Appendix A

EXPERIMENTAL SECTION

1. Isolation and Culture of Rat Cortical Astrocytes

Mixed cortical cell isolation for astrocyte culture was performed using postnatal P1 Wistar pups.

Before starting the dissection, 30 ml of astrocyte culture media (DMEM with high glucose, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin) was prewarmed to 37°C. One T75 flask was coated with 20 ml of poly-D-lysine (PDL) solution at a concentration of 50 µg/ml in cell culture-grade water and incubated for 1 hour at 37°C in a CO_2 incubator.

Following the guidelines outlined by the Committee for Control & Supervision of Experiments on Animals (CPCSEA), New Delhi, India, the animal was sacrificed by decapitation. The cranium was cut carefully from the neck to the snout, with two additional cuts: one anterior to the olfactory bulbs and one below the cerebellum to free the brain. Using flat-tip forceps to gently lift the cranial flaps, the brain was removed and placed in an HBSS-filled dish on ice. Under a stereomicroscope, the olfactory bulbs and cerebellum were removed using fine forceps.

To retrieve the cortices, the posterior end of the brain was held with fine forceps, and a midline incision was made between the hemispheres. A second set of forceps was inserted into the groove to peel the cortex away from the brain. The meninges were carefully dissected from the cortex hemispheres with fine forceps to prevent contamination by meningeal cells and fibroblasts. The prepared cortex hemispheres were transferred to a second dish with HBSS and returned to ice. Finally, each cortical hemisphere was cut into small pieces using sharp blades (approximately 4 to 8 times) and transferred to a 50ml falcon tube. Volume make-up was done with HBSS.

Tissue was incubated with 2.5 ml of 2.5% trypsin at 37°C in a water bath for 30 minutes, with occasional mixing every 10 minutes. It was then centrifuged at 300 × g for 5 minutes to pellet the tissue. The supernatant was carefully decanted, and the tissue pellet was dissociated into a single-cell suspension by adding 10 ml of astrocyte plating medium and pipetting 20–30 times with a 10 ml plastic pipette until fully dissociated. The volume was adjusted to 20 ml with an astrocyte plating medium. Dissociation into single cells was confirmed using a cell counter, yielding around 1×10^6 cells. The PDL was aspirated from the T75 flask, and the cell suspension was plated and incubated at 37°C in a CO_2 incubator[1].

The medium was changed 2 days after the plating of the mixed cortical cells and all 3 days thereafter. After 5 days of culture, the astrocyte culture was trypsinized and used for co-culture experiments.

2. Co-culture of Primary Neurons and Astrocytes inside the microfluidic device

We developed a microfluidic setup to culture neurons and astrocytes in a vertical orientation inspired by the work of Mingjian Shi and colleagues[2].

Microfluidic Devices were UV-sterilized and both the PDMS components and the glass surfaces of the µFD were coated with 20 µg/ml poly-L-ornithine and 5 µg/ml laminin for 12 hours at 37°Cto enhance cell adhesion. The excess coating was washed with deionized water for 2 hours, and the chambers were equilibrated with Minimum Essential Medium (MEM) containing 10% horse serum and 0.6% glucose. Astrocytes, isolated from P1 rat pups, were loaded at 25,000 cells per device). Devices were inverted for 2 hours to facilitate astrocyte attachment to chamber roofs, then incubated upright for 4–5 days until confluence. Media were replaced every 36 hours.

Primary neurons, isolated from P_0 Wistar rat pups, were loaded into the device at 25,000 cells per device and incubated for 3 hours at 37°C. Attached neurons were maintained in B27-supplemented Neurobasal™ media, conditioned by the confluent astrocyte monolayer. Media were replenished every 36 hours, and devices were kept at 37°C with 5% CO_2. The astrocyte and neurons were separated vertically by approximately 100 µm. After the incubation period, the cells were immunostained using GFAP (glial fibrillary acidic protein) and TUJ1 (beta III tubulin) antibodies to confirm the successful establishment of the co-culture model.

