

Multi-chamber culture device for connected neuronal aggregates

Preliminary Report

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Abstract

The study of neuronal connections is important for understanding the pathophysiology of neural networks and neurodegenerative diseases. Simple well plates or petri dishes fail to accurately model the 3D micro-physiological environment and geometries present in the human body. To aid the study of neural pathways and connections, the team proposes a device fabricated by milling a 384-well plate to create a series of individual cell culture chambers with extracellular matrix (ECM) filled channel connections to neuronal aggregates. A double-sided biocompatible adhesive will be used to seal the milled well plate to a thin transparent bottom substrate. This device will allow for the growth and interaction of a connected neuronal network while isolating the media surrounding each neuronal cell body. This device allows for simple treatment of isolated media and post-treatment imaging. Proposed testing evaluates the device's performance through media isolation, ECM loading, aggregate positioning, cell viability/cytotoxicity, and neurite growth assessments. The data collected will guide design improvements and maximize functionality. Successfully validating this platform provides researchers with a physiologically relevant model to study neural connectivity, evaluate hearing restoration strategies, and investigate cellular communication without cross-contamination of culture media.

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1. Introduction

a. Motivation and Societal Impact

In the United States alone, over 50 million people suffer from some level of hearing loss [1]. Within the next 25 years, this number is predicted to rise significantly on a global scale. The World Health Organization estimates that, globally, 2.5 billion people will have some form of hearing loss, whether mild or severe, and 700 million people will need rehabilitation for disabling hearing loss [2]. While hearing loss can be associated with many social challenges, such as difficulties in communication, it is also associated with an increased risk of cognitive decline, increasing the risk of diseases such as Alzheimer's [3]. Existing technologies such as hearing aids or cochlear implants provide considerable benefits; however, they do not fully restore normal hearing in most patients, highlighting the need for additional research into the biological limitations of current treatments [4] [5].

In more severe cases of hearing loss, cochlear implants are used to restore some hearing. They work by bypassing damaged cochlear hair cells to pass a series of electric signals directly to the auditory nerve. The brain interprets these signals as sound, giving the patient a relatively accurate representation of their environment [5]. However, the functionality of cochlear implants partially depends on the survival of spiral ganglion neurons, which are responsible for relaying auditory information. The degeneration of these neurons can limit the effectiveness of cochlear implants and other hearing restoration techniques [6]. Thus, current research has moved towards regenerative tissue engineering technologies and the "living electrode" approach, where cochlear anatomy and the integration of regenerated neurons are being prioritized [4] [7] [8].

Even with significant advancements being made in this field, the integration of regenerated neurons into existing auditory neuronal networks still proves to be a major challenge. Neuronal growth and survival do not automatically guarantee connectivity to an established network. To understand these biological barriers, some research studies are investigating how distinct neuronal populations interact and

form connections with one another in order to develop and evaluate regenerative therapies for hearing loss [9].

The proposed research device is intended to aid in this study of integration by providing a compartmentalized *in vitro* device to study interactions between distinct neuronal populations across extracellular matrix (ECM) channels. A device such as this one could assist researchers in studying neuronal network connectivity in more biologically accurate, three-dimensional systems, assisting in the long term goal to develop better treatments for hearing restoration and better strategies for auditory nerve regeneration [6] [9].

b. Competing Devices

Current methods for compartmentalizing neuronal populations are split into multiple areas of study. One focus looks at fluid flow and transport, specifically in microfluidic devices or organ-on-a-chip devices [9] [10]. These devices use small channels to control the flow and transport of media or nutrients; however, in certain studies, such as this one, this flow of media is not useful or beneficial [10]. Another focus specifically studies axonal growth and some interactions. These studies typically use Campenot chambers, which have the ability to keep media separated but often require the use of nerve growth factor or other growth stimulants. Additionally, most Campenot designs are less suitable for central nervous system studies and more suitable for peripheral nervous system neurons [10] [11]. Besides media flow and axonal interactions, studying the effects of different media on growth and function is also common. This focus uses microwell arrays, which fully compartmentalize neuronal populations and ensure suitable aggregate positioning. However, typical microwell arrays cannot establish connections between these distinct populations, meaning heavy modification would be required in order to create these connections without allowing media flow [12]. Thus far, there is no specific, specialized device that allows for neuronal connectivity without allowing media flow between compartments. The proposed device is intended to ensure compartmentalization as well as connectivity without the transfer of media.

