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Exploiting Click-Chemistry and Microfluidics to Map the Neuronal Itinerary of APP Processing and Amyloid-Beta Generation

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By

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Table of Contents

List of figures

Abbreviations

1. Alzheimer's disease- AD
2. Amyloid precursor protein- APP
3. Amyloid beta- Aβ
4. GWAS- Genome wide association studies
5. APP intracellular domain- AICD
6. Endoplasmic reticulum- ER
7. Plasma member- PM
8. Trans-Golgi network- TGN
9. Poly(dimethylsiloaxane)- PDMS
10. Phosphate buffer saline- PBS
11. Minimum Essential Media- MEM
12. Poly-D-lysine- PDL

Acknowledgements

I would like to thank my research advisors, Dr. Jin-Moo Lee for his support and Leah Czerniewski for her guidance throughout this project. Other members of the lab, Kelsey Bria, Qingli Xiao, Karen Smith, Ping Yan and Zachary Rosenthal have always been eager to help me out whenever I am stuck and have been an invaluable source of new ideas and inspiration. Anil Cashikar and Hanson lab for their help with antibodies. Ann Benz and Mennerick lab for their continuous advice and supply of rat hippocampal neurons. Rahul Gupta from IMSE has also played a pivotal role in helping me with fabrication process and Sandra Lam from George Lab for her help with microfluidics. I would also like to thank the members of my thesis committee for taking the time out to review my thesis.

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Abstract

Alzheimer's disease (AD) is a chronic neurodegenerative disease and is the sixth leading cause of death in the United States with approximately 5.5 million Americans diagnosed with it. The neuropathological hallmark includes extracellular senile plaques and intraneuronal neurofibrillary tangles. Recent GWAS studies have identified genes associated with AD, and have revealed several classes of genes implicated in disease pathogenesis. In particular, three general pathways associated with an increased risk of AD included: 1) cholesterol metabolism, innate immune system, and the membrane trafficking. Our lab has focused on intracellular trafficking as it relates to the processing of amyloid precursor protein (APP), the precursor protein for the Aβ peptide—a critical component of the senile plaque. Much is still unknown about the intracellular itinerary of APP, and the cellular location of Aβ production. Here, I describe the use of click-chemistry applied to an APP construct to further study APP processing. In addition, I describe work that I did to develop a microfluidic system to enable visualizing APP processing within the unique cytoarchitecture of primary neurons. Microfluidics helps in isolating the soma and axon to understand APP processing in these specific compartments.

CHAPTER 1

Introduction

Alzheimer's disease is a chronic neurodegenerative disease and is the sixth leading cause of death in the United States with approximately 5.5 million Americans diagnosed with it [1]. The major pathological hallmark for Alzheimer's is accumulation of insoluble deposits of amyloid-beta (Aβ) peptide. GWAS studies for AD showed various factors of which the genes in the intracellular pathway of APP has gained the most attention[2] . This peptide is obtained by the sequential proteolytic cleavage of amyloid precursor protein (APP) [3]. One of the challenges in understanding this disease has been in elucidating the trafficking and processing of Aβ. To understand this problem Aβ has been engineering by click-labelling [4] to visualize the trafficking.

1.1 Proteolytic processing of APP

The amyloid-beta (Aβ) peptide is made up of 37-43 amino acids, obtained by proteolytic cleavage of amyloid precursor protein (APP). APP is a type-I oriented membrane protein with its carboxyl (C) terminal in the cytosol and the amino (N) terminal in the lumen. APP is proteolytically processed at several different subcellular locations and is targeted by three different proteases – alpha (α), beta (β) and gamma (γ) secretases [3].

APP can undergo two processes- the amyloidogenic pathway that leads to the generation of Aβ and the anti-amyloidogenic pathway that prevents the generation of Aβ (figure 1.1)[3]. The amyloidogenic pathway produces Aβ by the consecutive action of β and γ secretase. The β-secretase activity initiates Aβ generation by shedding the ectodomain of APP

(APPsβ) and generates the C-terminal (βCTF) that is cleaved by the γ -secretase. Following the γ-secretase cleavage, Aβ is found in the extracellular fluids like plasma and cerebrospinal fluid [3].

