer was spun onto the wafer. However, it was very difficult to achieve repeatable results in the patterning of the PiRL®III material. The PiRL®III was successfully patterned by RIE once, but the results were not repeatable. Several wet etches of the PiRL®III were attempted (using MIF 319 and AZ 400K developers), but none of the wet etches were able to produce an acceptable pattern definition of the PiRL®III material. Also, it was extremely difficult to remove the PiRL®III material from the fluid channel. Any strong basic solution tended to remove the PiRL®III material, but the PiRL®III tended to break up into chunks instead of dissolve, posing serious issues in using the PiRL®III polyimide as a sacrificial material. Furthermore, the PiRL®III could only be
spun to a thickness of 12 µm in one coat, which was somewhat on the low end for the desired thickness of the fluid channel. Double coats of PiRL®III were spun on wafers in attempts to achieve a channel of at least 20 µm in thickness, but this double coating further complicated the patterning of the PiRL®III material.

After much experimentation, AZ 4620 was chosen as the sacrificial material to create the fluid channel in the microtower arrays. The AZ 4620 could easily be patterned to a thickness of 23 µm in one spin coat and the AZ 4620 could also be readily dissolved in acetone. It was hypothesized that if the patterned AZ 4620 layer could be capped with a protective material, then the patterned AZ 4620 layer would be isolated from the third SU-8 layer while the third SU-8 layer was spun on top of the wafer. Parylene C was chosen as the material to form a protective coating over the AZ 4620 sacrificial layer since Parylene C was biocompatible, could be uniformly deposited by CVD at reasonably low temperatures, and could be etched by RIE in an O₂ plasma. Moreover, the deposited Parylene C was free of pin holes and provided complete isolation between the patterned AZ 4620 sacrificial layer and the third SU-8 layer during the spinning of the third SU-8 layer. In fact, S. Takeuchi et. al. have fabricated Parylene flexible neural probes with hollow microfluidic channels, using Parylene as the structural material and AZ 4620 as the sacrificial material that later forms the hollow microfluidic channels in the neural probe devices [30].

However, in our application, the patterned AZ 4620 layer experienced some alterations during the processing of the third SU-8 layer, specifically during the UV light exposure of the third SU-8 layer. Since the third SU-8 layer and the Parylene C layer were transparent, the UV exposure of the third SU-8 layer would also expose the already
patterned AZ 4620 layer lying under the third SU-8 and Parylene C layers. Since AZ 4620 is a positive photoresist, the UV light exposure during the processing of the third SU-8 layer tended to break down the cross-linked areas of the patterned AZ 4620 photoresist, as was evidenced by a bubbling and sometimes a reflow of the patterned AZ 4620 layer. The bubbling of the AZ 4620 was controlled to a tolerable level by performing a 95 °C hardbake of the patterned AZ 4620 layer for 15 min. prior to the Parylene C deposition. Moderate bubbling of the patterned AZ 4620 layer still occurred even with the 95 °C, 15 min hardbake of the AZ 4620 layer, but the bubbling no longer distorted the overall shape or definition of the channel, and reflow of the patterned AZ 4620 photoresist no longer occurred. In the absence of the AZ 4620 hardbake, the bubbling and reflow of the AZ 4620 was so severe that the patterned AZ 4620 material would spread out and overflow past the first two patterned SU-8 layers, thus no longer providing a suitable shape for the fluid channel in the microtower array. After the third SU-8 layer was patterned, the exposed 2 µm Parylene C layer was etched away in a Plasma Therm RIE system (45 sccm O₂, 5 sccm CHF₃, 50 mTorr, 300 W, 12 min).

The sacrificial AZ 4620 material was removed by an acetone soak that ranged from 90 min to 2.0 hr, followed by a 15 min IPA rinse/soak. The IPA rinse/soak helped to remove any residues remaining from the acetone soak. Extending the sacrificial removal process to a 3.0 hr acetone soak resulted in delamination of the SU-8 layers in each microtower array, but a 2.0 hr acetone soak left the devices intact, robust, and with the desired hollow fluid channels. The sacrificial AZ 4620 layer could be successfully removed either before or after the 2-D linear microtower arrays were released from the Si substrate. However, complications in the 3-D vertical packaging and sealing of the
microtowers showed that it was best to not remove the sacrificial material prior to releasing the linear microtower arrays from the Si substrate (see Section 3.3: Vertical Packaging and Sealing Process).

3.2.7 Release Process

The 2-D linear microtower arrays were released from the Si substrate by placing the wafer in a Cr etchant (CR-7S, Cyantek) for approximately 3 hr. However, the time required to release the 2-D linear microtower arrays from the original Si substrate depended on the freshness of the Cr etchant being used and on the specific microtower design being released. The original microtower designs, which were in linear arrays of 8 or 16 microtowers, and had a center-to-center tower spacing of 600 µm, had released from the Si substrate with no difficulty. The new microtower designs, which were in linear arrays of 12 microtowers and had a center-to-center tower spacing of 200 µm, took longer to release from the Si substrate, but still released from the Si wafer without difficulty.

However, the brain slice microtower arrays, which were in linear arrays of 12 and had a center-to-center microtower spacing of 200 µm were very difficult to release from the Si substrate. The backside portion of the brain slice microtower arrays was designed with wide (150 µm) SU-8 channels that left only narrow sections (50 µm) of the Cr release layer exposed between adjacent microtowers. Hence, the design of the backside portion of the brain slice microtower arrays was not conducive to the final microtower array release process. The brain slice microtower arrays would release from the Si wafer after a week in the CR-7S etchant, but after such a long time in the Cr etch, the microtower arrays were damaged from the etchant and were no longer usable.
Ultrasonic agitation was attempted to assist in the release of the brain slice microtower arrays from the Si wafer. The sonication expedited the release of some of the brain slice microtower arrays, but the sonication also caused damage to the brain slice microtower arrays. Additionally, an alternate Cr etch was attempted using a 1:1 ratio of hydrochloric acid (HCl) and DI water, catalyzed with a piece of Al foil, but this Cr etch didn’t result in the release of any of the microtower arrays from the Si wafer. The start of the Cr etch could be visualized by rapid bubble formation using the Al foil catalyst, but the visual Cr etching reaction would subside in less than 1.0 min, and did not result in the releasing of the microtower arrays from the Si orifice plate. Even after 2.0 days of sitting in the diluted HCl solution, the devices had not released from the wafer.

However, the brain slice microtower arrays were eventually successfully released from the Si wafer by gently nudging the bottom portion of each brain slice device with tweezers. After the brain slice microtower arrays on the Si wafer had soaked in the CR-7S Cr etchant for 24 hours, the wafer was rinsed in DI water, then placed in a piece of glassware and covered with a shallow layer of DI water. Tweezers were then manually implemented to gently nudge each brain slice microtower array off of the Si substrate. This process worked, but it was not ideal since it was time consuming to serially remove each brain slice microtower array from the Si substrate, and the manual process sometimes induced damage to the bottom portion of the brain slice microtower arrays. Oftentimes, sections of the lower portion of the brain slice microtower arrays would remain on the Si substrate, while the remaining portion of the brain slice microtower array would release from the wafer.
To address the problems associated with the release of the brain slice microtower arrays, a thicker 5000 Å Cr layer (as opposed to the original 1600 Å Cr layer) was sputtered onto the Si wafer as a release layer in attempts to increase the flow of the Cr etchant underneath the brain slice microtower arrays to aid in their release. This thicker (5000 Å) sputtered Cr release layer improved the release process since it resulted in the brain slice devices releasing from the Si substrate after 3 days in the CR-7S. However, 3 days was still a lengthy release process time. In addition, thick Cr films tend to have a high residual stress [80], which can induce delamination of the Cr after subsequent materials are deposited onto the thick sputtered Cr layer. Slight delamination of the thick sputtered Cr was observed along the periphery of the Si wafer during fabrication of the microtower arrays, but the delamination wasn’t severe enough to be problematic in the fabrication of the microtower arrays.

The brain slice microtower array release problem was best solved by using an evaporator to deposit the Cr release layer as opposed to a sputtering system. Past literature has demonstrated that e-beam metal layers have superior quality to sputtered films and therefore perform better as release layers. Sputtering is a higher energetic process than evaporation, and therefore sputtered films tend to exhibit higher defects than films deposited using evaporation. A 1600 Å Cr release layer was deposited using either the e-beam evaporator or the PVD-75 Filament Evaporator, and resulted in successfully releasing the brain slice microtower arrays within 24 hr using the CR-7S Cr etchant.

S. D. Psoma et al. have assessed various metals and polymers used as sacrificial materials for releasing SU-8 structures from the substrate the SU-8 structures were originally fabricated upon. The results from the cited study showed that metals worked
better as sacrificial layers when releasing smaller SU-8 structures (up to 200 µm), with Cu providing the quickest release of the SU-8 structures, followed by Au, Al, and finally, Cr. However, this study used only sputtered metal films. The study by S. D. Psoma et al. also claimed that polymers (such as polystyrene) worked better as sacrificial layers when releasing larger SU-8 structures (up to 600 µm). [61]. Additionally, studies by C. Luo et al. cited the successful release of large SU-8 structures (with areas up to 50 cm²) using polystyrene as the sacrificial release material [62].

3.3 Vertical Packaging and Sealing Process

After the linear microtower arrays were released from the Si substrate, they were vertically packaged and structurally sealed into an upright, 3-D format. Silicon was chosen as the packaging substrate for the 3-D assembly of the microtower arrays, since Si is extensively used in microfabrication processes. Silicon can easily be patterned, etched, and used as a base substrate during microfabrication. Silicon orifice plates 1.5 cm × 1.5 cm × 500 µm were deep reactive ion etched (DRIE) using the Bosch process. Each Si orifice plate contained an array of through holes (8×8, 16×16, or 12×12) for the 3-D, vertical packaging of the microtower arrays. An SEM micrograph of a Si orifice plate, showing a cross-section of the through holes is shown in figure 3.9.
The center-to-center spacing of the through holes in the Si orifice plate was equal to the center-to-center spacing of the microtowers in a single linear array so that the microtowers could be easily slid into the Si orifice plate. The Si orifice plates could be batch fabricated in a Plasma Therm inductively coupled plasma (ICP) etching system, which facilitates mass fabrication since 32 Si orifice plates could be simultaneously created from a single 4 in Si wafer.

A schematic depicting three separate linear microtower arrays vertically packaged into a Si orifice plate is provided in figure 3.10. Once the linear microtower arrays were released from their original substrate by etching the Cr release layer, a single microtower array was picked up at its end containing the cross-arms, by means of a vacuum hose, and manually slid into the through holes in the Si orifice plate. The large Au tabs served as a structural stop to prevent the microtower arrays from sliding further through the Si orifice plate. The linear arrays of microtower structures were individually packaged in succession, one adjacent to the next, thus forming a complete (8×8, 12×12, or 16×16) 3-D array of vertical microtowers.
Since the diameters of the through holes in the Si orifice plate were slightly larger than the diameters of the microtowers, the microtower arrays had a slight tilt when initially packaged. The microtower arrays were corrected to a 90° angle with respect to the Si orifice plate with the aid of a tooling constructed by stereolithography (SLA). The SLA tooling was a comb-like structure, with teeth that slid between each adjacent linear microtower array that was packaged into the Si orifice plate, which physically straightened out each microtower array to a 90° angle with respect to the Si orifice plate (see Section 5.2: Packaging Results and Discussion).

Once the microtower arrays were placed into the Si orifice plate, and straightened out with the SLA jig, the microtower arrays were structurally sealed into place on the backside of the Si orifice plate using a 3:1 mixture of 184 PDMS and hexane. Hexane is used as a means to dilute the PDMS since the PDMS by itself is too viscous to flow between all the microtowers to create an appropriate seal. The PDMS/hexane mixture
creates both a structural and fluidic seal between the microtower arrays and the Si orifice plate holes. The PDMS/hexanes mixture is cured in an oven at 70 °C for 60 min. Subsequently, the AZ 4620 photoresist is removed from the microtower arrays by a 60 min acetone soak, followed by several IPA rinses, to expose the hollow fluidic channels in each microtower and cross-arm. Scanning electron micrographs of the topside of a packaged 8×8 microtower array are shown in figure 3.11. Figure 3.11a shows 1.5 mm tall microtower arrays packaged and sealed into a Si orifice plate. Figure 3.11b shows a close up of the packaged microtower arrays, showing the fluid channel openings at the top of the microtowers, side ports, and electrodes.

![Vertically packaged linear SU-8 microtower arrays](a)

![Fluid channel opening and side ports](b)

Figure 3.11. SEM micrographs of an 8×8 packaged microtower array. (a) 1.5 mm tall microtowers packaged and sealed into a Si orifice plate; (b) close up view of the microtowers and the top functional cross-members, showing the top fluid channel openings, fluid side ports, and electrodes.

The backside of the microscaffold device is displayed in figure 3.12, showing the lower portions of the microtower arrays projecting out the back of the Si orifice plate. Initially, the microtower arrays were sealed into the Si orifice plate with an insufficient PDMS/hexane seal (see figure 3.12a), which provided satisfactory mechanical stability to
each microtower array, but did not create a robust enough seal to prevent fluid from seeping through the Si orifice plate holes of the functioning fluidic system. However, applying a second PDMS/hexane coating to the backside of the Si orifice plate created an appropriate barrier to prevent leakage of any fluids through the Si orifice plate holes (see figure 3.12b).

After the linear microtower arrays were vertically packaged and sealed into the Si orifice plate, SU-8 “pitchfork” structures were slid into the packaged array of microtowers and sealed into place with PDMS. These pitchfork structures created additional cross-arms that connected adjacent linear microtower arrays to each other. SEM micrographs of the vertically packaged microtower arrays with the joining pitchfork structures are provided in figure 3.13. These additional cross-arms were structural components consisting of solid SU-8 that were hypothesized to aid in 3-D neuronal network formation once dissociated neuronal cells were plated on the device.
The SU-8 pitchfork structures were fabricated on top of a Cr release layer on a Si substrate, from a single layer of SU-8 2025 (80 µm thick). Once the SU-8 pitchforks were patterned and hardbaked, they were released from the Si wafer by etching the Cr release layer in CR-7S (Cyantek Corp.) etchant.

**3.4 Fluidic Packaging**

The integration of fluid functionality into the dissociated neuronal cell microscaffold system was a collaborative effort between the Frazier and the Glezer research labs at the Georgia Institute of Technology. A PDMS fluid manifold, which served as an interface between the microscaffold and the external fluid delivery system, was designed and fabricated to house the Si orifice plate and the packaged microtower arrays. The PDMS fluid manifold was designed in two pieces (a top piece and a bottom piece), which were created using 3-D paraffin wax molds. The wax molds were built in a 3-D rapid prototyping wax printer (Thermojet, 3-D Systems). Schematics of the top and
bottom wax mold pieces and photographs of the respective PDMS structures created using those wax molds are shown in figures 3.14a and 3.14b.

![Image](image_url)

**Figure 3.14.** PDMS fluid manifold. (a) Photograph of the top PDMS manifold piece. Insert shows a schematic of the 3-D wax mold for the top manifold piece. (b) Photograph of the bottom PDMS manifold piece. Insert shows a schematic of the 3-D wax mold for the bottom manifold piece.

The bottom PDMS manifold piece contained a recess into which the 1.5 cm × 1.5 cm × 500 µm Si orifice plate fit and was sealed into place with PDMS. Next, the top and bottom PDMS manifold pieces were aligned using four nylon screws and nuts (McMaster) at the four corners of the device, and the top and bottom manifold pieces were sealed together using PDMS. A schematic showing a 3-D cross-section of the fluid manifold integrated with the microscaffold is shown figure 3.15. Tubing for media delivery was attached to the inlet port at the base of the fluid manifold. The fluids first fill the pool at the bottom of the manifold, and then travel up through each of the tower lengths, through the tower cross-arms, and out the fluidic ports to perfuse the 3-D neuronal network being cultured within the active microscaffold system.
The fluid was delivered to the inlet port via a syringe pump. The initial characterization of the fluidic system was performed by imaging the flow of DI water dyed with food coloring through the microscaffold/fluid manifold package. Since the SU-8 is transparent, it was possible to observe the fluidic path of the colored DI water and determine whether there were any leaks in the PDMS/Si orifice plate seal. Furthermore, the fluidic functionality of the microscaffold/fluid manifold package was validated by running a mixture of polystyrene fluorescent beads (1.0 µm in diameter, Molecular Probes) and DI water through the microtower cross-arms and out the fluid ports in the towers. Microscopy confirmed the flow of the polystyrene beads through the microtower cross-arms and the fluid ports.