3. Immunocytochemistry and Fluorescence Imaging

Immunocytochemistry was performed to identify neuronal markers on neurospheres, primary neurons in monolayers, and SH-SY5Y-derived neurons. The cells or neurospheres were fixed with 4% formaldehyde for 1 hour and washed with PBS. Permeabilization was carried out using 0.2% Triton-X, followed by staining with primary antibodies, incubated overnight at 4°C. The primary antibodies used included anti-Tubulin beta III isoform (clone TU-20) and anti-GFAP. After primary antibody incubation, PBS washes were performed.

For double staining, a second primary antibody was applied, while single staining involved incubation with mouse or rabbit fluorochrome-labelled secondary antibodies for 2 hours at 37°C. After additional PBS washes, samples were mounted on clean glass slides for microscopy. Microscopic images were captured using an Olympus IX83 microscope equipped with an Andor iXon3 897 EMCCD camera, and images were processed using ImageJ software[3].

4. Mass Spectrometry and HPLC analysis of molecules

Mass spectra were acquired using an Agilent Technologies electrospray ionization (ESI) Q-TOF mass spectrometer (G6530C). High-performance liquid chromatography (HPLC) analysis was performed with a Shimadzu C18 column (150 × 4.6 mm, 5 µm particle size). The mobile phase comprised a gradient of 40–70% acetonitrile in an aqueous solution, with detection at 254 nm. The flow rate was maintained at 1 mL/min, and the column temperature was set to 30°C. Pressure ranged from 85 to 142 kgf. HPLC analysis confirmed that the purity of all compounds exceeded 95% [4].

5. Molecular Docking Studies

To find out the binding mode of the TDSB molecule, an AChE docking study was performed. We took the crystal structure of AChE (PDB ID: 2CKM). The PDB file was downloaded from the RCSB Protein Data Bank. All the water molecules and non-protein atoms were removed

from the protein for the docking study. The docking study was carried out in Autodock-Vina (software version 1.1.2).

For the docking study between the Aβ42 peptide and TDSB, blind docking was carried out in Autodock-Vina software (version 1.1.2).21 The receptor Aβ42 peptide was (PDB ID: 1IYT).

6. Cell viability Assay

In a 96-well plate, differentiated SH-SY5Y cells were treated with different concentrations of TDSB (Alzheimer's disease model) and $FeCl_3$, SG-B-24 (Ferroptosis model) (3.125, 6.25, 12.5, 25, and 50 µM). After 24 h of treatment, 50 µL of 5 mg/mL MTT dye was added. The cells were incubated for 4 h, and then, the accumulated formazan was dissolved using a solution of MeOH: DMSO (1:1). The absorbance of formazan was recorded using a multimode microplate reader at 570 nm[5].

7. Live/Dead Assay

SH-SY5Y cells were cultured and differentiated in our Xurography-based microfluidic devices at low and high seeding densities until they reached confluency. Cell viability was evaluated by staining cells with Calcein-AM/propidium iodide (PI). The cells were incubated with 5µM of calcein AM and 1 µM PI solutions (dissolved in colourless DMEM media) for 30 min at 37 °C and 5% CO2. The media was then discarded, and after one PBS wash, the cells were visualized under the inverted fluorescence microscope (IX83, Olympus) in an appropriate fluorescent channel.

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Appendix B

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CERTIFICATE

OF PARTICIPATION

This certificate is presented to

Subhadra Nandi

for participating in the International Workshop on Microfluidics
held at Shiv Nadar University, Greater Noida, during 24 – 28 February 2020

Dr. V. M. Rajesh
Convener

Dr. Susanta Roy
Co-Convener

Prof. Rupamanjari Ghosh
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Unveiling the Human Brain on a Chip: An Odyssey to Reconstitute Neuronal Ensembles and Explore Plausible Applications in Neuroscience

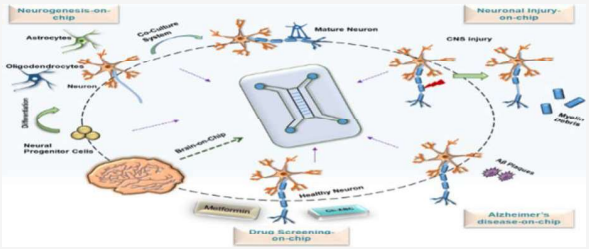
Subhadra Nandi,[#] Satyajit Ghosh,[#] Shubham Garg, and Surajit Ghosh*