c. Problem Statement

Common devices used in biological research, including well plates and petri dishes, are readily accessible, provide easy platforms for addition of cells, media, and reagents, and allow for observation using standard laboratory microscopes. However, they fail in their ability to model the complex physiological environments of the human body. In recent decades, there has been a large push toward the development of microphysiological systems used for research, for example, the Organ-on-a-Chip platforms [13]. The ability to model complex tissue environments is incredibly important for the study of the auditory pathway because of its dynamic environment. 2D systems completely isolate cells from the complex physical forces, specialized microenvironments, and 3D neural networks that are vital to the transduction of sound to the brain [14]. This device provides a more compartmentalized and dynamic platform to study the growth and interactions of neuronal cell bodies on a 3D platform. The device allows for growth and interaction of neuronal cell aggregates in a structured manner. The device separates neuronal aggregates into distinct chambers each holding their own culture media. The device allows for growth of neurons between chambers, but separates the media in each chamber, allowing for independent treatments of aggregates.

2. Background

a. Background Research

Micro Tissue Engineered Neural Networks (micro-TENNs) are living 3D constructs used to replicate the neuroanatomy of white matter pathways in the brain. Micro-TENNs are currently being developed as implantable micro-tissues for axon tract reconstruction and as *In vitro* experimental platforms [15]. The geometry of micro-TENNs replicates the anatomy of axonal tracts, and may be an effective substrate for targeted reconstruction of axonal pathways in the brain. Micro-TENNs consist of

neuronal aggregates with integrated axonal tracts encased in micro-columns made of hydrogels. The hydrogel column contains ECM to optimize neuronal survival and neurite extension [16].

b. Design Research

Any material that comes into contact with cells or media must not be cytotoxic, with cell viability greater than 70% relative to the control in accordance with ISO 10993-5 compatible in vitro cytotoxicity assay [17]. Optical transmittance of any material intended to be imaged through should be evaluated in accordance with ASTM E1348-22 [18]. Cell culture will be conducted in a Biosafety Level II laboratory in accordance with the rules and regulations outlined by the Centers for Disease Control and Prevention and the National Institute of Health [19].

c. Client Information

Dr. Jason A. Brant is a practicing physician at UW with double board certification in General Otolaryngology and Neurotology. He received his MD at Washington University and did his fellowship and residency at the University of Pennsylvania. Dr. Brant requests a cell-culture device containing three or four isolated chambers maintaining independent culture media with a neuronal cell aggregate seated at the base of each chamber and adjacent aggregates connected through ECM-filled channels [4].

d. Design Specifications

The device must separate media and neuronal aggregates into three or four separate chambers with no media transfer between chambers. The chambers must be connected by ECM channels that are 100-300 micrometers in diameter. The neuronal aggregates must sit in each chamber and must not float in the media. The device must allow for post-treatment imaging of the cells.

The neuronal aggregates must grow into the ECM columns reaching 50-70% growth within the first 7 days. At least 90% of cell constructs must be recovered, intact, for imaging. There must be no measurable media transfer between chambers. Further specifications can be found in Appendix A.