Figure 3.15. A 3-D cross-section of the microscaffold device encased in the PDMS fluid manifold. The fluid manifold allows for continuous perfusion of the neuronal network cultured within the active microscaffold system.
3.5 Electrical Packaging

The fourth specific aim of this dissertation involved electrical integration and characterization of the dissociated neuronal cell microtowers to stimulate/record 3-D neuronal networks growing on the active microscaffold system. This was achieved by forming 90° electrical connections between the vertical microtower leads and the planar substrate leads on the Si orifice plate to validate electrical functionality of the dissociated neuronal cell microscaffold.

The electrical integration of the microtower electrodes on the dissociated neuronal cell microscaffold occurred on the topside of the Si orifice plate. Each microtower contained two electrodes with corresponding insulated leads that connected to thick Au tabs (23 µm thick) at the base of each microtower. Additionally, Au/Sn-Ag solder pads were electroplated on both sides of each through wafer via on the Si orifice plate. An SEM micrograph of the Si orifice plate with the Au/Sn-Ag solder pads electroplated on both sides of each through hole is provided in figure 3.16.

![SEM micrograph of the Si orifice plate with Au/Sn-Ag solder pads electroplated on both sides of each through hole.](image)

Figure 3.16. An SEM micrograph of the Si orifice plate with Au/Sn-Ag solder pads electroplated on both sides of each through hole.
Figure 3.17 provides cross-sections of the metallized Si orifice plate fabrication process.

Figure 3.17. Cross-sections of the fabrication process for the Si orifice plate with electroplated Au/Sn-Ag solder pads. (a) A Au layer seed is sputtered onto a blank Si wafer and a thick (23 µm) photoresist electroplating mold is spun and patterned on the wafer. (b) Electroplated Au (5 µm thick) is deposited into the mold. (c) Electroplated Sn-Ag solder (5 µm thick) is electroplated on top of the electroplated Au. (d) The photoresist electroplating mold is removed. (e) The Au seed layer is etched in exposed areas. (f) A thick photomask is spun and patterned on the wafer for through wafer etching. (g) Through wafer vias are created in the Si using the Bosch process. (h) The photoresist mask is removed with acetone and IPA.
To form an electrical bond between the Au microtower tabs and the Au/Sn-Ag solder Si orifice plate pads, the solder needs to reflow, which begins to occur around 220 °C. Hence, the entire packaged device needed to be heated on either a hotplate or in an oven at 220 °C to reflow the solder and create an electrical bond. Experiments have shown that heating the packaged SU-8 microtower arrays to 220 °C for a short duration did not damage the SU-8 microtower arrays. Additionally, after heating at 220 °C, some bonding between the Au microtower tabs and the Au/Sn-Ag solder orifice plate tabs was observed, but further experimentation showed that a temperature of 250 °C was more conducive to uniform reflow conditions and still didn’t induce any damage to the SU-8 structures.

The packaged microtower arrays and Si orifice plate were heated to 250 °C in an oven to induce reflow of the Sn-Ag solder and form a 90° electrical bond between the vertical microtower leads and the Sn-Ag solder tabs electroplated on the Si orifice plate. Reflow of the Sn-Ag solder was also attempted on a hotplate, but the oven reflow process provided better results. Also, electroplating Sn-Ag solder on top of the Au microtower tabs was also experimented with, but this created further complications in the fabrication of the microtower arrays, and was deemed unnecessary to provide electrical functionality to the microtower electrodes (see Section 5.1.3: Electroplating). When the microtower arrays were vertically packaged into the metallized Si orifice plate, the Au tabs at the base of each microtower came into contact with their respective Au/Sn-Ag solder tabs on the Si orifice plate, as shown in figures 3.18a and 3.18b. Both Au and a small amount of Sn-Ag solder was electroplated onto the tabs at the base of the microtowers shown in figures 3.18a and 3.18b.
Originally, it was thought that electroplating the Sn-Ag solder on both the microtower tabs and the Si orifice plate tabs would assist in forming the 90° electrical bond. However, the Sn-Ag solder tended to plate in a non-uniform manner on the microtower tabs. The Sn-Ag electroplating surface was not smooth and exhibited a whiskering effect that resulted in extremely rough topography. In turn, the rough, non-uniform topography of the Sn-Ag solder microtower tabs complicated subsequent photolithography steps in the microtower array fabrication process. For example, the extreme topography of the Sn-Ag microtower tabs caused problems in patterning the Au leads near the base of each microtower (see Section 5.1.3: Electroplating). Hence, it was decided to only electroplate Au on the microtower tabs and to electroplate the Au and Sn-Ag solder on the Si orifice plate tabs. Therefore, the Sn-Ag solder reflow would only occur on the Si orifice plate and would reflow to the extent necessary to form a bond with the electroplated Au microtower tabs. Also, originally the Sn-Ag solder refloowed to such
an extent that all of the Sn-Ag solder tabs on the Si orifice plate reflowed into one another. Experiments to resolve this reflow issue were attempted by patterning an SU-8 well around each of the solder tabs to confine the reflow.

3.5.1 Multi Channel Systems Preamplifier Setup

The microtower arrays were integrated with an electrical packaging that had the potential to stimulate and record neuronal cultures being cultured on the microscaffold system. Integrating the developed microscaffold system with the commercially available Multi Channel Systems preamplifier setup, which can stimulate or record from 59 sites, and is widely used in 2-D electrophysiological neuronal cell studies, was thought to be the most efficient way to provide electrical functionality to the microscaffold system.

Figure 3.19a shows a 2-D MEA patterned on a 4.9×4.9 cm² glass slide that can plug directly in the Multi Channel Systems preamplifier setup. A 2-D neuronal network can be cultured on such an MEA, with the neuronal culture being contained within the ring seen at the center of the glass slide. Figure 3.19b shows a picture of the Multi Channel Systems preamplifier into which the MEA can be inserted to obtain cell stimulation/recording data from the network. The preamplifier contains 60 pins (59 active sites and one ground) that attach to each mega contact pad at the periphery of the MEA to electrically stimulate/record from the cultured neuronal network.
The electrically functionalized Si orifice plate had a design layout that mimics that of the 2-D MEA seen in figure 3.19a. However, the substrate was Si (with an SiO₂ insulation layer) instead of glass, and the active microscaffold system with its 3-D array of microtower electrodes, was centered inside the culture ring instead of the 2-D MEA seen in figure 3.19a. Hence, the electrically functionalized Si orifice plate had an array of through holes at the center of the culture ring into which each microtower array was vertically packaged, electrically bonded to the substrate, and sealed into place with PDMS.

3.5.1.1 Details of Interfacing with the Multi Channel Systems Preamplifier Setup

A set of five photomasks was used to fabricate the electrically functional Si orifice plates for the 8×8 microtower arrays. The photomasks were designed using AutoCAD software, and high resolution transparencies of each photomask layer were created by JD Photo (UK). The patterns from the high resolution transparencies were then transferred to Cr-on-glass photomasks in the MiRC Cleanroom. Each electrically
functionalized Si orifice plate was fabricated from a 3 in Si wafer. The original Si orifice plates that did not contain any electrical circuitry and were used exclusively for fluidic applications were only $1.5 \times 1.5 \text{ cm}^2$. However, the electrically functional Si orifice plates were significantly larger ($4.9 \times 4.9 \text{ cm}^2$), since they were designed to fit into the Multi Channel Systems preamplifier setup. Top views of the fabrication process for the electrically functioning Si orifice plates, showing each photomask are provided in figure 3.20.

![Fabrication Process](image)

**Figure 3.20.** Top views of the fabrication process for each of the five photomasks used to fabricate the MEA Si orifice plate for the 8×8 microtower array. (a) Electroplated Sn-Ag solder tabs; (b) patterned Au leads, (c) SU-8 well defined to confine the Sn-Ag solder during reflow, (d) through wafer vias etched for vertical packaging of the microtower arrays.
Cross-sections of the fabrication process for the electrically functionalized Si orifice plate that is compatible with the Multi Channel Systems preamplifier setup are provided in figure 3.21, and a detailed protocol of the fabrication process is provided in Appendix C.

Figure 3.21. Cross-sections of the fabrication process for the electrically functionalized Si orifice plate that is compatible with the Multi Channel Systems preamplifier setup. (a) Pattern 10 μm of Futurrex NR-9 8000 to define electrode patterns; (b) evaporate Ti/Au; (c) lift-off process in acetone to define substrate traces; (d) AZ 4620 lithography for through wafer vias; (e) DRIE to form through wafer vias; (f) remove photoresist and sputter Au seed layer; (g) AZ 4620 lithography to form an electroplating mold; (h) electroplate Sn-Ag solder tabs; (i) remove AZ 4620 electroplating mold in acetone; (j) etch Au seed layer.
A photograph of the electrically packaged microtower array is provided in figure 3.22, showing an 8×8 array of microtowers vertical packaged and sealed into the Si orifice plate that is compatible with the Multi Channel Systems preamplifier setup. The substrate traces and contact pads, SU-8 microtowers, and polycarbonate culture ring can be seen. After the microtower arrays were sealed into place on the backside of the Si orifice plate using PDMS, the Sn-Ag solder substrate tabs were reflowed at 250 °C to form an electrical bond with Au tabs at the base of each microtower on the front side of the Si orifice plate to functionalize the microtower electrodes. Insulation of the metal tabs and substrate traces was subsequently performed using PDMS. The electrically functional Si orifice plate design, shown in figure 3.22, enabled integration of 59 microtower electrodes out the 128 total microtower electrodes in the 8×8 microscaffold design.

Figure 3.22. Photograph of an 8×8 electrically packaged microtower array. The Sn-Ag solder tabs on the Si orifice plate have been reflowed to form electrical bonds with the Au tabs at the base of each microtower.
CHAPTER 4
EXPERIMENTAL METHODS

4.1 Initial Biological Tests on the Unpackaged SU-8 Microtower Arrays

The LaPlaca, Allen, and Brewer labs performed numerous experimental biocompatibility treatments on SU-8 bits in efforts to leach any lingering toxins out of the SU-8 and to aid in cell adhesion to the SU-8. The biocompatibility treatments that worked best on the SU-8 for culturing neurons were a 90,000 mJ/cm² ultra-violet (UV) light exposure, followed by a three day 150 °C vacuum oven bake, followed by a 60 sec O₂ plasma treatment, and a 60 sec IPA sonication. The UV light exposure and the vacuum oven bake helped to fully cure and hardbake the SU-8, while the O₂ plasma treatment rendered the SU-8 surface hydrophilic to aid in cell adhesion. Finally, the 60 sec IPA sonication removed any remaining residues from the hollow channels in the microtowers, and significantly improved cell survival on the SU-8 microtower arrays. Other treatments, including liquid CO₂ critical drying (Tousimis Super Critical Dryer) and a piranha etch (H₂SO₄ and H₂O₂ in a ratio of 3:1) were experimented with to enhance the biocompatibility of the SU-8 without significant success. Also, the liquid CO₂ treatment delaminated the multiple SU-8 layers that comprised the microtower arrays (see Section 5.4.1: Biocompatibility of SU-8), so the liquid CO₂ treatment could not be included in the biocompatibility protocol for the microtower arrays presented in this dissertation.

Biocompatibility of the 2-D linear microtower arrays was initially demonstrated by the Brewer lab at the Southern Illinois University School of Medicine by culturing primary hippocampal embryonic rat pup neurons on the unpackaged, released, hollow
microtower arrays. The microtower arrays were treated with a 90,000 mJ/cm² UV light treatment, a three day 150 °C vacuum oven bake, a 60 sec O₂ plasma treatment, and a 60 sec IPA sonication at the Georgia Institute of Technology prior to sending the microtower arrays to the Brewer lab for biological testing. Once the microtower arrays arrived at the Southern Illinois University School of Medicine, one set of unpackaged microtower arrays was subjected to the following protocol:

1. Rinse in 70% EtOH 2X, soak for 5 min
2. Rinse 2X in sterile water
3. 60 sec sonication in IPA, dry
4. 30 sec glow discharge treatment
5. Rinse 2X 70% EtOH
6. Cover overnight with 100 µg/mL poly-L-lysine (PLL)
7. Rinse 2X in sterile water
8. Plate at 50,000 neurons/cm² (500 cells/mm²)

and another set of microtower arrays was subjected to the same protocol with the exclusion of step 3 (60 sec IPA sonication) and step 4 (30 sec glow discharge treatment).

Hippocampal neurons to seed the microtower arrays were isolated from embryonic day 18 rats (BrainBitsLLC) according to established procedures [81]. Neurons isolated by digestion of the brain tissue in papain [Worthington, 2.0 mg/mL in Hibernate E (BrainBitsLLC)] were re-suspended in culture medium and applied to the microtowers at 500 cells/mm² of structure surface area in a 35 mm D culture dish. After 10 min in the incubator [Forma, 5% carbon dioxide (CO₂), 9% O₂], a fresh load of
neurons was again rinsed into the structure. After 30 min in the incubator, 3.0 mL of the medium was added to the dish to completely cover the structures.

Briefly, the culture medium was exchanged with Neurobasal. Cells were loaded with Calcein-AM (5 µg/mL, Molecular Probes) for 1 hr at 37 °C. After a rinse with warm Hibernate E low fluorescence (BrainBitsLLC) to preserve control of pH at ambient CO₂, neurons were imaged under a 20x/0.95 NA Olympus water immersion objective. Viable cell fluorescence was excited with blue light. Green fluorescence was collected on a Q imaging Retiga charge coupled device (CCD) camera. Z-stacks were collected and deconvolved with Image Pro+ Software.

Biocompatibility of the 2-D linear microtower arrays was also demonstrated by the Wheeler lab at the University of Illinois at Urbana-Champaign. Prior to cell plating, the planar microtower arrays received the following treatment at the University of Illinois at Urbana-Champaign:

1. A 100% EtOH soak for 20 hr
2. Rinse once DI water
3. Dry in an oven at 90 °C for 3 hr
4. Poly-D-lysine (PDL) coating (0.1 mg/mL in DI) by soaking the microtower arrays in a 1 mL micro-centrifuge tube containing PDL to ensure PDL coating on all the microtower surfaces
5. Cell plating of cortical neurons (BrainBitsLLC) at 30,000 cells/cm² (300 cells/mm²).

The Wheeler lab used the same culturing protocol as the Brewer lab to culture the neurons on the unpackaged, linear microtower arrays. However, the Wheeler lab plated
at a cell density of 300 cells/mm² (as opposed to 500 cells/mm² plated by the Brewer lab), and confirmed cell viability using SEM and phase contrast images (as opposed to live/dead cell fluorescent staining performed by the Brewer lab).

To fix the microtower structures with cultured neurons for SEM analysis, the following dehydration protocol was performed using a series of graded EtOH rinses:

1. Remove PBS
2. Add 25 % EtOH and wait for 5 min
3. Remove 25 % EtOH
4. Add 50 % EtOH and wait for 5 min
5. Repeat these steps for the 70% and 95% EtOH mixtures
6. For 100 % EtOH, repeat three times
7. Replace the 100 % EtOH solution with HMDS solution
8. Let the HMDS dry and then place the samples under vacuum at room temperature overnight
9. Samples removed from the vacuum for SEM analysis in the morning.

4.2 Fluid Flow Device Tests

The fluid was delivered to the inlet port at the base of the PDMS fluid manifold via a syringe pump. Initial characterization of the fluidic system was performed by imaging the flow of DI water dyed with food coloring through the microscaffold/PDMS fluid manifold package. Since the SU-8 microtowers were transparent, it was possible to observe the fluidic path of the colored DI water and determine whether there were any leaks in the PDMS/Si orifice plate seal. Furthermore, fluid functionality of the
microscaffold/fluid manifold package was validated by flowing a mixture of polystyrene beads (1.0 µm in diameter, Molecular Probes) and DI water through the fluid channels in the microtower cross-members, and out the fluid side ports in the microtowers. Microscopy confirmed the flow of the polystyrene beads through the microtower cross-members and the fluid ports.