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ABSTRACT: The brain is an incredibly complex structure that consists of millions of neural networks. In developmental and cellular neuroscience, probing the highly complex dynamics of the brain remains a challenge. Furthermore, deciphering how several cues can influence neuronal growth and its interactions with different brain cell types (such as astrocytes and microglia) is also a formidable task. Traditional *in vitro* macroscopic cell culture techniques offer simple and straightforward methods. However, they often fall short of providing insights into the complex phenomena of neuronal network formation and the relevant microenvironments. To circumvent the drawbacks of conventional cell culture methods, recent advancements in the development of microfluidic device-based microplatforms have emerged as promising alternatives. Microfluidic devices enable precise spatiotemporal control over compartmentalized cell cultures. This feature facilitates researchers in reconstituting the intricacies of the neuronal cytoarchitecture within a regulated environment. Therefore, in this review, we focus primarily on modeling neuronal development in a microfluidic device and the various strategies that researchers have adopted to mimic neurogenesis on a chip. Additionally, we have presented an overview of the application of brain-on-chip models for the recapitulation of the blood-brain barrier and neurodegenerative diseases, followed by subsequent high-throughput drug screening. These lab-on-a-chip technologies have tremendous potential to mimic the brain on a chip, providing valuable insights into fundamental brain processes. The brain-on-chip models will also serve as innovative platforms for developing novel neurotherapeutics to address several neurological disorders.



KEYWORDS: *Neuronal development, Microfluidic device, Neurodegenerative diseases, Drug Screening, Brain-on-chip, Neurogenesis-on-chip*

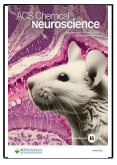
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1. INTRODUCTION

Development of the human brain involves a highly complex chain of dynamic self-organization and reorganization processes operating within a highly constrained, genetically organized but constantly fluctuating environment.[1] Brain development initiates from neuroblasts (produced by neural stem cells in the subventricular zone)[2] that migrate and terminally differentiate into the neurons and glia, the permanent cellular components of the brain.[3] This migratory phase is genetically programmed, during which neuroblasts encounter an array of distinct, yet highly dynamic micro-environments. This is followed by the development of budding neural networks to allow the communication of signals that arise as a result of the neural polarity and synaptic specializations. It gives rise to numerous functions such as integrated network communication, and regulation of metabolic, physiological, and cognitive functions, to unveil the physical and chemical signals that promote the development of individual neurons and guide the formation of axons, dendrites, as well as functional synapses.[4] This will eventually offer an enhanced perception of neural network development and the complex functions that take place within the brain.

Investigations at the subcellular level are crucial since the changes that occur during the development process guide the flow of information and its subsequent processing. However, the complex cytoarchitecture of neurons and their protrusions that remain densely packed inside the brain hinders this subcellular analysis *in vivo*. Although conventional cell culture methods with well-standardized protocols have been successful in providing tremendous insights into neural development, they often have certain shortcomings too. *In vitro* 2D cell culture methods require high quantities of samples and

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Brain-on-a-Chip

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Subhadra Nandi, Satyajit Ghosh, Shubham Garg, Ankan Sarkar, and Surajit Ghosh

Unravelling the microenvironment of the human brain to investigate the different pathophysiological conditions is a pivotal task. The demarcation of the central nervous system from the peripheral nervous system makes the human brain developmental process a more protracted one. Increasing number of various neurodegenerative diseases causes a major challenge to the management of global public health. Although traditional in vivo animal models and in vitro cell culture models facilitate the evaluation of the structural and functional aspects of the brain, they have certain limitations too. This calls for an innovative approach in the shape of Microfluidics Technology that can offer a single platform to culture neurons in a quasi-physiological confined microenvironment, to reconstitute neural framework under spatiotemporal regulation and external stimulation for modelling of neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and more followed by high-throughput analysis of effective drug delivery systems. In this chapter, we have highlighted the various structural and functional aspects of the human brain by the reconstitution of the central nervous system and the peripheral nervous system on chip. We have thereby highlighted the neurological disorders that are associated with the malfunctioning of both the nervous systems succeeded by high-throughput analysis of drugs on chip that have been screened till date. Therefore, these lab-on-chip technologies hold promises for mimicking the complex brain microenvironment imparting novel platform for disease modelling and drug screening.