3. Preliminary Designs

a. Glass with PDMS Cover

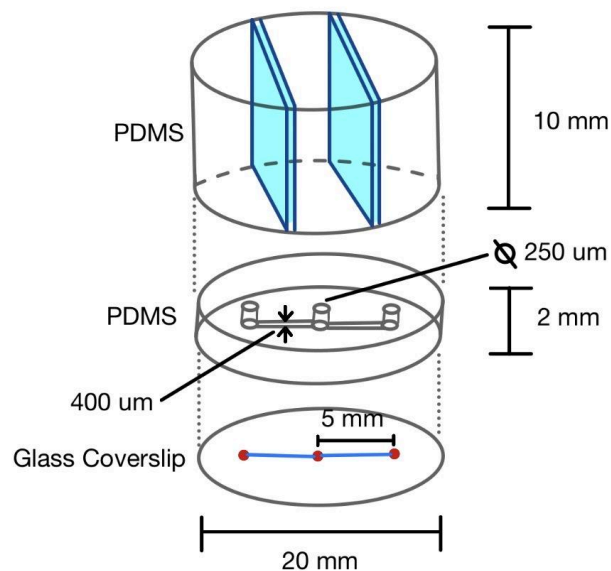


Figure 1: Glass with PDMS Cover Design

The “Glass with PDMS Cover” design (Figure 1) consists of a glass coverslip at its base and a PDMS cover for the top portion of the design. The glass coverslip is a circular glass piece on which the neuronal aggregates are placed and connected through micro-TENNS. There is a PDMS piece that is placed on top of the aggregates and has indentations to encapsulate the aggregates and their micro-TENNS, as well as keep cell culture media isolated for each neuronal aggregate. This design is highly feasible to use because of a simplified design concept consisting of two pieces – the glass coverslip

and the PDMS cover. The top PDMS cover allows for increased media volume and separation between chambers due to the height of the walls in between.

b. Milled 384 Well Plate

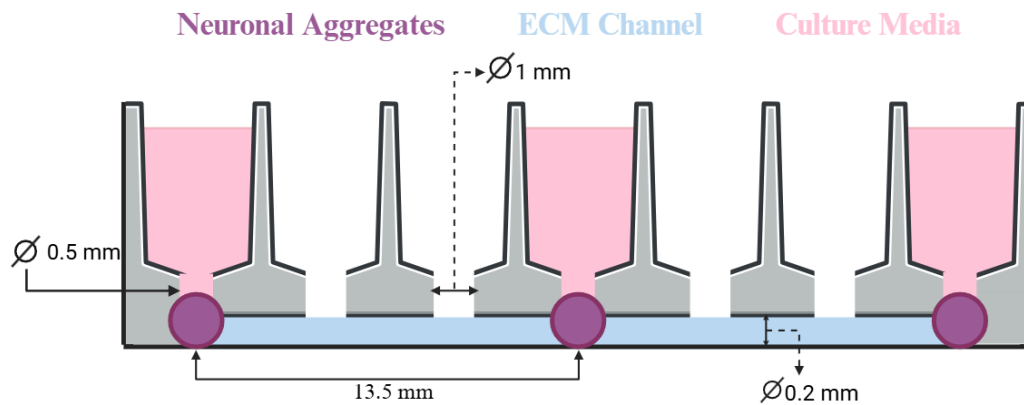


Figure 2: Milled 384 Well Plate Design

The Milled 384 Well Plate design (Figure 2) consists of a 384-well plate which has a milled base to create small channels connecting wells with each other. Each well has a milled hole which allows for placement of the neuronal aggregates, ECM addition, and cell culture media. In this particular design, the number of wells between the neuronal aggregates can be modified. This design is highly convenient to use as there is proper pipette access in each well of this 384-well plate. However, manufacturing this design is slightly complex, as it requires precisely milled channels and ports in a 384 well-plate which consists of smaller wells.

c. PDMS with Divots for Agarose

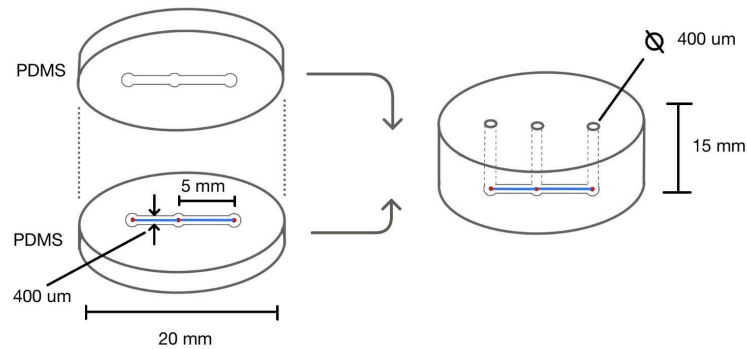


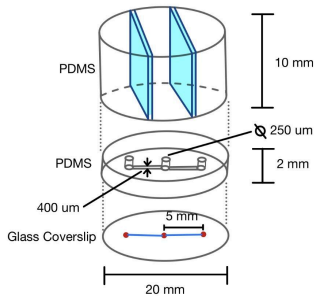
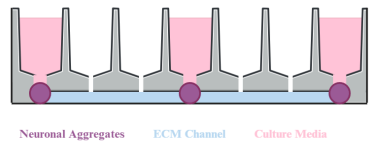
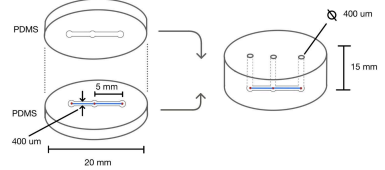
Figure 3: PDMS with Divots for Agarose Design