4.3 Biological Tests on the Packaged Microscaffolds with Perfusion (Compared to Cultures on the Packaged Microscaffolds without Perfusion)

The series of biocompatibility treatments (90,000 mJ/cm² UV light exposure, three day 150 °C vacuum oven heat treatment, 60 sec O₂ plasma treatment, and 60 sec IPA sonication) were performed on the packaged SU-8 microtower arrays after the AZ 4620 sacrificial photoresist was removed to yield the fluid channels, but prior to sealing the device into the PDMS fluid manifold. The Brewer lab at the Southern Illinois University School of Medicine performed numerous biological tests on the packaged microscaffolds with fluid functionality and compared cell viability data with fluid perfusion to cultures grown on glass coverslips and cultures grown on the packaged microscaffolds without perfusion. Additionally, the LaPlaca lab at the Georgia Institute of Technology cultured 3-D neuronal-astrocytic co-cultures on the packaged microscaffold systems without fluid perfusion. The LaPlaca lab used a Matrigel matrix to achieve a 3-D neuronal cell culture within the microtower array, while the Brewer lab cultured re-aggregate neuronal networks on the different planar microtower surfaces of the microscaffold in the absence of a matrix.
4.3.1 Biological Tests on the Packaged Microscaffolds with Perfusion (Compared to Cultures on the Packaged Microscaffolds without Perfusion) Performed by the Brewer Lab at the Southern Illinois University School of Medicine

Once the completed microscaffold systems were packaged into PDMS manifolds, the devices were sent to the Brewer lab at the Southern Illinois University for biological testing with fluid perfusion. Optimum perfusion rates for two different cell densities (1,000 cells/mm$^3$ and 10,000 cells/mm$^3$) were determined, and perfusion cell viability data was compared to cell viability data on unperfused glass coverslips and unperfused microscaffold (control) systems. Cell viability studies were performed at 3, 7, 14, and 21 DIV.

A glow discharge treatment was performed on the active microscaffold systems prior to cell plating, which helped to increase cell viability on the devices. The packaged microscaffolds were tilted at approximately a 15° angle during the initial 180 sec glow discharge treatment. The sample was then tilted approximately 15° in the opposite direction and received an additional 180 sec glow discharge treatment. A photograph of the tilted microscaffold undergoing the glow discharge treatment is provided in figure 4.1.
Inlet and outlet tubings were attached to the backside of the PDMS fluid manifold. Using a drummel, rectangular openings were drilled into the base of one plastic pe-tri dish and through the bottom and side of a separate pe-tri dish. The two pe-tri dishes were then sealed together using PDMS as shown in figure 4.2. The packaged microscaffold device was then placed in the top pe-tri dish and the tubing was run out the bottom and side openings in the dishes.

**Figure 4.1.** Photograph of the microscaffold device during the glow discharge treatment. The sample is tilted at approximately a 15° angle.

**Figure 4.2.** Photograph of the pe-tri dish setup with openings through which the fluid tubing was fed.
Also, a rectangular PDMS insert was fabricated to fit around the immediate periphery of the microtower arrays to assist in maximal cell plating on the microtower structures. The PDMS insert was used to contain the cells and media during cell plating on the microscaffolds, but the PDMS insert was removed 1.0 hr after the cell plating. Pictures of the PDMS insert and the packaged active microscaffold system are provided in figure 4.3.

![Figure 4.3. Photographs of the experimental cell culture setup showing (a) the packaged microscaffold device with the tubing running through the bottom and side openings in the petri dishes. The PDMS insert is shown next to the device; and (b) the active microscaffold device, housed in the petri dishes, with the PDMS insert placed around the immediate periphery of the microtower array.](image)

The experimental protocol for coating and plating on the active microscaffold systems with fluid perfusion is listed below.

**Protocol for coating and plating on the active microscaffold systems with fluid perfusion:**

1. Soak towers in 70% EtOH to sterilize
2. Rinse in water, aspirate
3. Tilt towers ~15° so that the side of the microtowers is facing out and perform 180 second glow discharge treatment
4. Tilt towers ~15° in opposite direction. Perform 180 sec glow discharge treatment on opposite side of the microtowers
5. Connect sterile fittings to bottom of manifold and plug ends
6. Rinse towers and fittings in EtOH to sterilize and wet
7. Rinse in sterile water
8. Place each microscaffold device in a new sterile dish, labelled with name
9. Place PDMS insert onto Si plate, around the microtower array (carefully) to create walls to contain the medium
10. Place enough PDL over each microscaffold to submerge it (100 µL). Coat overnight
11. Aspirate PDL, rinse in sterile water and allow to dry
12. Prime manifold and fittings with pH equilibrated medium
13. Rinse towers with pH equilibrated medium vigorously, forcing all air bubbles from microtowers
14. Tilt towers, draw off media and immediately apply half of total cell solution (50 µL) vigorously to ensure penetration to bottom of the microtower array
15. Tilt tower in opposite direction and apply remaining cell application (50 µL)
16. Plate glass control slips at the same time.
17. After at least one hr, remove PDMS insert and add pH equilibrated medium to fill the reservoir.

New methods were developed to ensure that the microtowers in the interior of the array received similar PDL rinsing treatments and neuron applications as the microtowers
around the periphery of the array. To prepare the microscaffold for neurons, the microtower assemblies were soaked in 70% EtOH overnight for sterilization. A vacuum exposure followed by re-application of EtOH ensured interior access of the EtOH. While wet, three cycles of rinsing in sterile water were followed by rinsing three times in 100 μg/mL PDL (Sigma). The last solution was left on the structures for at least 4.0 hr or overnight. Immediately before plating neurons, culture medium (Neurobasal/B27/0.5 mM glutamine/25 μM glutamate, Invitrogen) was rinsed into the structures five times.

Hippocampal neurons to seed the structures were isolated from embryonic day 18 rats according to established procedures [81]. Neurons isolated by digestion of the brain tissue in papain (Worthington, 2.0 mg/mL in Hibernate E (BrainBitsLLC) were re-suspended in culture medium applied to the structures at 500 cells/mm² of structure surface area in a 35 mm D culture dish. After 10 min in the incubator (Forma, 5% CO₂, 9% O₂), a fresh load of neurons was again rinsed into the structure. After 30 min in the incubator, 3.0 mL of medium was added to the dish to completely cover the structures. A perfusion rate of 0.05 μL/hr was used on the active microscaffolds where neurons were being cultured with nutrient perfusion. Live cells were either stained with Calcein-AM to fill the cytoplasm or Syto-16 to label live cell nuclei in denser cultures. Propidium iodide was used to stain dead cells. Neuron survival was counted as percent live cells/total cells.

4.3.1.1 Fluid Flow Rates

Fluid was delivered to the base of the microscaffold system via a syringe pump. Numerous perfusion rates were experimented with to determine optimum perfusion rates for the following two cell densities; (1) 1,000 cells/mm³, and (2) 10,000 cells/mm³. The initial biological experiments with fluid perfusion performed by the Brewer lab
determined that the optimum perfusion rate was somewhere between 0.05 µL/hr and 0.025 µL/hr with a 270 sec glow discharge treatment after 4 DIV for the two different cell densities (1,000 cells/mm\(^3\) and 10,000 cells/mm\(^3\)) under investigation. Further investigation showed that the cells showed the greatest viability at the lowest perfusion rate of 0.05 µL/hr. This perfusion rate was so low that drain/outlet port designed into the PDMS manifold was unnecessary since the excess media would tend to simply evaporate at such a low perfusion rate.

Short-term (three day) viability studies were initially performed to determine optimum perfusion rates for the cell culture. Once the optimum fluid flow rates were determined, longevity of the culture after 7, 14, and 21 days was determined (compared to no perfusion). Additionally, for both short and long-term studies, the optimum perfusion cell viability data was compared to cell viability data on unperfused glass coverslips and unperfused microscaffold systems.

The protocol for media flow on live cells using the active microscaffold system is listed below.

**Protocol for media flow on live cells:**

If live, healthy cells are present, connect manifolds to syringe pump.

1. Sterilize syringes and tubing by flowing EtOH
2. Rinse with sterile water
3. Prime tubing and syringes with pH equilibrated medium
4. Remove plug in manifold and connect primed tubing
5. Run tubing out of filter hole in back of incubator to syringes on pump
6. Pump rate - 0.05 µL/hr (one media change per 6.666 days)
7. Remove flow after designate time period.

A photograph of the incubator setup that houses the active microscaffold system during neuronal cell culture is provided in figure 4.4. The syringe pump that delivers media to the active microscaffold system can also be seen in figure 4.4. The tubing from the syringe pump enters the incubator from a hole in the backside of the incubator and connects to the base of the PDMS fluid manifold to deliver media to the cells being cultured within the active microscaffold system.

Figure 4.4. Photograph of the incubator and syringe pump experimental setup for delivering media to the neuronal networks cultured on the active microscaffold system.

SU-8 microtower stain after pumping over weekend (7 DIV)

1. Add 1 µL Syto-16 stock to cell culture area of manifold
2. Add 2 µL Propidium Iodide (4.6 mg/mL in HBSS)
3. Gently swirl dye solution around well and incubate for 15 min
4. Rinse in Hibernate minus phenol red until solution is clear (usually 1-2 rinses)
5. Imaging will be done in Hibernate
6. Count live cells and dead cells using different filters

7. Microscope pictures taken
   a. Count live/dead cells (survival) and compare with control.

**4.3.2 Biological Tests on the Packaged Microscaffolds without Perfusion Performed by the LaPlaca Lab at the Georgia Institute of Technology**

The LaPlaca lab at the Georgia Institute of Technology cultured passive diffusion 3-D neuronal-astrocytic co-cultures on the packaged microscaffold systems (in the absence of fluid perfusion). The LaPlaca lab used a Matrigel matrix to achieve a 3-D neuronal cell culture within the microscaffold structure. The goal of these passive diffusion co-cultures was to assess cell survival and distribution within the SU-8 microscaffold system. The culture consisted of cortical neurons (rat; E18) + cortical astrocytes (rat; P0; passage 4 - 5) mixed in a 1:1 ratio. Neurobasal/B27/G5 medium and Matrigel matrix (final concentration 7.5 mg/mL) was used. The passive diffusion cell density was 2,500 cells/mm³, and cell viability and distribution was assessed at 15 and 21 DIV, using confocal microscopy. Live and dead cell staining was performed using Calcein AM and ethidium homodimer-1.

**4.4 Electrical Device Tests**

Once electrical functionality was established, the active microscaffold system was immersed in saline and a probe station was utilized to measure the impedance of the microtower electrodes via the contact pads on the periphery of the Si orifice plate. Additionally, the Au microtower electrodes were electroplated with Pt black to lower the electrode impedance to a level that was conducive for stimulation/recording of the
cultured neuronal networks. The electrical testing of the active microscaffold system was performed using the impedance spectroscopy setup and the Pt black electroplating device in the DeWeerth lab at the Georgia Institute of Technology. A schematic showing the impedance measuring setup is provided in figure 4.5.

![Schematic showing the impedance setup used to test the viability of the microtower electrodes.](image)

Figure 4.5. Schematic showing the impedance setup used to test the viability of the microtower electrodes.

The electrically packaged microscaffold system (3-D MEA) was fitted into the Multi Channel Systems interface and impedance switching board. The polycarbonate culture ring surrounding the microtower array (3-D MEA) was filled with Hank’s Balanced Salt Solution (0.1 micron filtered, Invitrogen Corp., Carlsbad, CA), and the impedance of the 59 functionalized microtower electrodes was measured across a range of frequencies (1 mHz to 100 kHz), using a Stanford Research Systems SR785 (Stanford Research Systems, Sunnyvale, CA) two-channel dynamic signal analyzer. A serial connection from the impedance switching board to a computer with Matlab software
collected the impedance data from the 59 microtower tower electrodes. The 60 switches from the switching board to the MEA corresponded to the 60 mega contact pads at the periphery of the MEA, connecting to 59 functionalized microtower electrodes and one ground line. The three lines from the switching board to the signal analyzer allowed for impedance measurements over a wide frequency range. The general purpose interface bus (GPIB) used was a standard communication protocol that allowed communication between different instruments.

A photograph of the electrical testing setup is provided in figure 4.6, showing a 2-D commercial MEA fitted into the impedance switching board and Multi Channel Systems interface. The polycarbonate culture ring, surrounding the 2-D MEA, filled with saline solution, the serial connection to the computer, and the three lines connected to the signal analyzer can also be seen. The electrically active microscaffold system presented in this dissertation (shown in figure 3.22) was plugged into the impedance switching board and Multi Channel Systems interface setup to test the viability of the Au microtower electrodes by performing impedance spectroscopy.
After the functionality (impedance) of the microtower electrodes was validated across a range of frequencies, Pt black was electroplated onto the microtower electrodes using the Pt black electroplating device in the DeWeerth lab to increase the surface area of each electrode and therefore significantly lower the impedance of each electrode to a range suitable for neuronal stimulation and recording. A schematic of the electroplating setup is provided in figure 4.7.

Figure 4.6. Photograph of the impedance switching board and Multi Channel Systems preamplifier setup, interfaced with a 2-D MEA for neuronal cultures. The serial connection runs to a computer with Matlab software, and the three cables connect to a Stanford Research Systems SR785 two-channel dynamic signal analyzer.
The electroplating setup functioned such that when a switch closed to deliver current to the electroplating bath, microtower electrode, and the Pt coated mesh electrode, Pt ions are reduced to Pt metal at the microtower electrode surface, therefore depositing Pt black onto the microtower electrode surface. As the surface area of the microtower electrode increased (from the Pt black deposition), the impedance of the microtower electrode decreased. A 1 kHz sine wave was applied across the Pt black plating bath (contained within the polycarbonate culture ring) and reference resistor and changes (decreases) in the voltages were observed to confirm that the Pt black electroplating was occurring. The Pt black electroplating solution was composed of 1% chloroplatinic acid (Sigma C-3044, 8% diluted with DI water), 0.0025% hydrochloric acid, and 0.01% lead acetate in DI water.

A laptop communicated to a peripheral interface controller (PIC) microcontroller through a serial connection, with the ability to switch electroplating current to each of the functionalized microtower electrodes. A Keithley 2400 current source with sub-nanoamp
precision delivered current and measured voltage during the electroplating process, electroplating 16 microtower electrodes at a time. Past experiments in the DeWeerth lab determined that electroplating Pt black at 1 µA/(40×40 µm²) using this setup resulted good adhesion of the Pt black and resulted in electrodes with the desired impedance for neuronal stimulation/recording (in the kHz range). Therefore, the current density 1 µA/(40×40 µm²) was multiplied by the area of a microtower electrode (15×15 µm²) to determine a current value of I = 0.14 µA used for the Pt black electroplating for the microtower electrodes. A photograph of the Pt black electroplating device developed in the DeWeerth lab at the Georgia Institute of Technology [82] is provided in figure 4.8.

Figure 4.8. Photograph of the Pt black electroplating setup used to electroplate up to 16 microtower electrodes with Pt black at one time. After 16 microtower electrodes had been electroplated, the MEA was rotated 90° to electroplate the next set of microtower electrodes.
CHAPTER 5

RESULTS AND DISCUSSION OF FABRICATION, PACKAGING, AND BIOLOGICAL TESTING

5.1 Fabrication Results and Discussion

A significant research effort in this dissertation was directed towards the fabrication of the hollow SU-8 microtower arrays with integrated electrodes. Specifically, significant fabrication challenges arose in the lithography of the SU-8, the creation of the fluid channel, and the release of the completed SU-8 structures from the original Si substrate upon which they were fabricated. Also, finding the right synergy among the various materials used in the microfabrication of the SU-8 microtower arrays was crucial for the successful development of the active microscaffold system. For example, some of the sacrificial materials experimented with in the fabrication of the hollow fluid channel were not compatible with the processing of the SU-8, while other sacrificial materials had properties that were more conducive to the SU-8 processing. However, sometimes additional processing steps could be added to make these problematic sacrificial materials work with the developed SU-8 processing.