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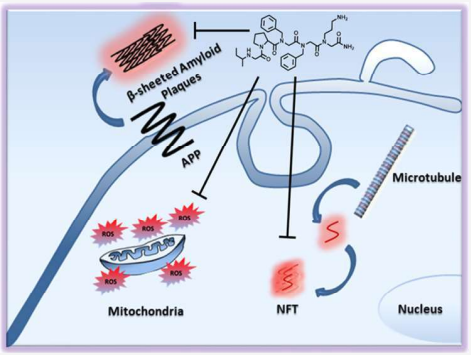
Short Peptoid Evolved from the Key Hydrophobic Stretch of Amyloid-β42 Peptide Serves as a Potent Therapeutic Lead of Alzheimer's Disease

Rajsekhar Roy,[#] Juhee Khan,[#] Krishnangsu Pradhan,[#] Prasunpriya Nayak, Ankan Sarkar, Subhadra Nandi, Surojit Ghosh, Heera Ram, and Surajit Ghosh*

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ABSTRACT: Amyloid-β 42(Aβ42), an enzymatically cleaved (1−42 amino acid long) toxic peptide remnant, has long been reported to play the key role in Alzheimer's disease (AD). Aβ42 also plays the key role in the onset of other AD-related factors including hyperphosphorylation of tau protein that forms intracellular neurofibrillary tangles, imbalances in the function of the neurotransmitter acetylcholine, and even generation of reactive oxygen species (ROS), disrupting the cytoskeleton and homeostasis of the cell. To address these issues, researchers have tried to construct several strategies to target multiple aspects of the disease but failed to produce any clinically successful therapeutic molecules. In this article, we report a new peptoid called RA-1 that was designed and constructed from the hydrophobic stretch of the Aβ42 peptide, ^{16}KLVFFA^{21}. This hydrophobic stretch is primarily responsible for the Aβ42 peptide aggregation. Experimental study showed that the RA-1 peptoid is stable under proteolytic conditions, can stabilize the microtubule, and can inhibit the formation of toxic Aβ42 aggregates by attenuating hydrophobic interactions between Aβ42 monomers. Furthermore, results from various intracellular assays showed that RA-1 inhibits Aβ42 fibril formation caused by the imbalance in AchE activity, reduces the production of cytotoxic reactive oxygen species (ROS), and promotes neurite outgrowth even in the toxic environment. Remarkably, we have also demonstrated that our peptoid has significant ability to improve the cognitive ability and memory impairment in *in vivo* rats exposed to $AlCl_3$ and D-galactose (D-gal) dementia model. These findings are also validated with histological studies. Overall, our newly developed peptoid emerges as a multimodal potent therapeutic lead molecule against AD.



KEYWORDS: *peptoid, Aβ42 aggregation, Alzheimer's disease (AD), AChE inhibition, neuroprotection, primary cortical neurons, ROS, microtubule stabilization*

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1. INTRODUCTION

Toxic aggregation of amyloid-β 42 (Aβ42) peptide in the extracellular region is considered the most important hallmark of Alzheimer's disease (AD).[1] Senile plaques formed of Aβ42 peptide deposited in the synaptic region cause impairment in the synaptic plasticity and finally cause neuronal death.[2] Previously, the nucleation of this Aβ42 peptide monomer only to the final fibrillary aggregates was thought to be the main reason, but recently, oligomers were found to be much more toxic. The propagation of aggregation of the monomer was thought to be initiated through the hydrophobic core, i.e., ^{16}KLVFFA^{21}.[3] Hence, several strategies were developed that revolved around blocking the interaction of hydrophobic cores of Aβ42 monomers. In this regard, short sequences of peptides called β-sheet breaker (BSB) peptided that can form direct interactions with the hydrophobic core and disrupt the aggregation at an early stage were synthesized. Soto and colleagues in their work took a pioneering approach to modulate a β-sheet breaker peptide by modifying the sequence ^{17}LVFFA^{21} and generating LPFFD, a short 5-amino acid peptide that can even disassociate preformed Aβ aggregates.[4] Furthermore, Chalifour et al. replaced the L-amino acids with their D-enantiomers and showed enhancement in the stability and also increased effectiveness against the aggregation.[5] Extrapolating this work, Chacón et al. modified LPFFD by adding acetyl protection to the N-terminal and amine protection to the C-terminal end.[6] By doing this, they increased the serum stability and blood−brain barrier permeability that enhanced the effect in *in vivo* mice models.