The PDMS with Divots for Agarose design (Figure 3) consists of two pieces of PDMS, in which divots are constructed using a master mold. The divots in both pieces of the PDMS allow for the feasible placement of the neuronal aggregates, as well as support the column geometry of the micro-TENNs. There are holes above the aggregates to ensure that the neuronal aggregates maintain contact with the culture media. This design allows for flexibility, as an additional PDMS piece can be fixed above to increase the media volume. In addition, fabrication in this design is simple as it only requires a master mold for the PDMS components.

4. Preliminary Design Evaluation

a. Design Matrix

Table 1: Design matrix evaluation of three preliminary designs

Design Criteria (Weight)	Glass slip w/ PDMS Cover		Milled Well Plate		PDMS w/ divots for agarose	
						
	Raw Score (X/5)	Weighted Score	Raw Score (X/5)	Weighted Score	Raw Score (X/5)	Weighted Score
Aggregate Stability/Positioning (30)	3/5	18	5/5	30	4/5	24
Media Isolation (25)	3/5	15	5/5	25	3/5	15
Imaging Ability/Cell Recovery (20)	4/5	16	5/5	20	2/5	8
Ease of Use (10)	4/5	8	4/5	8	3/5	6
Ease of Fabrication/Reproducibility (10)	3/5	6	2/5	4	3/5	6
Cost (5)	4/5	4	1/5	1	5/5	5
Total (100)	67		88		64	

b. Design Matrix Summary

The team selected criteria of aggregate stability and positioning, media isolation, imaging ability and cell recovery, ease of use, ease of fabrication and reproducibility, and cost. Aggregate stability and positioning was ranked as the most important criteria because in order for the neuronal aggregates to remain viable, they cannot float in the media. Axons must be able to enter the lumen to interact and connect through the channels of the device. Without this action, the main goal of the device is defeated. Media isolation describes the ability of the device to maintain separate and isolated environments within each chamber, allowing for different treatment to the neuronal cell bodies. This criteria also strives to minimize the diffusion and mixing of media between chambers. Imaging ability and cell recovery evaluates the ease of removing the neuronal aggregates from the device with minimal structural damage to the aggregates. Furthermore, the aggregates must be able to be imaged, whether it is through transferring them out of the device or through imaging them while still in the device. The ease of use criteria measures the accessibility of each chamber within the device via pipette, as well as the ease of placing the ECM filled channels, the initial placing of the cells, and the continuous changing of media. The ease of fabrication and reproducibility section evaluates the assembly of the design and its complexity, as it should be reproducible for further experimentation. Lastly, the cost criteria judges the affordability of the materials needed to fabricate the designs.

c. Proposed Final Design

Based on the criteria described above, the 384-well plate has ranked the highest in majority of the criteria, thus it is our final design choice. The design ranked the highest for aggregate stability/positioning because when the cells are funneled into the milled ports, the entire well plate is spun forcing the cells to be centrifuged down and attached to the well floor. This prevents the neuronal aggregates from floating into the media. As for media isolation, this design also ranked the highest because the chambers of this device are the wells of the plate itself so media cannot leak through the walls. Also, this design has the

largest distance between media chambers, and the distances can be changed. Additionally, the 384-well plate design has the best imaging ability because the neuronal aggregates can be imaged directly in their place within the chambers. There is no need for removing nor transferring the micro-TENNS, which prevents structural damage and increases the device's ability for cell recovery. The ease of use for this design is also ranked relatively high as no PDMS nor agarose tubing is necessary, and the majority of this design involves the well plate itself and its manipulation. The 384-well plate, however, did not rank the highest for both fabrication/reproducibility and cost. As for ease of fabrication/reproducibility, this design requires precise milling of ports and channels on a very small scale, which can be difficult to reproduce uniformly. Finally, for cost, the 384-well plates need to be bought and ordered, and they can become quite expensive depending on the amount needed. Regardless of these two obstacles, after discussion, this 384-well plate design is the most feasible and will yield the best results for our client's needs.