Biocompatibility had to be considered when choosing materials for the fabrication of the microtower arrays since the final device was intended for increasing the long-term viability of \textit{in vitro} neuronal cell cultures. This section of the dissertation provides the fabrication results and discussion for the realization of the planar microtower arrays, and includes subsections on material selection, SU-8 lithography, electroplating, and sacrificial layer fabrication.
5.1.1. Material Selection

One of the greatest challenges for BioMEMS is delivering a final product that is biocompatible. That is, the BioMEMS device should not elicit an unfavorable response from the living cells with which it is interacting. Additionally, the surrounding tissues and cells should not hinder the functioning of the BioMEMS device (biofouling). Also, an added challenge is that in general, the photolithographic materials used in the IC industry contain high solvent levels and are inherently toxic. Recent interest in the BioMEMS field has led to studies concerning the biocompatibility of numerous materials such as Si, various metals, and SU-8, which are often used in MEMS device fabrication [47, 48].

SU-8 was chosen as the structural material from which the microtower arrays were fabricated since SU-8 could be patterned into high aspect ratio structures using standard photolithography techniques, and its claims as a biocompatible material were rising. For example, SU-8 had been used in numerous BioMEMS applications, from the micromolding of PDMS structures to the creation of channels for microfluidic applications in cell sorting and capture [45, 46]. However, the biocompatibility of SU-8 received a significant amount of attention in the 3-D Nets project. The general findings from the 3-D Nets collaborations were that the SU-8 microtower array structures created from thin films of SU-8 tended to be biocompatible, while the SU-8 microtower array structures created from thick (≥ 700 µm) layers of SU-8 had to be coated with Parylene to not elicit an unfavorable response from the neurons cultured on the devices [83]. A post-processing biocompatibility protocol was performed on all of the SU-8 microtower structures prior to cell plating (see Section 4.1: Initial Biological Tests on the
Unpackaged SU-8 Microtower Arrays. Probably the most significant post-processing step for improving the biocompatibility of the SU-8 microtower arrays was the 60 sec IPA sonication, which aided in the removal of toxic residues from the microtower arrays. It was hypothesized that there could be a relation between the thickness of the SU-8 used in making the microtower array and the duration of the IPA sonication required for the removal of all toxic residues from the structures.

Experiments had been performed by other collaborators on the 3-D Nets project involving the creation of the microtower arrays using stereolithography (SLA). Although the SLA process was useful for creating 3-D structures, the SLA process produced microtower devices with limited resolution, and therefore couldn’t create microtower arrays with the desired dimensions. Also, a larger problem was that the resin used in the SLA process was highly toxic to the neurons. Methods to maneuver around the biocompatibility issue were attempted by coating the SLA structures with Parylene C or PDMS, but this was not an ideal solution. Typically, such outer Parylene coatings are not good for long term reliability and the biologists on the 3-D Nets project stressed that they preferred to receive a device that was inherently biocompatible as opposed to receiving a device that contained toxins that could potentially leach out through a protective, biocompatible, outer coating.

Also, cell viability experiments were performed in collaboration with the Brewer lab on SLA structures coated with PDMS, which showed that the SLA resin was toxic to neurons. One PDMS coated SLA structure was sterilized in EtOH and exhibited 43% viability, while another PDMS coated SLA structure was autoclaved and exhibited only 9% viability. The control viability was more than 80%. A graph showing the percentage
of cell survival vs. distance from the PDMS coated SLA structure that was sterilized in EtOH is provided in figure 5.1. The general trend is that the percentage of cell survival increases with distance from the SLA structure. For the PDMS coated SLA structure that was autoclaved, cell survival was constant throughout the well and did not show any dependence on distance from the SLA structure.

![Graph](image)

**Figure 5.1.** Percentage of cell survival vs. distance from the PDMS coated SLA structure that was EtOH sterilized. The general trend is that cell viability increases with distance from the SLA structure.

Many metals commonly used in microfabrication that are inexpensive such as Cu, Ni, Al, and Fe could not be used in the fabrication of the microtower arrays since they are toxic to neurons. However, metals such Au and Pt, although expensive, are biocompatible and were therefore more suitable for forming electrical connections in the microtower arrays. Au was chosen as the material for the microtower electrodes and leads since it was biocompatible and could easily be deposited using a metal sputtering
system. Also, the processing of Au has become fairly standard in microfabrication, so Au could be easily patterned using a KI/I₂ solution. Additionally, the KI/I₂ solution was mild enough to not attack the SU-8 microtower structures or the sacrificial Cr layer upon which the microtower arrays were fabricated. Furthermore, Au was chosen as the material for the large metal tabs at the base of each microtower because Au could be electroplated to various thicknesses using a standard commercial Au electroplating bath (Techni Au 25-ES, Technics Inc.) and a Pt mesh electrode (Pt gauge 52 mesh woven 0.1 mm diameter wire, 99.9%, 50×50 mm², Johnson Matthey Catalog Company).

A 1900 Å thick layer of Au was sputtered onto the Si wafer after the first SU-8 layer was patterned and hardbaked. Au has problems adhering to Si, so generally a Ti or Cr layer is deposited between the Si and Au to promote adhesion. The Cr release layer aided in adhesion of the Au layer to the Si wafer where the first SU-8 layer in the microtower array design wasn’t patterned. The sputtered Au layer served as an electroplating seed layer for the thick Au tabs, and then the Au traces and electrodes on each microtower were patterned from this same Au seed layer.

The 15×15 µm² microtower electrodes (two electrodes per microtower) were fabricated from Au. However, since the impedance of an Au electrode is too high for neuronal stimulation and recording, the Au microtower electrodes were electroplated with Pt black after the active microscaffold system was electrically packaged into a functioning MEA that was compliant with the Multi Channel Systems preamplifier setup. Typically, Au electrodes for neuronal stimulation/recording are platinized with Pt black since Pt black has a rough and non-uniform topography that yields a high surface area, and therefore greatly lowers the impedance of the electrodes [68]. The Pt black
electroplating was performed on the electrically packaged microtower array, and the Pt black was selectively electroplated onto functionalized microtower electrodes using the Pt black electroplating setup in the DeWeerth lab at the Georgia Institute of Technology.

Cr, although not considered to be a biocompatible metal, was chosen as the sacrificial layer upon which the planar microtower arrays were fabricated. Cr was a reasonable choice as a sacrificial release layer since it could easily be deposited via a sputtering or evaporation system, and its use as a sacrificial material is well-established [61, 62, 64, 65]. Also, since the Cr was only used as a release layer, there was no Cr in the completed microtower arrays, so it was reasoned that the toxicity of Cr would have little or no effect on the biocompatibility of the final device. The commercial Cr etchant (CR-7S, Cyantek) was used to release the planar microtower arrays from the Si substrate, and a 15 min DI water rinse was performed on the released microtower arrays to clear any acid residues from the microtower arrays.

5.1.2. SU-8 Lithography

The SU-8 lithography was fairly straightforward for the fabrication of the original microtower arrays that had either 8 or 16 microtowers in a linear array, as can be seen in figure 5.2, showing a microscope image of the first SU-8 layer with well defined side ports, microtower tabs, and serrated edges of the microtower walls that aided in adhesion of the subsequent SU-8 layers.
However, challenges arose with the SU-8 lithography in the brain slice microtower array design. The brain slice microtowers were originally designed to have side ports with dimensions of 4.0×4.0 µm², 4.5×4.5 µm², 5.0×5.0 µm², 5.5×5.5 µm², 6.0×6.0 µm², and 7.0×7.0 µm². However, the SU-8 lithography process could not produce the side ports than had dimensions less than 6.0×6.0 µm² with adequate resolution, which resulted in these smaller side ports being clogged with SU-8, as seen in figures 5.3a and 5.3b.

As shown in figure 5.3b, the original brain slice microtower design had larger side ports on the second SU-8 layer than on the first SU-8 layer (by 4×4 µm²), to aid in alignment of the two SU-8 layers. Initially, it was reasoned that the first SU-8 layer was being overexposed, which led to clogging of these smaller ports. Therefore, numerous experiments were performed that varied the prebake parameters, UV exposure times, post exposure bake parameters, and development times, in attempts to produce the smaller side ports with adequate resolution.
When the exposure time was greatly decreased and the develop process was performed with violent agitation, sometimes the smaller side ports could be produced with adequate resolution, but the results always varied across the wafer. In some places on the wafer the side ports would be nicely patterned and in other places on the same wafer the side ports would be clogged. The non-uniformity of the SU-8 lithography was the result of the violent manual agitation that was performed on the wafers, which made it difficult to achieve repeatable, uniform results.

However, when the develop process was performed with only a mild agitation, the resulting side ports were always clogged with SU-8. Also, if the develop process with violent agitation was performed for more than two min (or with mild agitation for more than three min), then the microtower arrays would delaminate from the wafer. Also, if the SU-8 was underexposed, then catastrophic delamination of the SU-8 structures was observed, which is a typical response from underexposed SU-8 [66].

Figure 5.3. Images of clogged side ports in the brain slice microtower arrays. (a) An SEM micrograph showing underdeveloped side ports in the first SU-8 layer. (b) A microscope image showing Au electrodes and leads, where the leads are insulated between the first and second SU-8 layers. Both open and clogged side ports can be seen.
The frequent delamination of the SU-8 structures and the non-uniform lithography results across a single wafer led to the conclusion that the SU-8 had to be underexposed or overdeveloped to achieve such small features from a 5 µm thick layer of SU-8 2005. Since it wasn’t possible to achieve uniform resolution from the SU-8 lithography across a single wafer, it was decided to increase the size of each side port in the brain slice microtower design by 4.0×4.0 µm². Therefore, a revised first SU-8 layer photomask was designed and fabricated that contained brain slice microtower arrays with side ports having dimensions of 8.0×8.0 µm², 8.5×8.5 µm², 9.0×9.0 µm², 9.5×9.5 µm², 10.0×10.0 µm², and 11.0×11.0 µm². All other design parameters on the photomask remained the same. Also, sometimes the side ports in the brain slice microtower arrays would become clogged, not because the first SU-8 wasn’t properly developed, but because the second SU-8 layer wasn’t fully developed, as seen in figure 5.4.

![Clogged side ports from the underdeveloped second SU-8 layer](image)

**Figure 5.4.** SEM micrograph of clogged SU-8 side ports in the brain slice microtower arrays, which resulted from underdeveloped SU-8 from the second SU-8 layer.
As a result, the second SU-8 layer often had to be overdeveloped to the point that it was near delaminating to be sure that the side ports were clear of any unwanted SU-8. Occasionally the second SU-8 layer was accidentally overdeveloped or over-agitated to the point that the second SU-8 layer would delaminate in some places, resulting in brain slice microtower arrays like the one seen in figure 5.5, which had fully open side ports, but a somewhat collapsed Au electrode. The collapsing of the Au electrodes is the result of the delamination of the second SU-8 layer, since the Au electrode and its corresponding lead should have been sandwiched between the first and second SU-8 layers, as seen in figure 5.3a.

![SEM image of a brain slice microtower with the revised, larger, open side ports. The Au electrode in this image is somewhat collapsed from overdeveloping and delamination of the second SU-8 layer.](image)

Figure 5.5.  SEM image of a brain slice microtower with the revised, larger, open side ports. The Au electrode in this image is somewhat collapsed from overdeveloping and delamination of the second SU-8 layer.
SEM micrographs of a completed, planar (2-D) SU-8 microtower array still attached to the original Si wafer upon which it was fabricated are provided in figures 5.6a and 5.6b. The sacrificial photoresist had been removed from these planar microtower arrays prior to SEM imaging, so the hollow fluid channels are present. A remaining strip of Parylene C can be seen in figure 5.6b. After the third SU-8 layer was patterned, the exposed 2 µm Parylene C layer was etched in a Plasma Therm RIE system (45 sccm O₂, 5 sccm CHF₃, 50 mT, 300 W, 12 min). This dry etch successfully etched the exposed Parylene C layer except for where the Parylene C covered the patterned the AZ 4620 sacrificial layer.

Figure 5.6. SEM micrographs of a completed planar microtower array from the original design, showing (a) the top (third) SU-8 layer and electroplated Au tabs. The third SU-8 layer has no fluid side ports or openings for microtower electrodes. (b) The fluid channel opening at can be seen. The serrated edges of the first and second SU-8 layer and a remaining strip of Parylene C can also be observed.

The AZ 4620 sacrificial layer was designed to project out of the top and bottom of each microtower by 300 µm to assist in the removal of the sacrificial material, and even though the Parylene C covering the projected portion of the patterned AZ 4260 was not protected by the third SU-8 layer and was therefore exposed to the O₂ plasma etch, that
specific projection of the Parylene C typically was not etched away. The reasons for the O₂ plasma selectively not etching all of the Parylene C that covered the projected strip of AZ 4620 are unknown. However, the remaining strip of Parylene C that sometimes remained and projected out the front and back of each microtower didn’t interfere with the removal of the sacrificial material that defined the hollow channel. Additionally, the remaining Parylene C strips that projected from the backsides of the microtowers typically broke off of the backsides of the microtowers when the microtower arrays were manually inserted into the Si orifice plates during the 3-D vertical packaging process.

Originally, there were adhesion problems between the Parylene C layer and the third SU-8 layer, as seen in figure 5.7, where slight delamination can be observed between the third SU-8 layer and the Parylene C layer. However, the delamination issue between the Parylene C and the third SU-8 layer was remedied by performing a 15 sec O₂ plasma treatment (Plasma Therm RIE) on the Parylene C layer prior to spinning the third SU-8 layer on the wafer. This O₂ plasma treatment made the Parylene surface hydrophilic, which aided in adhesion of the third SU-8 layer to the Parylene C surface.

![Figure 5.7. An SEM micrograph showing the slight delamination issue between the Parylene C layer and the third SU-8 layer. The delamination issue was resolved by performing an O₂ plasma treatment on the Parylene C layer to render the surface hydrophilic.](image-url)
5.1.3. Electroplating

Both Au and Sn-Ag electroplating were experimented with in the fabrication of the microtower arrays. Originally, only Au (Techni-Au 25ES, Technics Inc.) was electroplated onto the large tabs at the base of each microtower. In the fabrication of the original 8×8 and 16×16 microtower arrays, the Au microtower tabs (40×120 µm²) were electroplated to a thickness of 23-25 µm. Each Au tab connected to an Au microtower lead that was 20 µm wide. It was thought that the thicker the Au tabs were electroplated, the more robust they were, and the less likely they were to rip off from the microtowers during the packaging. Also, it was originally assumed that thicker Au tabs would have a greater probability of intersecting the metal tabs on the Si orifice plate, thus providing a higher yield of functioning microtower electrodes in the packaged device.

However, if the Au tabs were electroplated too thick, then the elevated topography of the Au tabs made it difficult to pattern the Au microtower leads in the vicinity of the Au tabs. If the Au tabs were electroplated too thick, then the two Au leads running along the length of the microtower either tended to short out at the base of the microtower or disappear completely at the base of the microtower, thus creating an open circuit. This effect was predominantly apparent in the brain slice microtower design, where the Au microtower leads were only 10 µm wide and had a 30 µm center-to-center spacing on each microtower (each brain slice microtower was only 50 µm wide at the base). Figure 5.8 and 5.9 provide examples of the complications associated with the Au lead lithography on the brain slice microtowers when the Au tabs were electroplated too thick (23 µm). Figure 5.8 shows that the Au leads on the brain slice microtowers have been almost completely etched array, but the in some places between the electroplated Au
tabs, there is still sputtered Au remaining that connects the two electroplated Au tabs.

Figure 5.9 provides another microscope image of this complication with the Au lead lithography, showing shorted out tabs at the base of each microtower, in-tact Au microtower electrodes, but missing Au microtower leads that have been overetched along the length of each microtower.