In the anti-amyloidogenic pathway, APP is cleaved nearly in the middle of the Aβ region by α-secretase giving rise to α-CTF, followed by γ-secretase cleavage resulting in the generation of a small 3kDa peptide called p3. Apart from p3 and Aβ, γ-secretase also generates APP intracellular domain (AICD) that is involved in nuclear signaling [3].

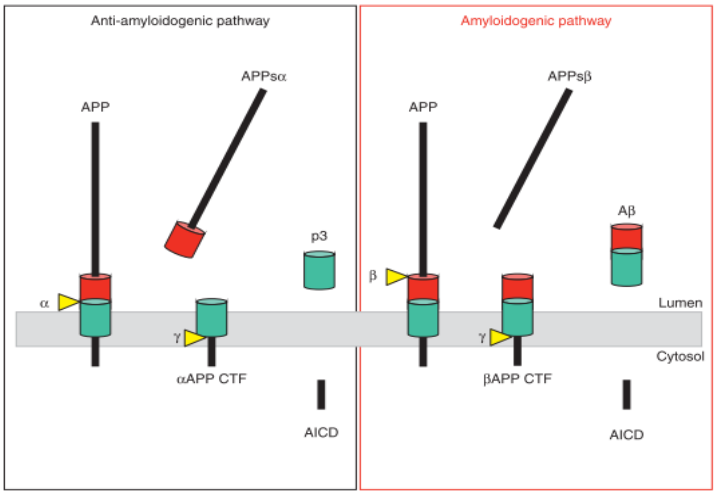

Figure-1.1: Anti-amyloidogenic and amyloidogenic processing of APP

1.2 Cellular trafficking of APP

In non-polarized mammalian cell lines, APP transits from endoplasmic reticulum (ER) to the plasma member (PM). During this transit, APP is post-translationally modified. in the Golgi apparatus and the trans-Golgi network (TGN). Membrane APP that hasn't been shed (due to alpha-secretase cleavage) is internalized due to the presence of the "YENPTY" motif near the C-terminus. Following this, APP is sent to endosomes and a fraction of the

endocytosed molecules is recycled to the cell surface, recycled to the TGN, or trafficked down the lysosomal pathway [3].

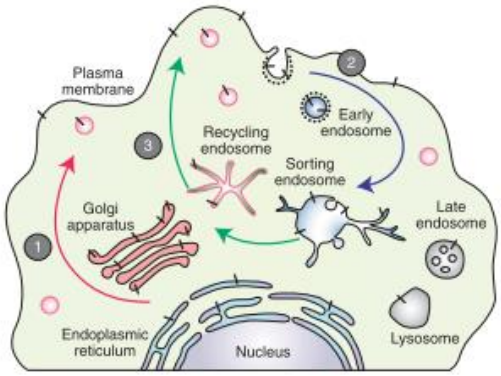

Figure-1.2.a: Cellular trafficking of APP in non-polarized cells.

In neurons, the trafficking is complicated due to the various compartments in the neurons-soma, axon and dendrites. APP trafficking in the neuronal soma is similar to that of the non-neuronal cell, that is, it transports from ER to Golgi and TGN. After leaving the TGN, APP is transported to axons and dendrites in post Golgi transport vesicles. APP delivery to axons makes use of fast axonal transport system but there are several theories with respect to its processing [3].