5. Fabrication

a. Materials

Well plate: A Corning 384-well clear, flat-bottom polystyrene microplate was selected as the base material of the device due to its optical clarity, compatibility with microscopy, and established use for cell culture. The plate is tissue-culture treated to promote cell attachment and is sterile, making it suitable for cell culture applications [20]. The plate will be modified to create three chambers and ECM connections while maintaining compatibility with standard cell culture workflow.

Double-sided biocompatible adhesive: ARcare 90445Q, a thin, double-sided medical-grade adhesive manufactured by Adhesives Research, was selected to bond the modified well plate to the transparent bottom substrate [21]. The adhesive has an approximately 81 μm thickness and has demonstrated favorable bonding strength, cell compatibility, and stability under cell culture conditions [22]. This double sided adhesive will create a seal between the modified well plate and transparent bottom substrate, creating enclosed channels for the ECM and aggregates to occupy. Openings will be laser cut

into the adhesive to align with the chamber and channel geometries, allowing neuronal connections while maintaining separation of the culture media.

Transparent bottom substrate: A thin, transparent substrate will be bonded to the underside of the modified well plate to provide a continuous bottom surface for the neuronal aggregate chambers and ECM channels. Polystyrene film and glass are being considered due to their established use in cell-based microfluidic devices. Previous research has demonstrated the use of polystyrene films in adhesive-bonded cell culture platforms, as well as glass coverslips for neuronal culture and microscopy [23]. The final material will be selected based on optical clarity, cytocompatibility, bonding compatibility, and structural integrity.

b. Methods

CAD Design and Preparation: The device geometry will be developed using Autodesk Fusion to define the neuronal aggregate chambers and connecting ECM channels. The design will incorporate openings through each well for aggregate placement and ECM loading via pipette while maintaining media separation between adjacent media chambers. Detailed CAD drawings will be provided in Appendix B.

Well Plate Modification: A clear polystyrene well plate will be modified using precision CNC milling. The corresponding toolpaths from the CAD file will be generated and converted into G-code. The G-code will then be uploaded to a Tormach 770M CNC Mill to control the cutting process. The well plate will then be secured into the milling machine to cut out the desired openings and channels from the CAD file. Following machining, the plate will be cleaned to remove residual polystyrene debris and inspected to verify the dimensions and quality of the milled features.

Adhesive Preparation: ARcare 90445Q double-sided adhesive film will be cut to match the underside geometry of the well plate. Openings will be precisely laser cut into the adhesive to align with neuronal aggregate chambers and ECM channels.

Transparent Substrate Preparation: A transparent bottom substrate will be prepared to match the bonding area of the modified well plate. The substrate will be cleaned to remove contaminants and inspected for scratches or surface defects that could interfere with cell adhesion and imaging capabilities.

Surface Treatment: Prior to device assembly, the modified well plate and transparent bottom substrate will undergo oxygen plasma treatment to modify their surface properties. Plasma treatment will be used to promote surface hydrophilicity, improving interactions between the device's surface and cell culture components [24].

Device Assembly: The patterned adhesive film will be aligned with the modified well plate and applied using care and uniform pressure to establish adhesion. The transparent bottom substrate will then be positioned beneath the adhesive and bonded to form a continuous bottom surface. The assembled device will then be inspected for proper alignment or visible gaps that could compromise functionality.

UV Sterilization: Following fabrication and assembly, the completed device will be exposed to UV light for 15 minutes prior to cell culture to reduce the risk of microbial contamination [25]. The device will then be maintained under sterile conditions until use.

6. Testing and Results

Media Isolation: The design's ability to separate media and provide individual treatments to separate neuronal aggregates will be tested through two methods; a physical separation test and a fluorescent tracer test. Both tests can be completed on devices containing ECM and devices without ECM to determine if media isolation is achieved by inhibiting bulk flow or diffusion.