Figure 5.8. Microscope image depicting problems in the Au lead lithography and etching that were the direct result of overelectroplating the Au microtower tabs. This image shows overetching of the Au microtower leads, and shorting between the Au electroplated tabs.
These problems with etching the Au microtower leads were less prevalent on the original microtower array design and on the revised microtower array design for dissociated cultures since these microtowers had larger widths (120 µm and 80 µm, respectively) than the brain slice microtowers (50 µm). Like the brain slice microtowers, the leads on the revised microtowers for dissociated cultures were also only 10 µm wide, but their center-to-center spacing was 38 µm (as opposed to 30 µm on the brain slice microtowers). The leads on the original microtower array design were 20 µm wide and had a 60 µm center-to-center spacing. The larger the width and center-to-center spacing of the Au microtower leads, the less likely were complications with the electroplating to interfere with the patterning of the microtower Au leads. On the microtower arrays for dissociated neuronal cultures, the Au leads would tend to narrow at the vicinity of the
thick electroplated Au tabs, but shorting of the electroplated tabs or overetching of the Au microtower leads did not occur. A microscope image of the brain slice microtowers, showing how overplating the Au tabs would cause disconnects in the microtower Au leads near the vicinity of the Au tabs is provided in figure 5.10. This effect was from the diffraction of UV light during the photolithography process, which complicated the patterning of features when high-aspect ratio structures were present.

![Microscope image depicting problems in the Au lead lithography. Electroplating the Au tabs too thick caused disconnects in the Au microtower leads in the vicinity of the electroplated Au tabs.](image)

Figure 5.10. Microscope image depicting problems in the Au lead lithography. Electroplating the Au tabs too thick caused disconnects in the Au microtower leads in the vicinity of the electroplated Au tabs.

Also, sometimes the sacrificial channel tended to disappear in the vicinity of the Au tabs if the Au tabs were electroplated too thick. Again, this effect was especially magnified in the brain slice microtower design where the microtowers, leads, and channels had much smaller dimensions. The complications in the Au lead lithography and sacrificial layer due to the thick Au tabs were remedied by only electroplating the Au microtower tabs to a thickness of 17 µm on the brain slice microtower arrays and the revised microtower arrays (since they were fabricated on the same photomask set).
Figures 5.11a and 5.11b show the brain slice microtower arrays with properly etched Au leads and electrodes, which were attainable by limiting the Au electroplating of the microtower tabs to 17 µm (as opposed to 23 µm).

![Image of microtower arrays with properly etched Au leads and electrodes.](image)

Figure 5.11. Microscope images depicting the brain slice microtower arrays with (a) properly etched Au leads and Au tabs that aren’t overplated; and (b) properly etched Au leads and electrodes, properly electroplated Au tabs, and the patterned AZ 4620 sacrificial layer that later forms the hollow fluid channel.

Experiments were performed where Sn-Ag solder was also electroplated onto the microtower tabs on top of the electroplated Au, since it was thought that having Sn-Ag solder on both the microtower tabs and the Si orifice tabs would facilitate integration of the microtower electrodes. However, the Sn-Ag solder didn’t electroplate as smoothly and uniformly as the Au, as seen in 5.12. The Sn-Ag solder often produced “whiskers” and “tree-like” shapes that projected both horizontally and vertically out past the desired dimensions of the feature being electroplated (see figure 5.12b).
The undesirable whiskers on the Sn-Ag microtower tabs caused some of the microtower tabs to short out with each other, as seen in figure 5.13, especially on the revised microtower arrays for dissociated neuronal cultures, since the edge-to-edge spacing of the tabs on adjacent microtowers was only 30 µm.

Figure 5.12. SEMs of the microtowers after the microtower tabs have been electroplated, but prior to etching of the microtower Au leads, showing how the Au electroplates smoother than the Sn-Ag solder. The electroplated microtower tabs and the first SU-8 layer can be seen. (a) Smooth, electroplated Au tabs on the original microtower design; (b) Sn-Ag solder tabs on the brain slice microtowers, displaying the whiskering effect.

Figure 5.13. Microscope image of a revised dissociated microtower array, showing the Sn-Ag solder tabs electroplated past the desired dimensions, and induced shorting of adjacent microtower tabs.
The non-uniform plating and whiskering effect of the Sn-Ag solder also interfered with the subsequent patterning of the Au microtower leads and the sacrificial channels near the vicinity of the Sn-Ag microtower tabs. As a result, it was decided that it was unnecessary to electroplate Sn-Ag solder onto the microtower tabs to form a 90° electrical connection between the Au microtower tabs and the Sn-Ag Si orifice plate tabs. Therefore, only Au was electroplated onto the microtower tabs, while Sn-Ag was electroplated onto the Si orifice plates to enable electrical functionality of the microtower electrodes.

5.1.4 Sacrificial Layer Fabrication

There were many challenges presented in the fabrication of the hollow fluid channels in the SU-8 microtower arrays. Experiments were performed using a variety of sacrificial materials to define the hollow channels in the SU-8 microtower arrays. The sacrificial material had to be deposited at low enough temperatures (≤ 300 °C) to not damage the first and second SU-8 layers that had been previously defined, be relatively thick (17-23 µm), have a high quality, be compatible with the subsequent wafer processing, and be easily removed from the microtower arrays to yield the desired hollow channels. Additionally, the ideal sacrificial material would also be photodefinable to minimize the number of processing steps.

As mentioned in Section 3.2.6 (Fluid Channel Fabrication), several thick photoresists and polymers could be patterned nicely in the shape of the fluid channels, but once the third SU-8 layer was spun on top of the patterned sacrificial layer, the SU-8 would mix with the sacrificial material and destroy the previously defined fluid channel
pattern. The mixing of SU-8 with patterned photoresist layers has been observed by other researchers [61] and has prevented many from pursuing the use of photoresists as sacrificial materials in SU-8 structures. Hardbaking of the sacrificial photoresist prior to spinning on the third SU-8 layer still didn’t prevent the observed mixing of the two materials, and caution also had to be taken to not hardbake the sacrificial photoresist to the point that it would be difficult to later remove from the completed structure.

PiRL®III (Brewer Science) was a sacrificial polyimide material experimented with that when patterned, did not mix with the third SU-8 layer when it was spun onto the wafer. However, the PiRL®III was not photodefined, and was very difficult to pattern. Both wet and dry etching of the PiRL®III material was attempted, but the results typically yielded an unacceptable pattern and the results weren’t always repeatable. Furthermore, the PiRL®III material was very difficult to remove from the completed SU-8 structures. Strong basic solutions tended to remove (or etch) the PiRL®III material, but the PiRL®III didn’t dissolve and would instead break up into smaller pieces, which was problematic for clearing out the fluid channels.

Initial attempts at creating hollow SU-8 structures by experimenting with various sacrificial materials were first performed using a microneedle array photomask set. This microneedle array photomask set was previously used for fabricating hollow metallic microneedles [76]. Figure 5.14a shows an SU-8 microneedle with PiRL®III patterned as the sacrificial material that defined the hollow channel. Figure 5.14b shows the microneedle after an attempt at removing the PiRL®III polyimide from the SU-8 microneedle structure using ammonium hydroxide (NH₄OH; pH = 11.6).
Figure 5.14a shows one of the most successful results from numerous efforts to adequately pattern the PiRL®III polyimide, which was achieved using an RIE (Plasma Therm) etch. However, cracks can be seen in the PiRL®III layer, which often would occur during the processing of the PiRL®III material. Repeated attempts at achieving the same well-defined PiRL®III pattern were met without success. As can be seen in figure 5.13b, the NH₄OH mostly cleared the PiRL®III from the SU-8 structure, leaving a hollow channel, but it also left visible residues. Additionally, the NH₄OH tended to damage the SU-8 structures.

The AZ 4620 photoresist was the best material experimented with in terms of forming a thick (17-23 µm), photodefensible layer that had a low processing temperature (110 °C), and could also be easily dissolved and cleared from the completed structures. However, once the third SU-8 was spun onto the wafer, it would always mix with the patterned AZ 4620 layer, even if the AZ 4620 layer was hardbaked prior to the spinning of the third SU-8 layer. It was hypothesized that if the patterned AZ 4620 layer could be capped with a protective coating to prevent it from coming into physical contact with the
third SU-8 layer during the third SU-8 layer spinning process, then the AZ 4620 could function as the sacrificial material for defining the fluid channel.

S. Takeuchi et. al. fabricated Parylene flexible neural probes with hollow channels, using AZ 4620 photoresist as the sacrificial material to form the hollow channels [30]. Even though the neural probes created by S. Takeuchi et. al. contained no SU-8, the fabrication process and results presented in the creation of the Parylene neural probes suggested that Parylene could serve as the protective coating to isolate the patterned AZ 4620 layer from the third SU-8 layer. Furthermore, Parylene C is a biocompatible material, can be deposited at low temperatures using a CVD process, yielding a high quality, conformal coating of various thicknesses.

A conformal, 2 µm Parylene C layer was deposited onto the wafer after the sacrificial AZ 4620 photoresist was patterned, and successfully served as an isolation layer, and prevented mixing of the third SU-8 layer with the patterned AZ 4620 sacrificial photoresist. However, during the processing of the third SU-8 layer, the UV exposure tended to break down the AZ 4620 photoresist (since the SU-8 was transparent and the AZ 4620 is a positive photoresist). The patterned AZ 4620 tended to bubble (outgas) during the UV exposure of the third SU-8 layer, and the AZ 4620 would reflow out past its desired dimensions as can be seen in figure 5.15a. However, performing a 15 min hardbake on a hotplate at 95 °C on the patterned AZ 4620 sacrificial layer prior to the Parylene C deposition controlled the bubbling (or outgassing) of the AZ 4620 to a tolerable level, as can be seen in figure 5.15b.
5.2 Packaging Results and Discussion

Once the linear microtower arrays were released from the Si substrate on which they were originally fabricated, the microtower arrays were vertically packaged (in a 3-D format) to form either 8×8, 12×12, or 16×16 arrays of microtowers. Each linear microtower array was picked up by a vacuum hose and manually inserted into a Si orifice plate (500 µm thick), and sealed in place on the backside of the Si orifice plate using a 3:1 ratio of 184 PDMS and hexane.

5.2.1 Packaging Results and Discussion for the 8×8 Microtower Array

A photograph of a single linear microtower array (containing 8 microtowers) vertically packaged and sealed into a Si orifice plate is provided in figure 5.16.

Figure 5.15. Microscope images showing the bubbling of the AZ 4620 sacrificial layer induced by the UV exposure of the third SU-8 layer (a) in the absence of a sacrificial layer hardbake; and (b) with a 15 min sacrificial layer hardbake for on a hotplate at 95 °C.
The 184 PDMS was too viscous to travel in between all of the microtowers to adequately seal an 8×8, 12×12, or 16×16 array, so the PDMS was diluted with hexane in a 3:1 ratio for the sealing of multiple microtower arrays in a single Si orifice plate. The PDMS/hexane mixture provided both a structural and fluidic seal between the microtowers and the Si orifice plate, and a double seal PDMS was required to form a fluidic seal without any leaks between the microtowers and the Si orifice plate (see Section 3.3: Vertical Packaging and Sealing Process).

Special care had to be taken to ensure that the PDMS/hexane mixture didn’t travel down the length of the microtowers and clog the entry port at the base of each microtower, as seen in figures 5.17a and 5.17b. Figure 5.17a shows a microtower where the inlet port is completed covered with the PDMS/hexane seal, and figure 5.17b shows a microtower where the PDMS/hexanes seal has run all the way down the length of the microtower and could have potentially clogged the inlet port at the base of the packaged microtower.
It was undesirable for the PDMS/hexane mixture to drip down the length of the microtowers since it could potentially lead to clogging of the microtower inlet ports, so the device was placed on its side during the PDMS/hexane sealing and curing process (in an oven at 60 °C) to prevent the PDMS/hexane mixture from dripping down the length of the microtowers. An N2 gun was used to disperse the PDMS/hexane mixture between all of the packaged microtowers, and care also had to be taken to not blow the PDMS/hexane mixture down the length of the microtowers with the N2 gun. Also, it was found that packaging and sealing the microtower arrays with the AZ 4620 layer still inside the fluid channel reduced the amount of clogged microtowers in the final device. Therefore, the release of the sacrificial channel with acetone followed by IPA was performed after the packaged microtowers were sealed in place with PDMS.

After a packaged microtower array was sealed with the PDMS/hexane mixture and the sacrificial layer was removed, each device was inspected under a light microscope to insure that the majority of the inlet ports at the bottom of each microtower

Figure 5.17. SEMs showing the bottom of packaged microtowers where (a) the PDMS/hexane mixture ran down the length of the microtower and clogged the inlet port; and (b) the PDMS/hexane mixture ran down the length of the microtower but did not clog the inlet port.
and exit ports at the top of each microtower were open. Figure 5.18 shows light microscope images of the top horizontal cross-member in a packaged linear microtower array, showing an open top exit port (figure 5.18a) and a top exit port that was clogged (figure 5.18b) during the sealing process with the PDMS/hexane mixture. Just as significant consideration had to be taken to ensure that the PDMS/hexane mixture didn’t clog the inlet port at the base of each microtower; significant consideration also had to be taken to ensure that the PDMS/hexane mixture didn’t clog the side ports or the exit port at the top of each microtower.

Figure 5.19 provides SEM micrographs of both the top exit port and bottom inlet port from a packaged and sealed microtower array, where the inlet and exit port are unclogged and free of the PDMS/hexane sealing mixture, showing results from when the PDMS/hexane sealing process was properly performed.
The holes in the Si orifice plate had larger diameters than the microtowers, which helped when manually inserting each microtower array into the Si orifice plate. However, once packaged, the microtower arrays were tilted with respect to the each other and the Si orifice plate. To straighten out the vertically packaged microtower arrays, a jig was fabricated using SLA, which contained a slit to hold the Si orifice plate, and also contained an array of comb-like teeth that slid in between adjacent packaged linear microtower arrays. The comb-like teeth on the SLA structure straightened out each packaged microtower array so that the packaged linear microtower arrays formed 90° angles with respect to the Si orifice plate. Also, the structural design of the SLA jig forced the Au tabs at the base of each microtower to make contact with the Si orifice plate. The SLA jig was designed using Solid Edge software, and a schematic of the SLA jig is provided in figure 5.20.

Figure 5.19. SEM images showing a properly packaged a sealed (unclogged) microtower from (a) the top exit port; and (b) bottom inlet port. The sacrificial photoresist was removed after the sealing was performed.
Stereoscope images of an 8×8 tilted microtower array that was packaged and sealed into the Si orifice plate without the SLA jig are provided in figure 5.21. Figure 5.21a provides a side view of the packaged 8×8 microtower array, showing that most of the linear microtower arrays did not form 90° angles with respect to the Si orifice plate when they were packaged and sealed without the SLA jig. Figure 5.21b shows that there are places where the Au microtower tabs do not make contact with the Si orifice plate, which would cause problems with electrical integration since the Au microtower tabs must make contact with the Sn-Ag tabs on the Si orifice plate to functionalize the microtower electrodes. The Au tabs at the base of each microtower needed to be bonded to the Sn-Ag tabs and Au traces patterned on the Si orifice plate to provide electrical...
functionality to the microtower electrodes, so having the Au tabs in contact with the Si orifice plate was crucial to electrically functionalize the active microscaffold system.

**Figure 5.21.** Stereoscope images of the microtower arrays that were packaged and sealed without using the SLA jig, showing (a) the tilted microtower arrays; and (b) places where the Au tabs at the base of each microtower do not come into contact with the Si orifice plate.

Stereoscope images of an 8×8 straightened microtower array that was packaged and sealed into the Si orifice using the SLA jig are provided in figure 5.22. Figure 5.22a provides a side view of the packaged 8×8 microtower array, showing that the linear microtower arrays form 90° angles with respect to the Si orifice plate when they are packaged and sealed using the SLA jig. Figure 5.22b shows that when the SLA jig is used during the packaging and sealing of the microtower arrays, the Au microtower tabs make contact with the Si orifice plate, which is necessary for functionalizing the microtower electrodes.
After the linear microtower arrays were vertically packaged and sealed into the Si orifice plate, the solid SU-8 pitchfork structures were slid into the packaged microtower array and sealed into place with PDMS. These pitchfork structures served as additional cross-members that connected adjacent linear microtower arrays to each other, and were hypothesized to aid in 3-D neuronal network formation once dissociated neuronal cells were plated within the active microscaffold system. A stereoscope image of an 8×8 microtower array (1.5 mm in height) packaged into the 3-D format with the joining pitchfork structures is provided in figure 5.23. The microtower leads, fluid side ports, and Au tabs can also be seen. An additional stereoscope image of the packaged microscaffold system with the joining SU-8 pitchfork structures is provided in figure 5.24. For biological studies with fluidics only, the microtower arrays were sealed on the backside with a double PDMS/hexane coating since there were no substrate traces that required insulation when the microtower electrodes weren’t functional (as shown in figures 5.21 and 5.22).