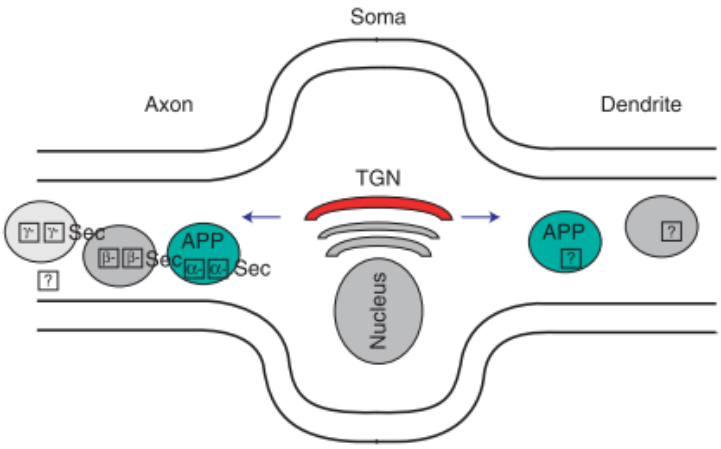

Figure-1.2.b:APP and secretases transport in neurons.

1.3 APP processing in axons

APP processing in neurons is still not understood due to the complex system of compartmentalizing and transport. Two models had been proposed as to the processing of APP in axons. One model postulated by Muresan *et.al.* 2009, states that APP is processed in the soma and the cleaved products gets sorted into axons. This is supported by finding that the endocytic sorting pathway for the β secretase: BACE-1, is limited largely to the soma. But a recent paper by Thinakaran *et.al*.2015 found BACE-1 enriched in axons[5]. Another model proposes local processing in axons/synapses with extracellular APP fragments. This model is supported by the evidence that there is colocalization of processing enzymes together with APP in vesicles and evidence that synaptic activity in vivo increases due to endocytosis-dependent Aβ release in the brain. From these finding it can be deduced that Aβ secretion is dependent on synaptic activity and endocytosis, implicating synapses as the site of processing and secretion [6].

A recent paper by Niederst *et.al* 2015, used compartmentalized culture system to study the processing. A microfluidic system was used to study Aβ generation. The samples from the axonal compartment was collected and Aβ measured. The Aβ fragments were identified using immunoassay. The amount of protein secreted per axon was calculated as by multiplying the volumes from both soma and axons with APP fragment concentration and divided by number of axons per chamber. To estimate the number of molecules secreted per axon, Avogadro's number was multiplied with the fragment concentration. Total axonal concentration was calculated by subtracting the APP amount from the somal chamber. They compared mouse and human hippocampal neurons and found the result to be consistent. They determined that about 70-80% of APP is being processed in the soma[6]. One of the concerns is to understand whether APP fragments are endocytosis- independent or dependent. They found that axonal Aβ generation is dependent on endocytosis sorting and is a major pathway. This was found by using dynasore, a dynamin inhibitor, reduced the amount of axonal APP suggesting that it is endocytic dependent. [6].

So far, the sorting of APP fragments was understood but not for full length-APP (FL-APP). They found that FL-APP can enter the axon in endocytosis independent pathway [6].

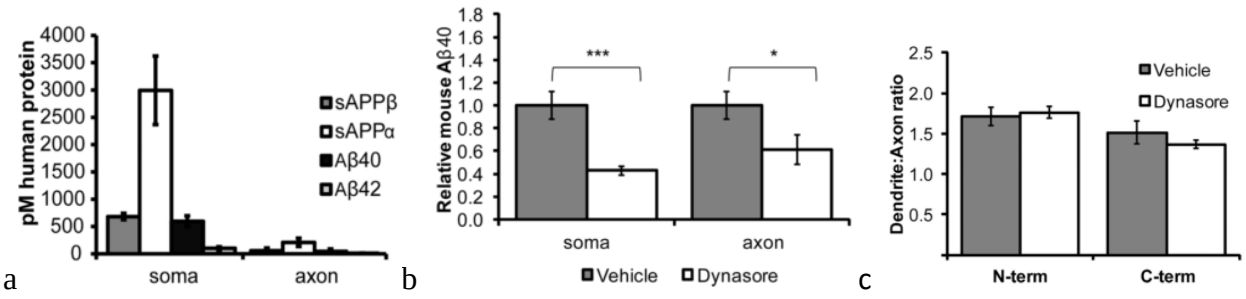

Figure 1.3.a: a) Comparison of APP and it's fragments in soma versus axon

b) Graph indicating endocytosis as major pathway for axonal Aβ generation

c) Graph indicating that FL-APP sorting is endocytosis independent

They also found that there are multiple pathways for axonal sorting of APP, Aβ and BACE1 sorting [6].