In the physical isolation test, food dye of a known volume will be added to one of the wells. The movement of the food dye through the channel will be characterized by distance over time if visible by eye or using a microscope. If not visible, the movement of food dye through the channel will be characterized by change in volume over time.

In the fluorescent tracer test, a water soluble fluorescent tracer of known concentration will be added to one of the wells. The remaining wells of the device will be filled with tracer free media. Fluorescence will be measured over time in the tracer-free wells and concentration will be calculated.

ECM Loading: Loading of the ECM will be tested qualitatively. ECM will be pipetted through the specified ports allowed to flow across the channel and polymerize. A successful design will contain ECM that polymerizes across the entire channel without gaps or air bubbles.

Aggregate Positioning: Aggregate positioning will be tested qualitatively. Aggregates will be centrifuged directly into the well bottom using an inverted pyramid construct. A successful design will contain aggregates that sit securely at the bottom of their well without floating into the media above.

Cell Viability/Cytotoxicity: To demonstrate the ability of the device to support cell growth, cell viability testing will be conducted. Neuronal and glial cells will be seeded along with a cell tracker at a density of the clients choosing in the ECM. After polymerization, media will be added to all wells. Cultures will be maintained in the incubator for 14 days, with viability measured at 0, 7, and 14 days. A viability of 85% will be considered sufficient [26].

Neurite Growth through ECM: The growth of the neurites from the aggregates into the channel lumen will be measured in distance over time. A successful device will allow for neurite connection within fourteen days. Assuming bidirectional growth, that is a rate of 0.482 mm/day using the geometry of the proposed final design.

7. Discussion

Potential sources of error arise during device fabrication and loading. During device fabrication, the well plate will be milled on a 770M Tormach CNC mill with a great positional accuracy ($\leq 1.09 \mu\text{m/cm}$) [27]. The largest source of error will arise in the placement of the double sided adhesive as this is performed by hand and must align perfectly with the geometry of the milled channels. Sources of error may also occur during ECM and aggregate loading as improper technique can lead to air bubbles within the device, impeding the ability of aggregates to grow toward one another.

A key ethical consideration of this project is the environmental impact of the device. As with the majority of research materials, this device is single use because reuse of the device introduces uncertainties in sterility, biocompatibility, and mechanical integrity. Research waste, including cell culture devices such as well plates, is usually autoclaved and disposed of, ending up in landfills. In total, bioscience research is responsible for almost 2% of global plastic waste [28]. This environmental impact must be taken into account when considering the eventual use of the product. Since the product is a small-scale device used by a singular research laboratory, the overall environmental impact is much less when compared to commercialized research devices. With that being said, steps will be taken to optimize the use of the device and ensure a maximum functional unit ratio.

Another environmental cost associated with the device is the process or sterilization. The device must be sterilized before use to reduce the risk of contamination by microorganisms. Environmental impacts and hazards vary by sterilization technique, and the two most applicable for this project are steam autoclave sterilization and UV sterilization. Neither produce toxic chemical byproducts, however steam sterilization uses large amounts of energy and water, creating notably higher impacts on the environment [29]. For these reasons, among practical engineering reasons, UV sterilization will be used for this product.

8. *Conclusions*

This project aims to meet the need for a physiologically relevant culture platform for study of the neuronal auditory pathway. Current models, including well plates and petri dishes, are insufficient to model the complex geometries and microtissue environments of the central nervous system. The proposed device solves this problem through the modification of commercially available 384-well plates. In milling these plates, a specified 3D culture geometry is created where addition of ECM allows for connection of neuronal aggregates and simultaneous distinct chemical treatment of said aggregates. The device will be used to study how chemical treatment of one aggregate affects its cellular activities, growth, and interaction with other adjacent aggregates, modeling the real cellular responses of the central nervous system.

Moving forward, fabrication of the milled 384-well plate will begin first with modeling in Autodesk Fusion CAD software. Next, the well plate will be milled on a CNC mill, and assembled with its other components to create a working device. The device will undergo various testing procedures to verify that it is supportive to cell growth, and that sufficient media isolation is achieved. Lastly, modifications will be made in response to the results discovered during testing.