Figure 5.22. Stereoscope images of the microtower arrays that were packaged and sealed using the SLA jig, showing (a) the straightened microtower arrays; and (b) the Au tabs at the base of each microtower making good contact with the Si orifice plate.
Figure 5.23. StereoScope image of an 8×8 array of 1.5 mm tall microtowers and pitchforks packaged and sealed into a 3-D format, thus yielding the active microscaffold system.

Figure 5.24. StereoScope image of the topside of the microscaffold system packaged into a 3-D format with the joining SU-8 pitchfork structures.
In packaging the microscaffolds that were used for biological studies with fluidics only (no functioning microtower electrodes), the linear microtower arrays were vertically packaged with the sacrificial photoresist still in the microtower channels. Then, after the microtower arrays were straightened and sealed both structurally and fluidically with the PDMS/hexane mixture, the sacrificial photoresist was removed from the channels. Once vertically packaged, the lowest side port on the 8×8 microtower arrays was located 100 µm above the surface of the Si orifice plate, so removing the sacrificial material after the PDMS sealing resulted in the least amount of clogging in case the PDMS/hexane sealing mixture traveled up the length of the microtowers.

However, for the electrical packaging of the microtower arrays, the order of the processing steps had to be rearranged. Initially, the microtower arrays (with the sacrificial photoresist still in the fluid channels) were packaged and sealed into the Si MEA orifice plate on the backside with the PDMS/hexane mixture. Then the Sn-Ag reflow was performed at 250 °C for 2 min, and the substrate leads and microtower tabs were insulated on the front side of the Si orifice plate using the PDMS/hexane mixture. After the PDMS sealing and Sn-Ag reflow were performed, attempts were made to remove the sacrificial photoresist from the channels using a 90 min acetone soak followed by a 15 min IPA rinse. However, it was difficult to remove all of the AZ 4620 photoresist from the channels after the reflow, which had been performed at 250 °C. Also, the initial backside PDMS sealing procedure caused problems since some of the PDMS traveled through to the front side of the Si MEA orifice plate where it lodged between the Sn-Ag substrate tabs and the Au microtower tabs. Since PDMS is an insulator, the PDMS that lodged between the Sn-Ag substrate tabs and the Au microtower
tabs prevented the formation of electrical bonds at these specific sites. However, the first electrically active microscaffold was packaged in the sequence listed above, and reasonable electrical data was still gathered from the system (see Section 5.5: Electrode Characterizations).

The electrical packaging scheme was revised so that the sacrificial photoresist layer was removed from the microtower arrays prior to packaging them in the Si MEA orifice plate. Once the hollow microtower arrays were vertically packaged into the Si MEA orifice plate, the Sn-Ag solder substrate tabs were reflowed at 250 °C to form an electrical bond with the Au microtower tabs. After the reflow was performed, the microtower arrays were sealed into place on the backside of the Si orifice plate using a double PDMS/hexane coating, and then the substrate traces and metal tabs on the front side of the Si MEA orifice plate were insulated with the PDMS/hexane mixture. This sequence of packaging steps provided the highest viability of functioning electrodes, but increased the chances of a microtower getting clogged since in this sequence, since the microtowers were hollow during the PDMS/hexane sealing procedure. However, the FEMLab modeling performed on the microtower arrays (see Section 2.1.2.3: FEMLab Fluidic Model) showed that the overall fluid dynamics of the system were not greatly affected if one of the microtowers in a linear array was clogged.

5.2.2 Packaging Results and Discussion for the 12×12 Brain Slice Microtower Array

The brain slice microtower arrays were packaged in a similar manner to the microtower arrays for dissociated cultures. Figure 5.25 provides an SEM micrograph of
a single brain slice microtower array packaged and sealed into a Si orifice plate with the PDMS/hexane mixture.

Figure 5.25. SEM micrograph showing a single linear array of brain slice microtowers packaged and sealed into a Si orifice plate using the PDMS/hexane mixture.

The fluid ports and electrodes can be observed on the packaged brain slice microtower array in figure 5.25. There are some visible cracks in the PDMS seal, but this is because only one coat of the PDMS/hexane mixture was used to seal this microtower array into place. All of the devices used for fluid testing had two coats of the PDMS/hexane mixture placed on the backside of the Si orifice plate to seal the microtower arrays into place. Figure 5.26 provides an SEM micrograph of a fully packaged 12×12 brain slice microtower array, packaged into the 3-D format to yield the active microscaffold system for thick brain slice cultures.
Figure 5.27 provides SEM micrographs of the backside of the brain slice microscaffold device. A view of the microtower arrays and the fluid inlet ports is provided in figure 5.27a, while figure 5.27b shows a close up view of the microtowers intersecting the Si orifice plate and the PDMS/hexane fluid and structural seal. Figure 5.27b shows that there are no leaks in the PDMS/hexane seal between the microtowers and the Si orifice plate.

Figure 5.26. SEM micrograph showing a 12×12 brain slice microtower array packaged into a 3-D format, yielding the active microscaffold system for thick brain slice cultures. The brain slice microtowers project 1.0 mm from the surface of the Si orifice plate.
5.3 Fluid Flow Experiments

The microtower arrays that were packaged and sealed into the 1.5 cm × 1.5 cm × 500 µm Si orifice plates were subsequently packaged into the PDMS fluid manifolds (see Section 3.4: Fluidic Packaging) for fluid flow experiments. The development of the PDMS manifold was a collaborative effort between the Frazier and Glezer labs at the Georgia Institute of Technology. The bottom PDMS manifold piece contained a recess into which the 1.5 cm × 1.5 cm × 500 µm Si orifice plate fit and was sealed into place with PDMS. Next, the top and bottom PDMS manifold pieces were aligned and sealed together with PDMS. Tubing for media delivery was attached to the inlet port at the base of the manifold. The fluids first filled the pool at the bottom of the manifold, and then traveled up through each of the microtower lengths, through the cross-members, and out the fluid ports to perfuse the 3-D neuronal network proliferating within the active microscaffold system.
The fluid was delivered to the inlet port via a syringe pump. Initial characterization of the fluidic system was performed by imaging the flow of DI water dyed with food coloring through the microscaffold/fluid manifold package. Since the SU-8 was transparent, it was possible to observe the fluidic path of the colored DI water and determine whether there were any leaks in the PDMS/Si orifice plate seal. Furthermore, fluid functionality of the microscaffold/fluid manifold package was validated by running a mixture of polystyrene beads (1.0 µm in diameter, Molecular Probes) and DI water through the functional microtower cross-members and out the fluid ports along the length of the microtowers. Microscopy confirmed the flow of the polystyrene beads through the functional microtower cross-members and fluid ports.

The first few devices with functioning fluidics were examined in the SEM to confirm that the inlet and outlet ports on each microtower were open and that the PDMS/hexane mixture had formed an adequate (fluidic) seal between each microtower and the Si orifice plate, since leaks in the Si orifice plate would have affected the overall fluid dynamics of the microscaffold system. Initially, when only a single PDMS/hexane coating was used to seal the microtowers to the Si orifice plate, numerous leaks were found in the Si orifice plate. However, performing a double PDMS/hexane coating on the backside of the Si orifice plate remedied the Si orifice plate leakage problem. Also, each active microscaffold system that was sent to the Brewer lab at the Southern Illinois University School of Medicine for biological testing with fluidics was examined using light microscopy to ensure that the majority of the inlet ports and outlet ports on each microtower were open. Active microscaffold systems that were determined to have at
least 80% functioning fluidics were considered tolerable for performing biological cell culture studies with fluid perfusion.

5.4 Biological Experimental Results and Discussion

Primary hippocampal embryonic rat pup neurons were cultured on the unpackaged microtower arrays by both the Brewer lab at the Southern Illinois University School of Medicine and the Wheeler lab at the University of Illinois at Urbana-Champaign to validate biocompatibility of the microtower arrays. Furthermore, the Brewer lab at the Southern Illinois University School of Medicine performed neuronal cultures on the packaged microscaffold systems with and without nutrient perfusion, and found that the active fluidics in the microscaffold system increased cell survival. Also, the LaPlaca lab at the Georgia Institute of Technology cultured 3-D neuronal-astrocytic co-cultures on the packaged microscaffold systems using Matrigel matrix, but no nutrient perfusion.

5.4.1 Biocompatibility of SU-8

Numerous sources cite SU-8 as being biocompatible [13, 14, 59, 60, 61, 62], but the collaborators from the 3-D Nets project found this claim to not necessarily be true. First of all, the SU-8 was inherently hydrophobic, so initially there were problems with the neurons adhering to the SU-8. However, the cell adhesion problem was solved by performing a 60 sec O₂ plasma treatment (Plasma Therm RIE) on the SU-8, which made the surface hydrophilic and aided in adhesion of the neurons to the surface of the SU-8 structures. The Allen, LaPlaca, and Brewer labs experimented with numerous
biocompatibility treatments using SU-8 bits to determine a biocompatibility protocol that would render the SU-8 biocompatible.

The biocompatibility treatments that worked the best for culturing neurons on the microscaffold structures presented in this dissertation were a 90,000 mJ/cm² UV light exposure, followed by a three day 150 °C vacuum oven bake, followed by a 60 sec O₂ plasma treatment, and a 60 sec IPA sonication. The UV light exposure and the vacuum oven bake helped to fully cure and hardbake the SU-8, while the O₂ plasma treatment rendered the SU-8 surface hydrophilic to aid in cell adhesion. Finally, the 60 sec IPA sonication removed any remaining photoresist residues in the microtowers, and significantly improved cell survival on the SU-8 microtower arrays.

Also, SEM analysis on the packaged microtower arrays before and after the 60 sec IPA sonication confirmed that the 60 sec sonication in IPA did not damage the microscaffold structures. For cell cultures performed on the packaged microscaffolds with nutrient perfusion, the biocompatibility treatments mentioned above were performed after the SU-8 microtower arrays were vertically packaged and sealed into the Si orifice plate, and after the sacrificial photoresist had been released from the channels, but prior to packaging the device into the PDMS fluid manifold.

Other treatments such as liquid CO₂, glow discharge, and a piranha etch, were experimented with to enhance the biocompatibility of the SU-8 without significant success. The liquid CO₂ treatment (Tousimis Super Critical Dryer), which was performed on hollow microtower arrays that were still attached to the original Si wafer upon which they were fabricated, caused damage to the microtower arrays by delaminating the third SU-8 layer from the Parylene C layer as seen in figure 5.28. As a
result, the liquid CO₂ treatment was eliminated as part of the biocompatibility protocol. Also, a 15 sec O₂ plasma treatment (Plasma Therm RIE) was added to the microfabrication processing after the Parylene C deposition but prior to the spinning of the third SU-8 layer, which aided in adhesion of the third SU-8 layer to the Parylene C layer.

The most significant of the biocompatibility treatments seemed to be the 60 sec IPA sonication. The IPA sonication removed any remaining residues from the microtower arrays. The microtower arrays that had a 90,000 mJ/cm² UV treatment, followed by a three day vacuum oven bake at 150 °C, followed by an O₂ plasma treatment and a 60 sec IPA sonication displayed a significant increase in cell viability compared to the microtower arrays that were processed with the 90,000 mJ/cm² UV treatment a, followed by a three day vacuum oven bake at 150 °C, followed by an O₂ treatment, and no IPA sonication. Therefore, the IPA sonication, which removed any remaining residues from the

![Image](image.png)

**Figure 5.28.** An SEM micrograph showing the delamination between the Parylene C layer and the third SU-8 layer, caused by the liquid CO₂ treatment. The liquid CO₂ treatment was eliminated from the biocompatibility protocol, but a 15 sec O₂ plasma treatment was added to the fabrication process to increase the adhesion between Parylene C and third SU-8 layers.
microtowers, was a crucial step in rendering the SU-8 microscaffolds biocompatible for neuronal cell culture.

5.4.1.1 Biological Results and Discussion from the Initial Biological Tests Performed on the Unpackaged SU-8 Microtower Arrays

Biocompatibility of the unpackaged microtower structures was initially confirmed by culturing primary hippocampal embryonic rat pup neurons on the unpackaged microtower arrays by the Brewer lab at the Southern Illinois University School of Medicine using the two protocols listed in Section 4.1: Initial Biological Tests on the Unpackaged SU-8 Microtower Arrays. Significant cell growth was observed on both sets of microtower arrays after four to six days in culture.

Images of the neurons growing on the surface of the planar microtower arrays can be seen in figures 5.29 and 5.30. Figure 5.29 shows neuronal growth on the first set of microtower arrays that had the second IPA sonication and the glow discharge treatments as part of their protocol. Also, it can be observed in figure 5.29 that the third SU-8 layer had delaminated from the microtower array structure. This delamination was the result of adhesion problems between the Parylene C and the third SU-8 layer, which was later resolved by performing a 15 sec O₂ plasma treatment on the Parylene C layer prior to spinning the third SU-8 layer on the wafer. Figure 5.30 shows neuronal growth on the second set of microtower arrays that had the second IPA sonication and glow discharge treatments excluded from their protocol.
The second IPA sonication performed at the Southern Illinois University School of Medicine showed no improvement in cell viability, so the second 60 sec IPA treatment (performed at Southern Illinois University School of Medicine) was removed from the

Figure 5.29. Images of neuronal cell viability on the planar SU-8 microtower arrays that received the second 60 sec IPA sonication and the 30 sec glow discharge treatment, showing (a) cultured neurons and two Au microtower leads; and (b) cultured neurons and an Au microtower electrode and corresponding lead.

Figure 5.30. Images of neuronal cell viability on the planar SU-8 microtower arrays that did not receive the second 60 sec IPA sonication, and did not receive the 30 sec glow discharge, showing (a) cultured neurons and two Au microtower leads; and (b) cultured neurons and two Au microtower leads (alternate view).
processing protocol. Therefore, only one 60 sec IPA sonication treatment was performed on the SU-8 microscaffold structures prior to cell plating, and the sonication was performed at the Georgia Institute of Technology prior to shipping the devices to Southern Illinois University School of Medicine. It was unclear to what extent the glow discharge improved cell viability, so a glow discharge treatment was still performed on all of the microscaffold structures prior to cell plating.

Figure 5.31 provides additional images of neuronal cell viability on the SU-8 microtower arrays. Figure 5.31a shows the neuronal network growing on a planar microtower structure, while figure 5.31b shows deconvolved Z-stack images of neurons growing on the one of the SU-8 cross-members.

Figure 5.31. Images of neuronal cell viability on the SU-8 microtower arrays. (a) Neuron plating density is 50,000 cells/cm². A 15×15 µm² Au electrode and its corresponding insulated lead for electrical stimulation/recording can also be seen. (b) Deconvolved Z-stack images of neurons growing on the microtower array.

Biocompatibility of the unpackaged microtower arrays was also confirmed by the Wheeler lab at University of Illinois at Urbana-Champaign. However, the Wheeler lab used SEM and phase contrast images to confirm cell viability while the Brewer lab
quantified cell viability using live/dead cell staining (see Section 4.1: Initial Biological Tests on the Unpackaged SU-8 Microtower Arrays). Also, the Wheeler lab used a lower cell plating density (300 cells/mm², as opposed to the 500 cells/mm² used by the Brewer lab). The Wheeler lab provided the microscope images shown in figures 5.32 and 5.33, showing neuronal growth on the planar microtower arrays at 4 DIV.

Figure 5.32. Neuronal cell growth at 4 DIV on a planar microtower array. The soma (cell bodies) and processes can be observed in addition to the detail of the microtower array structure.
Neurons were successfully cultured on the unpackaged SU-8 microtower arrays by the Wheeler lab for 17 days. SEM micrographs of neuronal growth on an SU-8 microtower are provided in figure 5.34, where it can be seen that the fluid ports and electrodes do not seem to hinder cell growth. Also, as can be seen in figure 5.34, taken after 17 days in culture that the neurons grew well on both the SU-8 insulation/structural material and on the exposed Au electrodes (15×15 µm²).