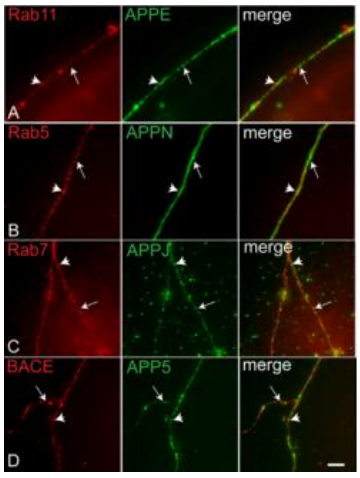

Figure 1.3.b: Colocalization of APP, Aβ and BACE1 with subcellular markers

One of the key questions that remains unanswered is the site of Aβ generation and it's trafficking in the neurons. To understand, Das *et.al* 2013, designed an assay called OptiCAB to find the sites of interaction of APP and BACE1 to better understand the first

cleavage event leading to Aβ generation. As with the previous study, they found soma to be region of maximum interaction of APP and BACE-1. They found that APP and BACE-1 was co-transported in axons and can interact during transit. Immobile APP and BACE-1 particles were found near the dendrites and presynaptic boutons [7].

The result from this paper indicate that APP and BACE-1 interact in both endocytosis and biosynthetic compartments, in recycling endosomes distributed throughout the neuronal processes and in Golgi-derived vesicles in which they are co-transported. It also implicates recycling microdomains (dendritic spines and presynapses) as important sites of APP and BACE-1 convergence. Also show that APP endocytosed from the plasma membrane enters late endosomes or lysosomes [7].

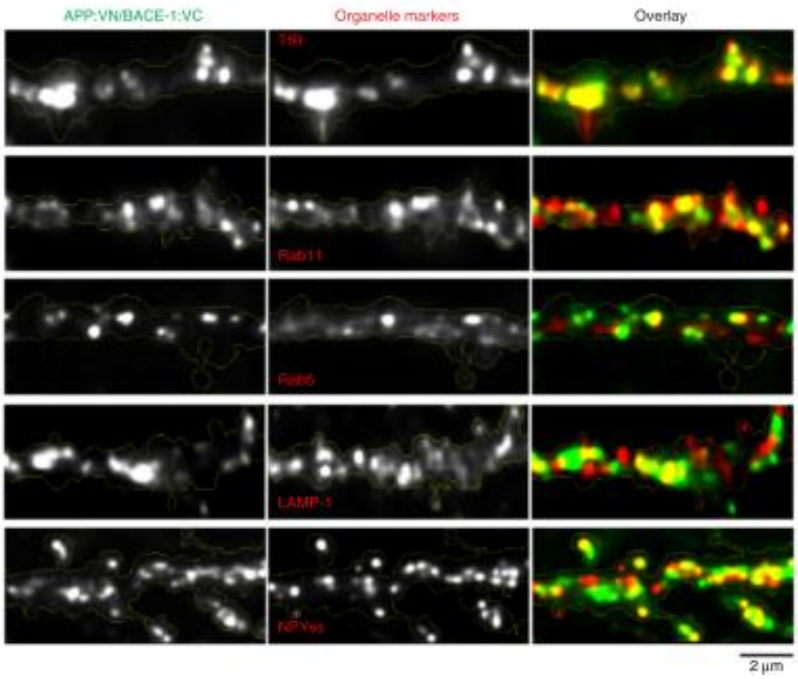

Figure 1.3.c: Colocalization of APP-BACE1 in dendrites with recycling endosomes (TfR & Rab11), early endosomes (Rab5), lysosome (LAMP1), Golgi (NPYss)