Ultimately, as the scientific community continues to realize the necessity for creation of physiologically relevant research tools, this device will serve among the growing number of microtissue modeling platforms used in breakthrough biological research. With continued engineering and testing, devices like these have the potential to uncover many unexplored aspects of cellular communication, tissue development, and disease progression. Through these discoveries, this technology will pave the way for more productive and impactful biomedical research.

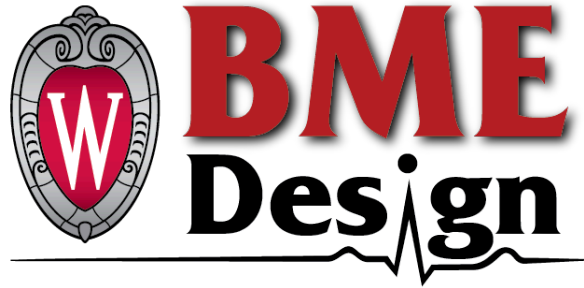
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Appendix A. PDS



Multi-chamber culture insert for connected neuronal aggregates

Product Design Specification

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Function

Common devices used in biological research, including well plates and petri dishes, are readily accessible, provide easy platforms for addition of cells, media, and reagents, and allow for observation using standard laboratory microscopes. However, they fail in their ability to model the complex physiological environments of the human body. In recent decades, there has been a large push toward the development of microphysiological systems used for research, for example, the Organ-on-a-Chip platforms [1]. The ability to model complex tissue environments is incredibly important for the study of the auditory pathway because of its dynamic environment. 2D systems completely isolate cells from the complex physical forces, specialized microenvironments, and 3D neural networks that are vital to the transduction of sound to the brain [2]. The multi-chamber culture device provides a more dynamic platform to study the growth and interactions of neuronal cell bodies on a 3D platform. The device allows for growth and interaction of neuronal cell aggregates in a structured manner. The device separates neuronal aggregates into distinct chambers each holding their own culture media. The device allows for growth of neurons between chambers, but separates the media in each chamber, allowing for independent treatments of aggregates.

Client requirements

- The device must create three to four distinct chambers.
- The device must keep the media separate between chambers.
- The device must accommodate extracellular matrix (ECM) filled channels connecting the neuronal aggregates in each chamber.

Design requirements

1. Physical and Operational Characteristics

a. Performance requirements

- i. The device must create three to four distinct chambers.
- ii. The device must keep the media separate between chambers with no measurable transfer, while maintaining ample media exposure to individual aggregates.
- iii. The device must accommodate ECM filled channels connecting the neuronal aggregates in each chamber.
- iv. The chambers must be accessible by pipette.
- v. The device must allow for post-treatment imaging of cells; the device must either be transparent or easily disassembled to allow for imaging. Disassembling of device cannot damage culture structures.

b. Safety

- i. The device itself does not pose any inherent danger to others; however, the most caution should be taken whilst fabricating and loading the device.
- ii. Proper PPE must be worn while working with cells, reagents, and media.
- iii. ECM must avoid extreme temperatures, direct sunlight, and strong oxidizing agents, as they are incompatible [3].
- iv. Cell culture and ECM must be handled in a biosafety cabinet, as proper ventilation is required [4].
- v. Agarose, a polymer that forms into a gel used for neuronal aggregate channels, is generally non-toxic. Regardless, proper PPE is needed, and caution should be taken when using high heat with it [5].

c. Accuracy and Reliability

- i. Neuronal aggregates must be linked together through ECM filled channels, but the medium within each chamber must stay separate.
- ii. Neuronal aggregates must not float. They must sit at the bottom of the well plate, or no axons enter the lumen.
 1. Axons must grow to at least 50-70% within the first 7 days of culture.
- iii. Aggregates must be within 200 μm of the target through each feed in 90% of chambers.
- iv. At least 90% of constructs must be recovered, have their tract intact, and the chamber from which they originated must be known.
- v. The device must have evaporation control so as to not lose medium over time, and for successful cell cultures.
- vi. Viability of aggregates must be equal or better than a free-floating control.

d. Life in Service

- i. The device and neuronal aggregates must be able to survive weeks in a sterile environment at 37°C, containing 5% carbon dioxide.
- ii. ECM remains stable for a minimum of three months from its date of shipment, when stored at -70°C [3].
- iii. Agarose gel columns must remain moist, and are stable at room temp.
- iv. The device will be single-use.