Figure 5.33. Neuronal cell growth at 4 DIV on a planar microtower array. The soma (cell bodies) and processes can be observed, in addition to an Au electrode and fluid side ports.
Interestingly, cells occasionally grew so as to partially cover the fluid ports (20×20 µm²). Also, the neural fibers tended to grow along the edge of the microtower structures, which implies that electrodes positioned along of the edge of the microtower structures should be considered in future microtower designs. Figure 5.35a provides an SEM micrograph showing the neural fibers following the edge of a microtower structure. Also, figure 5.35b shows that the neurons grow well on the differing planes of the microtower arrays. An additional SEM micrograph of the primary hippocampal neurons growing on the surface of the planar microtower arrays is provided in figure 5.36.

Figure 5.34. SEM micrograph of neuronal growth at 17 DIV on an SU-8 microtower. The microtower Au electrodes and fluid side ports can also be seen.
Figure 5.35. SEM micrographs showing that (a) the neural fibers tend to follow the edges of the SU-8 microtowers, and (b) the neurons grew well on the differing planes of the microtower arrays.

Figure 5.36. SEM Micrograph of a neuronal network at 17 DIV cultured on a planar SU-8 microtower array.
5.4.2 Biological Results and Discussion of Nutrient Perfusion

A perfusion rate of 0.05 µL/hr was used on the active microscaffold systems when re-aggregate neuronal cultures were being performed with nutrient perfusion. Live cells were either stained with Calcein-AM to fill the cytoplasm or Syto-16 to label live cell nuclei in denser cultures. Propidium iodide was used to stain dead cells. Neuron survival was counted as percent live cells/total cells. Cell viability was assessed at 7, 14, and 21 DIV. Figure 5.37 provides a comparison of fluorescent stains of live neurons growing on the active microscaffold system, both with and without nutrient perfusion [84].

![Substrate of perfused towers](image1)

![Perfused Towers](image2)

![Unperfused Towers](image3)

![Perfused Towers](image4)

Figure 5.37. Stains of live neurons that were cultured on the active microscaffold system. The Sytox green stained the live nuclei of denser cultures at the indicated times both with and without nutrient perfusion. The red propidium iodide was used to stain dead cells.
The graph provided in figure 5.38 shows that the fluid perfusion capabilities of the microscaffold system improved neuronal cell survival on the microscaffold at 14 and 21 DIV. The perfused (0.05 µL/hr) and unperfused (0 µL/hr) cell data acquired from culturing on the active microscaffold system was also compared to neuronal growth on a glass slide [84].

![Figure 5.38](image)

**Figure 5.38.** Graph of cell survival vs. culture age on perfused microscaffolds, unperfused microscaffolds, and glass slides. The cell survival rate is higher on the perfused microscaffolds at 14 and 21 DIV compared to unperfused microscaffolds and compared to the glass control. The perfusion rate was 0.05 µL/hr.

The biological data presented in this section of the dissertation shows that nutrient perfusion (0.05 µL/hr) from the active microscaffold system increases cell survival. However, there is some speculation as to whether dead cells with poor adhesion get flushed away and therefore are not quantified in the live/dead cell staining.
5.4.3 Biological Results and Discussion of the Passive Diffusion 3-D Neuronal-Astrocytic Co-Cultures on the Packaged SU-8 Microscaffold Systems

The LaPlaca lab performed passive diffusion neuronal-astrocytic co-cultures on the packaged microscaffold systems using the methods presented in Section 4.3.2: Biological Tests on the Packaged Microscaffolds without Perfusion Performed by the LaPlaca Lab at the Georgia Institute of Technology. Passive diffusion 3-D neuronal-astrocytic co-cultures (1:1 ratio) were grown on the packaged microscaffold systems and cell viability was assessed at 15 and 21 DIV using live/dead cell staining. Matrigel matrix was also used in these cultures to assist in 3-D neuronal network formation. The goal of these experiments was to assess cell survival and distribution within the microscaffold system.

The 3-D neuronal-astrocytic co-cultures displayed ~ 80% viability. Confocal microscope images of the 3-D co-cultures on the packaged microscaffold systems are provided at 15 and 21 DIV in figures 5.39 and 5.40, respectively. The live cells appeared green and the dead cells appeared red. It can be observed that the cells and matrix adhered directly to the SU-8 microtower walls. Additionally, processes can be seen that branch between adjacent linear microtower arrays, displaying a true 3-D neuronal network within the microscaffold system.
Cell adhesion directly to the SU-8 cross-member

Cells distributed throughout matrix with cell and matrix adhesion to the SU-8 microscaffold

Figure 5.39. Confocal images of neuronal-astrocytic co-culture viability at 15 DIV on the packaged SU-8 microscaffold system.

Processes branching between adjacent linear microtower arrays

SU-8 cross-members

Figure 5.40. Confocal images of neuronal-astrocytic co-culture viability at 21 DIV on the packaged SU-8 microscaffold system.
5.5 Electrode Characterizations

The electrically packaged microscaffold system functionalized 59 microtower electrodes and was compatible with the commercially available Multi Channel Systems preamplifier setup. Each SU-8 microtower contained two Au electrodes for extracellular stimulation/recording of the neurons, which connected to corresponding Au leads insulated within the microtower walls, and connected to electroplated Au tabs at the base of each microtower. A 90° bond was formed between the electroplated Au tabs and the Au substrate traces using the reflow of electroplated Sn-Ag solder at 250 °C. Subsequently, the Au tabs and substrate traces were insulated using PDMS. The polycarbonate culture ring that surrounded the 8×8 packaged microtower array was filled with phosphate buffered saline (PBS) and impedance measurements were performed on the 59 functionalized Au microtower electrodes using the Multi Channel Systems preamplifier setup in the DeWeerth lab at the Georgia Institute of Technology.

Impedance measurements on the 59 integrated microtower electrodes from the first electrically functional microscaffold system are provided in the graph in figure 5.41. The graph in 5.41 shows that there were a significant number of functioning electrodes in the electrically packaged microtower system, which are labeled in the graph. The open electrodes could have resulted from places where the PDMS/hexane seal became lodged between the Sn-Ag substrate tabs and the Au microtower tabs, and prevented an electrical bond from forming. Also, the open electrodes could have resulted from places where the substrate leads were overetched and therefore did not connect to the Sn-Ag substrate tabs. The lowest line on the graph in figure 5.41 is assumed to be a measurement from an exposed Au microtower tab that wasn’t adequately insulated with PDMS. The large
surface area of the exposed microtower tab (40 µm × 100 µm × 23 µm) accounted for the
decrease in the measured impedance. The area of each Au microtower electrode was
only 15×15 µm², and the measured level of impedance for the Au microtower electrodes
was in a reasonable range (~ 10⁶ Ω). Also, it is possible that the lowest line on the graph
in figure 5.41 is the impedance measurement from the ground line, which has a much
larger area than the microtower electrodes.

![Graph of impedance vs. frequency](image)

**Figure 5.41.** Graph of impedance vs. frequency, providing impedance measurements on the 59 functionalized microtower electrodes from the electrically packaged microscaffold system.

A color chart showing the magnitude of electrode impedance at 1 kHz is provided
in figure 5.42. There are 64 color blocks in the graph in figure 5.42, but only 59
electrodes and 1 ground to take measurements from, so the blocks at the four corners (dark blue) can be eliminated. The red blocks are representative of open microtower electrodes, and the pale blue blocks are representative of the functioning microtower electrodes. Counting the pale blue blocks in figure 5.42 shows that there were 34 functioning microtower electrodes (out of a possible 59) on this electrically packaged microscaffold device. However, in this device, some of the Sn-Ag solder tabs that were located on opposing sides of a single microtower were shorted together, so there really may have only been 17 functioning microtower electrodes on this device if we assume that all of the microtower tabs on either side of a single microtower were shorted together.

Figure 5.42. Magnitude of electrode impedance at 1 kHz.
Figure 5.43 provides electrode impedance measurements from a separate, electrically functionalized microscaffold device, where figure 5.43 provides impedance measurements on the Au microtower electrodes (prior to Pt black electroplating).

The impedance measurements provided in figure 5.43 were taken over a frequency range of 100 Hz to 100,000 Hz. Using the standard double layer electrical model, the impedance, given by equation 57, is determined by the passive elements in the circuit [i.e., the resistance (R) and double layer capacitance (C)], with contributing factors being R and 1/(jωC) in parallel.

\[
Z = R \parallel \frac{1}{j\omega C} \quad (57)
\]
At high frequencies (as $\omega \to \infty$), the impedance from the capacitive component becomes negligible, and the impedance levels out to a constant (DC) value, providing more of a resistive model given by the resistance of the electrode. At low frequencies (as $\omega \to 0$), the $1/(j\omega C)$ term dominates, providing mostly a capacitive based impedance. The impedance curves provided in figures 5.43 and 5.44 follow the resistive and capacitive based impedance models at high and low frequencies, respectively. The phase of the impedance was not measured, but the phase goes from -90° back towards 0°.

There are essentially two groups of curves on the graph in figure 5.43. The upper group of curves, with higher impedance values for the given frequencies, represents open microtower electrodes (electrodes that did not get properly bonded). The lower group of curves, with lower impedance values for the given frequencies, represents functioning microtower electrodes (electrodes that were properly bonded). As illustrated in the graph in figure 5.43, the impedance values of the open and functioning Au microtower electrodes at 1 kHz are $9 \times 10^5$ Ω and $5 \times 10^5$ Ω, respectively. Using equation 57 with the values $Z = 5 \times 10^5$ Ω and $\omega = 2\pi(10^3 \text{ Hz})$, the double layer capacitance value for the functioning electrodes can be calculated to be $C = 3.18 \times 10^{-10}$ F.

The impedance of Au electrodes is inherently too high (~ $10^6$ Ω) to perform electrical stimulation/recording measurements in neuronal cell culturing applications, so the Au microtower electrodes on the electrically packaged microscaffold system were electroplated with Pt black using the electroplating setup in the DeWeerth lab at the Georgia Institute of Technology. The rough topography of electroplated Pt black yielded an electrode with an increased surface area, which significantly lowered the electrode impedance by several orders of magnitude. The electroplating setup in the DeWeerth lab
was compatible with the Multi Channel Systems preamplifier setup and could electroplate up to 16 electrodes at one time. During the electroplating process the Pt black electroplating solution was contained within the culture ring that surrounded the packaged microscaffold device, and a Pt coated electrode was placed in the electroplating solution. The graph in figure 5.43 provides impedance measurements from the Au microtower electrodes prior to the Pt black electroplating, while the graph in figure 5.44 provides impedance measurements from that same device after Pt black has been electroplated on the microtower electrodes.

![Graph of impedance vs. frequency](image)

**Figure 5.44.** Graph of impedance vs. frequency, providing impedance measurements on the 59 functionalized Pt black microtower electrodes from the electrically packaged microscaffold system (after Pt black has been electroplated on the microtower electrodes).

It can been seen that the group of curves for the open Au electrodes is the same in both figures 5.43 and 5.44, which makes sense since the open Au electrodes in the microscaffold system couldn’t have been electroplated with Pt black. It can be seen that
some of the functioning Au microtower electrodes weren’t electroplated with Pt black for some reason, since their impedance curves also look identical in both figures 5.43 and 5.44. However, the impedance curves of the electrodes that were successfully electroplated with Pt black have significantly lower impedance values as a result of the increased surface area of the rough Pt black that has been deposited on the Au microtower electrodes. Furthermore, it can be seen that the impedances of the Pt black electrodes vary over a wide range, which means that the impedance values of the electrodes are not matched. Ideally, we would like to monitor the impedance of each electrode as it is being electroplated with Pt black so that all of the impedance values are matched. The lowest curves seen on figure 5.44 are the result of overplating on those particular electrodes, which results in the Pt black “mushrooming” over the 15×15 µm² electrode area, therefore increasing the base area of the electrodes, which significantly lowered the measured impedance of those electrodes.
CHAPTER 6

CONCLUSIONS AND FUTURE WORK

An active microscaffold system with integrated fluid perfusion and electrical stimulation/recording functionalities for in vitro culturing of re-aggregate neuronal cultures was designed, fabricated, packaged, and characterized. Several versions of the active microscaffold system were designed, fabricated, and packaged. Each active microscaffold system consisted of an array of microtowers (8×8, 12×12, or 16×16), ranging in height from 1-2 mm. The microtowers were initially fabricated in linear arrays (containing 8, 12, or 16 microtowers per linear array) with fluid and electrically functional cross-members that connected adjacent microtowers in the linear array. The linear microtower arrays were fabricated in a planar (2-D) fashion on top of a Si substrate, released from the Si substrate, and subsequently packaged into a 3-D format to yield the active microscaffold system. Additionally, structural SU-8 pitchforks that formed bridges between adjacent linear microtower arrays were added to the microscaffold system to allow for increased neuronal network connectivity.

Each microtower had a fluid channel and multiple side ports that provided perfusion functionality for gas exchange and biochemical control of the extracellular environment throughout the 3-D culture. The 8×8 microtower arrays that were 1.0 mm or 1.5 mm in height were the most conducive to biological testing, since it was more difficult to plate the neurons in the center of the 16×16 microtower arrays, and the 2.0 mm tall microtower arrays were difficult to image. Use of the active microscaffold system was demonstrated by culturing primary hippocampal rat pup neurons and characterizing cell viability within the system with and without nutrient perfusion.
Biocompatibility of the unpackaged SU-8 microtower arrays was initially validated by the Brewer lab at the Southern Illinois University School of Medicine and the Wheeler lab at the University of Illinois at Urbana-Champaign. The 3-D Nets collaborators made a significant research effort in relation to the biocompatibility of SU-8. The Brewer, LaPlaca, Allen, Frazier, and Wheeler labs collaborated to determine a biocompatibility protocol that was appropriate for the SU-8 microscaffold structures. Numerous sources have praised SU-8 for its biocompatibility, but the 3-D Nets collaborations found this claim to not necessarily be true. The active microscaffold structures presented in this dissertation had to undergo a series of biocompatibility treatments including a 90,000 mJ/cm² UV light treatment, a three day vacuum oven heat treatment at 150 °C, a 60 sec O₂ plasma treatment, and a 60 sec IPA sonication prior to cell plating to ensure biocompatibility of the completed microscaffold structures.

The neuronal cell data acquired by the Brewer lab demonstrated that nutrient perfusion (0.05 µL/hr) improved cell survival on the active microscaffold system at 14 and 21 DIV compared to cells cultured on the active microscaffold systems without nutrient perfusion and compared to cells cultured on glass slides [84]. The cell data with nutrient perfusion that was gathered on the active microscaffold system was a collaborative effort between the Frazier and Glezer labs and the Georgia Institute of Technology and the Brewer lab at the Southern Illinois University School of Medicine. Neuronal cell data with fluidics was not analyzed on the revised active microscaffold systems for re-aggregate cultures that contained a 12×12 array of microtowers, even though the fluid perfusion functionality of the revised 12×12 active microscaffold system delivered more uniform fluid volume flow rates than the original 8×8 active
microscaffold system from which all the cell data with active fluid perfusion was performed and analyzed. Additionally, the center-to-center microtower spacing in the revised 12×12 active microscaffold system was only 200 µm (as opposed to the 600 µm center-to-center microtower spacing in the 8×8 active microscaffold system). The decreased center-to-center microtower spacing in the revised 12×12 active microscaffold system could potentially have resulted in the branching of neuronal processes between adjacent microtowers and linear microtower arrays in the absence of an additional Matrigel matrix, thus delivering a better representation of a 3-D in vitro neuronal network.

It has been debated whether or not the neuronal cell cultures performed on the active microscaffold systems with nutrient perfusion in collaboration with the Frazier, Glezer, and Brewer labs can be classified as true 3-D cultures. These cultures were performed in the absence of an additional matrix (i.e., Matrigel), so the neuronal processes didn’t form branching connections between adjacent microtowers or linear microtower arrays. However, the neuronal networks in these cultures grew on the differing planar surfaces of the microscaffold structure, thus increasing the complexity of neuronal network connectivity from the standard 2-D neuronal network connectivity observed on 2-D MEAs. Additionally, these cultures performed by the Brewer lab used the fluid perfusion functionality of the active microscaffold system and demonstrated that the nutrient perfusion functionality of the active microscaffold system increased cell survival at 14 and 21 DIV.