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Targeting Chondroitin Sulfate Proteoglycans: An Emerging Therapeutic Strategy to Treat CNS Injury

Nabanita Mukherjee, Subhadra Nandi, Shubham Garg, Satyajit Ghosh, Surojit Ghosh, Ramkamal Samat, and Surajit Ghosh*

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ABSTRACT: Chondroitin sulfate proteoglycans (CSPGs) are the most abundant components of glial scar formed after severe traumatic brain injury as well as spinal cord injury and play a crucial inhibitory role in axonal regeneration by selective contraction of filopodia of the growth cone of sprouting neurites. Healing of central nervous system (CNS) injury requires degradation of the glycosamine glycan backbone of CSPGs in order to reduce the inhibitory effect of the CSPG layer. The key focus of this Viewpoint is to address a few important regenerative approaches useful for overcoming the inhibitory barrier caused by chondroitin sulfate proteoglycans.

KEYWORDS: *Central nervous system injury, axonal regeneration, glial scar, chondroitin sulfate proteoglycan, heparan sulfate proteoglycan, chondroitinase ABC enzyme*

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Central nervous system (CNS) injury results in the formation of severe glial scar circumnavigating the zone of injury. Glial scar is composed of highly proliferating and migrating glial cells, and the majority of them are reactive astrocytes along with microglia and oligodendrocyte precursors. The formation of glial scar reduces the chance of spreading inflammatory responses throughout the brain by precluding the injured zone from other parts. Along with these glial cells, upregulated expression of other extracellular matrix (ECM) molecules like GAG (glycosaminoglycan) proteoglycans, e.g., chondroitin sulfate proteoglycans (CSPGs), heparan sulfate proteoglycans (HSPGs), dermatan sulfate proteoglycans (DSPGs), and keratin sulfate proteoglycans (KSPGs) is also well documented. Depending upon the native protein chain structure, types of polysaccharide units, and number of sulfate substitutions, these proteoglycans have various developmental and regulatory roles in the central nervous system. Glial scar composed of these proteoglycans creates a thick protective sheet surrounding the injury site in order to seal the disrupted blood-brain barrier rapidly, but this apparently protective glial scar and its ECM associated proteoglycans, mainly CSPGs, are some of the major inhibitory factors which lead to dilapidated axon regeneration of severed neurons. Thus, treatment of CNS injury becomes complicated due to these two contrasting factors. It has been observed that the disrupted blood-brain barrier remains permeable until after 10−14 days of injury and CSPGs are secreted rapidly (within 24 h) at the site of injury and can persist for many months. Proteoglycan homeostasis plays a crucial role throughout the development of the brain and regulates the growth and direction of newly generated neurons. It has been found that CSPGs are upregulated in embryonic rat brain and impart repulsive guidance to the growing axons contrary to the attractive guidance mediated by HSPGs. The CSPG level gradually decreases throughout the brain development phase and exacerbates only after major CNS injury. These altogether indicate that proper balance of CSPGs and other proteoglycans is the key to neuronal development. Chondroitin sulfate (CSPG) consists of a glycosaminoglycan (GAG) moiety which is basically a disaccharide unit containing glucuronic acid and *N*-acetyl galactosamine. Aggrecan, brevican, phosphacan, and versican all are various members of the CSPG family. These GAG residues are attached to the oxygen atom of the serine residues of the main protein chain via O-xylose linkage to form CSPG proteoglycans. Snow et al.[1] first observed that CSPG proteoglycans specifically inhibit neurite outgrowth of developing neurons in vitro as well as in vivo and its inhibitory effect creates major regeneration barrier after severe TBI and spinal cord injury.[1] Further studies reveal that the glycosaminoglycan residues of chondroitin sulfate proteoglycan interact with several neuronal developments inhibiting receptors like Nogo receptor1 and 3,[2] protein tyrosine phosphatase (RPTP),[3] and leukocyte common antigen-related phosphatase (LAR) and thereby restrain regeneration of axons. In various in vitro experiments, it is concluded that the neurite growth is severely jeopardized on the interface of growth-inducing laminin and aggrecan proteoglycan, resulting in a dystrophic end bulb of the growth cone. CSPGs not only perturb the growth of injured neurons, but they also impart massive barriers in cell transplantation therapy too. CSPGs also