e. Shelf Life

- i. The device must last for up to 3-4 weeks in order to fully carry out scientific experiments and maintain continuous cell culture of neuronal aggregates [6].

f. Operating Environment

- i. The device must maintain functionality at the standard cell culture conditions, which is approximately 37°C in a humidified 5% CO₂ incubator [6].
- ii. No cytotoxic material must make contact with the neuronal cells being cultured.

g. Ergonomics

- i. The device must allow pipette access into every ECM loading channel.
- ii. The culture chambers must be accessible in order to allow for cell feeding which can occur daily to every other day.
- iii. The device should be manually manipulated using standard laboratory tools as required without damaging the device itself and the neuronal aggregates [7].

h. Size

- i. If the device is an insert, it must fit inside a well of diameter 22.09 mm [8].
- ii. The device must have a culture chamber wall height such that each chamber can contain 50 to 250 µL of media.
- iii. The ECM filled channels must have a lumen diameter between 100 and 300 µm.
- iv. The ECM filled channels must have an outer diameter of 400 µm.

i. Weight

- i. The top of the device must have a weight less than 15 g to minimize compressive loading on neuronal aggregates and channels.

j. Materials

- i. The material must not be cytotoxic, with cell viability greater than 70% relative to the control in accordance with ISO 10993-5 compatible in vitro cytotoxicity assay [9].
- ii. The material must withstand UV sterilization.
- iii. Material within the optical path must be optically transparent at wavelengths required for imaging, or the device must allow for disassembling so that the cells can be imaged.
 1. Optical transmittance should be evaluated in accordance with ASTM E1348-22 [10].
- iv. The material must maintain dimensional and chemical integrity for the entire culture period at 37°C and 5% CO₂.

k. Aesthetics, Appearance, and Finish

- i. The cell culture surface of the device must be smooth and hydrophilic.

2. Production Characteristics

a. Quantity

- i. If the device is an insert, only one functional master mold will need to be fabricated so that an insert can be cast.
- ii. If the device is an insert, multiple inserts can be cast to accommodate multiple well-plates during concurrent studies.
- iii. If the device is milled, one CAD file for CNC milling will need to be generated.

b. Target Product Cost

- i. No specific budget was provided by the client.
- ii. Related designs cite polydimethylsiloxane (PDMS), a common material used in cell culture related devices, costs to be under 2-3 US dollars per cast [11] [12]. Costs of the master mold can range from 5-20 USD, depending on material [13].

3. Miscellaneous

a. Standards and Specifications

- i. The device must adhere to the dimensions listed in Standards ANSI/SLAS 1-2004 through ANSI/SLAS 4-2004 for 12-well microplates, specifically focusing on well depth and area [14].
- ii. The device must withstand the mammalian tissue culturing conditions listed above as well as be compatible with device materials. Assay guidelines can be found in ASTM E3231-19 [15].
- iii. If PDMS is used, contact-related cytotoxicity must be evaluated using cell viability tests outlined in ISO 10993-5 and ASTM F813-20, specifically sections 12 and 13 [9] [16].
- iv. Cell culture will be conducted in a Biosafety Level II laboratory in accordance with the rules and regulations outlined by the Centers for Disease Control and Prevention and the National Institute of Health [17].

b. Customer

- i. The customer intends for this device to aid in studying different areas of the auditory nerve system and the interactions between auditory nerves.
- ii. The fabrication of this product should be easily scaled to accommodate the need for experimental replications.

c. Patient-related concerns

- i. The device will be a research tool so there are no patient-related concerns.

d. Competition

- i. There are multiple microfluidic culture platforms in use in other labs studying neuronal interactions [18].

- ii. eNUVIO's OMEGA96-ACE Triple-Chamber Neuronal Co-Culture Microplate allows for the study of *in vitro* co-cultures and neuronal propagation [19].
- iii. Patent US11939560B2 describes a microfluidic culture platform that separates fluids between interconnected departments allowing researchers to selectively treat specific sections of the neural network for study [20].

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Appendix B. Autodesk Fusion Drawings