Three-dimensional passive diffusion co-cultures (neurons and astrocytes) were performed on the active microscaffold systems with the addition of Matrigel in a
collaborative effort between the Frazier and LaPlaca labs. In these passive diffusion cultures, neuronal processes were observed that grew outwards and branched between adjacent linear microtower arrays, thus forming a true 3-D neuronal network. However, these 3-D neuronal cultures did not utilize the fluid perfusion properties of the active microscaffold system, and were performed mainly to assess 3-D cell survival and distribution within the microscaffold system in conjunction with Matrigel matrix.

Additionally, an active microscaffold system with integrated perfusion and electrical stimulation/recording functionalities for in vitro culturing of thick (≤ 1 mm) brain slices was designed, fabricated, and packaged. The brain slice active microscaffold system consisted of a 12×12 array of microtowers with integrated fluid perfusion and electrical stimulation/recording functionalities. Active microscaffold systems with fluid perfusion functionality for chronic culturing of thick slices of brain tissue have been delivered to the Potter lab and will undergo biological testing with nutrient perfusion. Past studies from the Frazier lab have demonstrated that active nutrient delivery from a glass capillary and PDMS fluid perfusion device increased the viability and structural integrity of thick in vitro brain tissue slices [9, 85, 86], and the brain slice active microscaffold system presented in this dissertation will enable similar studies with enhanced precision and with the addition of electrical stimulation/recording functionalities.

Future work includes obtaining electrical stimulation/recording data from neuronal networks cultured on the active microscaffold system presented in this dissertation in collaboration with the Brewer lab at the Southern Illinois University School of Medicine. The active microscaffold system with electrical functionality was
designed to be compatible with the Multi Channel Systems preamplifier setup to rapidly obtain impedance data from the functioning 3-D array of microtower electrodes. Additionally, obtaining neuronal electrical stimulation/recording data from the active microscaffold system will be simplified by using the Multi Channel Systems preamplifier setup. Furthermore, rapid comparisons between 2-D MEAs and the 3-D MEA on the active microscaffold system with fluid perfusion and electrical stimulation/recording functionalities can be made using the Multi Channel System preamplifier setup. However, a maximum of 59 out of the total 128 microtower electrodes could be functionalized using the Multi Channel Systems preamplifier setup in conjunction with the 8×8 microtower arrays.

Also, an additional project has spun off from the research presented in this dissertation involving the study of in vivo brain tissue regeneration after the surgical removal of brain tumors or hematomas. The revised microtower arrays that were originally designed for re-aggregate in vitro neuronal cultures (containing 12 microtowers per linear array) will be used in an in vivo project that is a collaborative effort between the Frazier and Brewer labs. These linear microtower arrays will be spliced into smaller linear arrays (yielding 6 microtowers per linear array), packaged into a 3-D format with fluid perfusion functionality, and attached to the skull of a live rat. The implanted active microscaffold system will interface with an area of the rat brain that has been removed to study brain tissue regeneration in vivo after a section of the brain has been removed (due to surgical removal of tumors or hematomas).
APPENDIX A

Planar SU-8 Microtower Array Fabrication Process (Original 8×8 and 16×16 Microtower Array Designs)

1. Begin with a bare Si wafer

2. Sputter 1600 Å of Cr onto the Si wafer at a rate of 600 Å/min using the Unifilm Sputter. This serves as a sacrificial layer when releasing the microtower arrays from the Si wafer.

3. Define 1st SU8 layer (SU-8 2005)
   - spin @ 500 rpm, 100 rpm ramp, 5 sec
   - 3000 rpm, 300 rpm ramp, 30 sec
   - prebake on hotplate @ 65 ºC, 1 min
     95 ºC, 3 min
   - expose with UV @ 90 mJ
   - post-exposure bake (PEB) on hotplate @ 65 ºC, 1 min
     95 ºC, 1 min
   - develop in PGMEA, 2 min
   - IPA rinse, 2 min
   - N2 dry
   - hardbake on hotplate @150 ºC, 30 min

4. Sputter 1900 Å of Au onto the wafer at a rate of 700 Å/min using the Unifilm Sputterer.

5. Lithography for electroplating (Clariant AZ 4620 photoresist)
   - spin @ 300 rpm, 100 rpm ramp, 15 sec
     900 rpm, 300 rpm ramp, 20 sec
   - prebake on hotplate @ 110 ºC, 2 min
   - expose with UV @ 810 mJ
   - develop in 40 mL AZ 400K developer and 100 mL DI water for 2 min 40 sec
   - DI water rinse
   - N2 dry

6. Electroplate Au tabs using gold plating solution
   - 55 ºC, 4.5 hr, 300 rpm
   - DI water rinse
   - N2 dry

7. Strip AZ 4620 with acetone, followed by IPA, DI water, N2 dry

8. Lithography for Au leads (Shipley 1827 photoresist)
   - spin @ 3000 rpm, 500 rpm ramp, 20 sec
   - prebake on hotplate @ 100 ºC, 2.5 min
expose with UV @ 540 mJ
develop in MF 319 (TMAH) for 60 sec
DI water rinse
N₂ dry

9. Au lead etch
   KI solution, ~3 min
   DI water rinse
   N₂ dry

10. Define 2nd SU8-2005 Layer (This SU8 layer insulates the Au leads)
    spin @ 500 rpm, 100 rpm ramp, 5 sec
    1000 rpm, 300 rpm ramp, 30 sec
    prebake on hotplate @ 65 ºC, 2 min
    95 ºC, 5 min
    expose with UV @ 140 mJ
    post-exposure bake (PEB) on hotplate @ 65 ºC, 1 min
    95 ºC, 2 min
    develop in PGMEA, 6 min
    IPA rinse, 2 min
    N₂ dry
    hardbake on hotplate @ 150 ºC, 30 min

11. Define Sacrificial Layer (Clariant AZ 4620 photoresist) that will later be cleared out to make the microtowers hollow
    spin @ 300 rpm, 100 rpm ramp, 15 sec
    900 rpm, 300 rpm ramp, 20 sec
    prebake on hotplate @ 110 ºC, 2 min & 45 sec
    expose with UV @ 810 mJ
    develop in 80 mL AZ 400K developer and 200 mL DI water for 2 min
    change the developer to a new, clean ratio of 80 mL AZ 400K developer and 100 mL DI water for an additional 1.5min
    DI water rinse
    N₂ dry

12. Parylene Run (This prevents the sacrificial resist from mixing with the 3rd SU8 layer)
    2 µm Parylene C deposition (CVD Process)
    12a. Perform a 15 sec O₂ plasma treatment in the on the Parylene layer. (45 sccm O₂, 300 W, 50 mT). This O₂ treatment makes the Parylene layer hydrophilic and improves the adhesion between the Parylene and 3rd SU8 layer.

13. Define 3rd SU-8 layer (SU-8 2025)
    spin @ 500 rpm, 100 rpm ramp, 5 sec
    1000 rpm, 300 rpm ramp, 30 sec
    prebake on hotplate @ 65 ºC, 2 min
expose with UV @ 140 mJ  
post-exposure bake (PEB) on hotplate @ 65 °C, 5 min  
95 °C, 15 min  
develop in PGMEA, 8 min  
IPA rinse, 2 min  
N₂ dry  
no hardbake this time since we still need to clear out the sacrificial photoresist  

14. Cr Etch (CR-7S) to release the towers from the substrate (this typically takes at least 3 hr)  
15 min DI water rinse.
APPENDIX B

Planar SU-8 Microtower Array Fabrication Process
(Revised 12×12 Design for Dissociated Neuronal Cultures and the 12×12 Brain Slice Microtower Arrays)

1. Evaporate 1600 Å of Cr on a blank Si wafer using the PVD75 Filament Evaporator

2. 1st SU-8 layer (SU-8 2005)
   a. 500 rpm/100 rpm ramp/5 sec
   b. 3000 rpm/300 rpm ramp/30 sec
   c. Prebake on hotplate @ 65 °C for 1 min
   d. Prebake on hotplate @ 95° C for 1 min
   e. Expose on MA-6 (CI-1 = 4.5-4.8 mW/cm²) for 9sec (~40.5 mJ/cm²), 20 µm alignment gap
   f. PEB on hotplate @ 65 °C for 1 min
   g. PEB on hotplate @ 95 °C for 1 min
   h. Wait 5 min
   i. Develop in PGMEA (P-Type Thinner, Shipley) for 2 min with a lot of agitation
   j. IPA rinse for 2 min
   k. N₂ dry
   l. Hardbake on hotplate @ 150 °C for 30 min

3. Sputter 1800 Å of Au @ a rate of 700 Å/min using the Unifilm Sputterer

4. Au tab lithography (17 µm of AZ 4620)
   a. 300 rpm/100 rpm ramp/5 sec
   b. 1200 rpm/300 rpm ramp/20 sec
   c. Prebake on hotplate @ 110 °C for 2 min
   d. Align and expose on MA-6 (CI-2 = 18-22 mW/cm²) for 35 sec (~770 mJ/cm²)
   e. Develop in 80 mL AZ 400K : 200 mL DI water for 2 min
   f. Rinse with DI water
   g. Continue developing in the same developer (80 mL AZ 400K : 200 mL DI water) for 1-2 min (until it looks good)
   h. DI water rinse
   i. N₂ dry

5. Shipley 1827 on the backside of the wafer
   a. 3000 rpm/500 rpm ramp/30 sec
   b. Place in vertical wafer holder and bake in 95 °C oven for 5 min

6. Electroplate thick Au tabs (~11-16 µm) using Techni-Gold 25 E S plating bath (the target is 13 µm of electroplated Au)
   a. Quick DI water sonication (1 sec)
b. 55 °C, 300 rpm, ~0.75 mA, 2 hr and 20 min
c. DI water rinse
d. N₂ dry

7. Clean off the front and backside of the wafer using acetone, 2 min IPA rinse, N₂ dry

8. Au lead lithography using Shipley 1813 photoresist
   a. 3000 rpm/1000 rpm ramp/30 sec
   b. Prebake on hotplate @ 100 °C for 2 min and 45 sec
   c. Align and expose on the MA-6 (CT-2 = 18-22 mW/cm²) for 6 sec
   d. Develop in MF-319 (TMAH) for 30 sec with no agitation. Agitate for the next 20-30 sec. Take the wafer out of the developer after a total of 50-60 sec
   e. DI water rinse
   f. N₂ dry

9. Au lead etch
   a. Etch the Au leads in KI until the leads are nicely patterned (2-5 min)
   b. DI water rinse
   c. N₂ dry

10. Clean the resist off the wafer using acetone, followed by an IPA rinse, N₂ dry

11. 2nd SU-8 layer (SU-8 2005)
    a. 500 rpm/100 rpm ramp/5 sec
    b. 2000 rpm/300 rpm ramp/30 sec
    c. Prebake on hotplate @ 65 °C for 1 min
    d. Prebake on hotplate @ 95 °C for 1 min
    e. Align and expose on MA-6 (CT-1 = 4.5-4.8 mW/cm²), 9 sec, (40 µm alignment gap)
    f. PEB on hotplate @ 65 °C for 1 min
    g. PEB on hotplate @ 95 °C for 1 min
    h. Develop in PGMEA (P-Type Thinner) for 2 min and 5 sec with a lot of agitation
    i. IPA rinse for 2 min
    j. N₂ dry
    k. Hardbake on hotplate @ 150 °C for 30 min

12. Place the wafer in Au etch solution to remove any residual Au that couldn’t be removed during the previous Au lead etch (30 sec to a few min, or until the unwanted Au is etched away)
    a. DI water rinse
    b. N₂ dry

13. Sacrificial Layer (23 µm of AZ 4620)
    a. 300 rpm/100 rpm ramp/15 sec
b. 900 rpm/300 rpm ramp/20 sec
c. Prebake on a hotplate @ 110 °C for 2 min and 15 sec (let the resist significantly cool and harden or else it will stick to the photomask)
d. Align and expose on MA-6 (CI-2 = 18-22 mW/cm²) for 45 sec (~990 ml/cm²), 60 µm alignment gap
e. Develop in 80 mL AZ 400K : 200 mL DI water for 2 min
f. Rinse with DI water
g. Continue developing in a new combination of 80 mL AZ 400K : 200 mL DI water for 1-2 min (until the developing is completed)
h. DI water rinse
i. N₂ dry

14. Parylene deposition (2 µm)
   a. 1 g of Parylene C yields a 2 µm layer of Parylene

15. O₂ plasma treatment to make the Parylene hydrophilic
   a. Use lro_resi.prc recipe for 15 sec (45 sccm O₂, 50 mT, 300 W, LOVAC)

16. 3rd SU-8 layer (SU-8 2025) (~80 µm)
   a. 500 rpm/100 rpm ramp/5 sec
   b. 1000 rpm/300 rpm ramp/30 sec
c. Prebake on hotplate @ 65 °C for 5 min
d. Prebake on hotplate @ 95 °C for 15 min
e. Let cool so the wafer isn’t tacky for the exposure (the resist needs time to significantly cool and harden or else it will stick to the photomask)
f. Align and expose on MA-6 (CI-1 = 4.5-4.8 mW/cm²) for 90 sec, 90 µm alignment gap PEB on hotplate @ 65° C for 1 min
g. PEB on hotplate @ 95°C for 4 min
h. Develop in PGMEA (P-Type Thinner) for 8 min, IPA rinse, N₂ dry

17. Parylene etch in the Plasma Therm RIE
   a. lro_pryl.prc recipe for 12 min (45 sccm O₂, 5 sccm CHF₃, 50 mT, 300 W)

18. Cr etch in CR-7S to release the tower structures
   a. Takes up to 24 hr

19. DI water rinse of the released structures for 15 min.
APPENDIX C

Fabrication Process for the Electrically Functional Si Orifice Plates Designed to Be Compatible with the Multi Channel Systems Preamplifier Setup

1. Begin with a bare Si wafer

2. Pattern 10 µm of Futurrex NR-9 8000 to define electrode patterns
   Spin @ 2000 rpm, 500 rpm ramp, 30 sec
   Prebake in a convective oven @ 100 ºC
   Expose with UV @ 300 mJ
   Post-exposure bake (PEB) in a convective oven @ 100 ºC
   Develop in RD6 developer, 3min
   DI water rinse, 2min
   N₂ dry

3. Evaporate 0.015 µm of Ti followed by 0.5 µm of Au using the e-beam evaporator

4. Lift-off process to define substrate traces
   Soak sample in acetone for 24 hr
   IPA rinse, 2min
   DI water rinse, 2min
   N₂ dry

5. Lithography for through wafer via etching (14 µm of Clariant AZ 4620 photoresist)
   Spin @ 1500 rpm, 500 rpm ramp, 30 sec
   Prebake on hotplate @ 110 ºC, 2 min
   Expose with UV @ 300 mJ
   Develop in 40 mL AZ 400K developer and 100 mL DI water for 2 min
   DI water rinse
   N₂ dry

6. DRIE of through wafer vias using the Plasma Therm ICP
   Mount patterned wafer onto of a 4 in carrier wafer using Cool Grease
   1000 cycles of the Bosch Process in the ICP

7. Strip AZ 4620 with acetone (24 hr soak)
   IPA rinse, 2 min
   DI water rinse, 2 min
   N₂ dry

8. Deposit Au seed layer (0.1 µm) using the DC Sputterer

9. Pattern 35 µm thick electroplating mold using AZ 4620 photoresist
   Spin @ 500 rpm, 200 rpm ramp, 30 sec
   Prebake on hotplate @ 95 ºC, 30 min
   Expose with UV @ 600 mJ
Develop in 40 mL AZ 400K developer and 100 mL DI water for 4 min
DI water rinse
N₂ dry

10. Electroplate Sn-Ag tabs (25 µm thick)
    Room temperature, no agitation, 2 hr
    DI water rinse
    N₂ dry

11. Strip AZ 4620 photoresist off the wafer
    Acetone soak, 24 hr
    IPA rinse, 2 min
    DI water rinse, 2 min
    N₂ dry

12. Au seed layer etch
    KI/I₂ solution, 3 min
    DI water rinse, 2 min
    N₂ dry
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Changes in Bulk Sn, and Eutectic Sn-Ag in Bulk and in Joints, from Aging and Thermal


[69] B.W. Kristensen, J. Noraberg, P. Thiebaud, M. Koudelka-Hep, and J. Zimmer,
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