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Three-Dimensional Microfluidic Platform with Neural Organoids: Model System for Unraveling Synapses

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ABSTRACT: Unraveling the large number of various signals in the brain under the influence of physical and chemical cues that govern the formation of individual neurons, axons, dendrites, and their functional synapses during the development of neural network is a challenging task. To understand this task, microfluidic devices equipped with microchannels for reconstitution of cell/tissue-culture environments have been studied. Microfluidic devices are emerging as powerful tools in neurobiology, since they are capable of controlling and manipulating the microenvironment of the brain in a precise manner. They can enhance the physiological relevance of three-dimensional (3D) cell culture by allowing spatial control over fluids in micrometer-sized channels. Recent technological advancement in designing microfluidic platforms for studying neural communication, disease progression, and detection of neurotransmitters enhance our fundamental knowledge and understanding. However, more such advanced and innovative interventions are required. This Viewpoint focuses on highlighting a few of them with future scope of further advancement in this field.

KEYWORDS: *Microfluidic device, neural organoid, synapse, neurotransmitters, 3D cell culture*

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Understanding the function and dynamics of the brain is an exciting field of research due to its highly complex and sophisticated networks, which is still considered to be similar to a locked black box. Unraveling this extremely complex machinery requires innovative strategies, which can mimic the physical environment of the brain as well as offer multimodal efficient structural and functional components to study and reconstitute the miniature of Brain Organoid-On Chip. Early development of the brain originates from neuroblasts that migrate and differentiate into the neurons and glia of brain parts.[1] During this process, neuroblasts encounter an array of complex microenvironments followed by the development of neural polarity and early neural networks for communication of signals.[1] It originates various functions such as integrated network communication and regulation of metabolic, physical, and cognitive functions, which offers a better understanding of complex brain functions. Moreover, it is crucial to understand the mechanistic insights of the formation of individual neurons, axons, dendrites, and their functional synapses during the development of neural networks and a large number of various signals under the influence of physical and chemical cues. To understand this task, microfluidic devices equipped with microchannels for reconstitution of cell-culture environments with channels of micrometer-scale dimensions have been studied. Microfluidic devices are emerging as powerful tools in neurobiology, since they are capable of controlling and manipulating the microenvironment of cells in a precise manner. In this approach, the introduction of engineering technologies for solving biological questions offers the fabrication of microfluidic based systems, where one can study the function of individual brain cells as well as their circuit or network in a stable microculture system in a laboratory. Recently, microfluidic based systems have been explored for understanding the various neurological functions as well as reconstitution of disease models which enriched our understanding and knowledge. Here, we highlight a few recent key developments in this area as follows: Park et al. showcased an interesting model system where they reconstituted a three-dimensional (3D) human triculture system for understanding neurodegeneration and neuroinflammation in Alzheimer's disease (AD) (Figure 1).[2] They created an engineered model

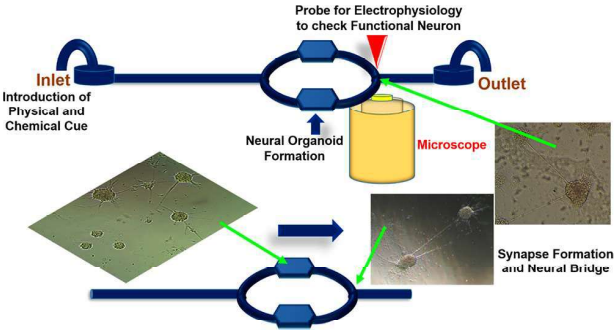


Figure 1. Cartoon represents design of 3D microfluidic platform for culture of neural organoid, real time monitoring of synapse formation and evaluation of functional neuron along with imaging.

system of neuron, astrocyte, and microglia interaction in the AD environment, which allows one to understand the recruitment of human microglia, neuroinflammatory response, and damage to neurons or astrocytes. To accomplish this model, they created a human triculture system, where neurons and astrocytes differentiated first and then adult microglia cells at different stages of AD were added. This fascinating, recently

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