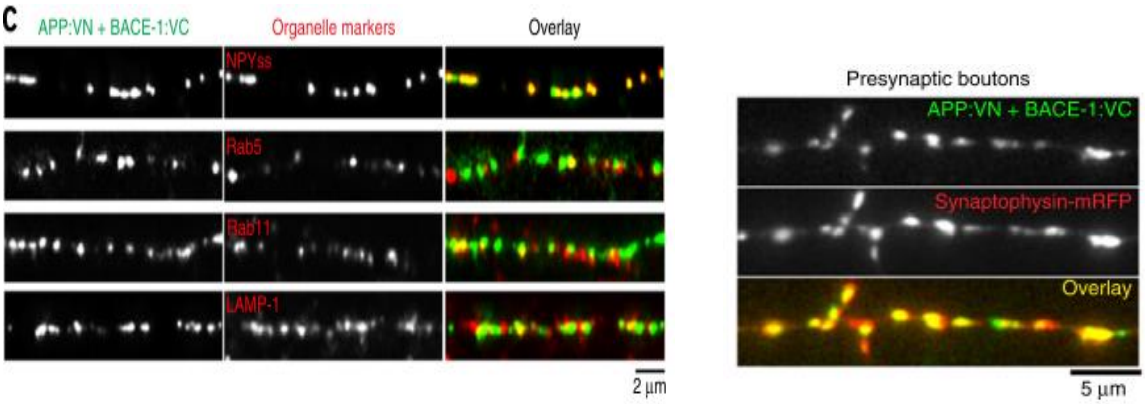

Figure 1.3.d: Colocalization of APP-BACE1 in axons and presynaptic boutons with recycling endosomes (TfR & Rab11), early endosomes (Rab5), lysosome (LAMP1), Golgi (NPYss)

These studies show an indirect relation to the possible subcellular location of Aβ and it's trafficking is still unknown. To address this issue, APP was click-labelled in the Aβ region to help in identifying it's trafficking. This works consists of validation of the clicked-labelled Aβ and fabrication of microfluidic devices to help in neuronal studies.

1.4 Microfluidics

Microfluidics is the science and technology of systems that process and manipulate small amounts of fluids in a geometrically constrained scale. It provides several advantages in analysis as it exploits both it's small scale and the characteristics of fluids in the micro-channels. Some of these advantages include decreased cost of manufacture, use ad disposal, decreased time of analysis, reduced consumption of reagent and analytes, reduced production of potentially harmful byproducts, increased separation efficiency and portability and decreased weight and volume [8]. It has uses in molecular analysis,

biodefense, molecular biology, microelectronics, clinical diagnostics and drug development [9].

Fluid dynamics inside the microfluidics gives a unique advantage to them. The Reynold's number in micro-channels is less than 2000 and flow is laminar. In these conditions gravity is less important, and the surface forces become dominant to influence the flow. The mixing of fluids in this system is only by diffusion. Electro-osmotic flow can be observed for charged fluids [10].

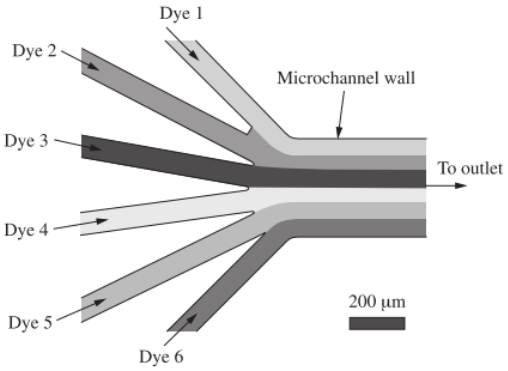

Figure 1.4.a: Laminar flow seen in micro-channels

Most microfluidic systems are carried out in polymers like poly(dimethylsiloxane) or PDMS. The properties of PDMS has various advantages that makes it favorable for microfluidics. Some of these properties include its elastomeric nature, it's optical transparent and can absorb wavelength in 240-1100 nm range, it acts as an insulator, it's non-toxic and biocompatible. It is permeable to gases and compatible with water and polar solvents [10].

The surface chemistry of PDMS can be changed easily to exploit it for our uses. PDMS is hydrophobic, that consists of repeating units of $–O-Si(CH_3)_2$. When it is exposed to oxygen, air, plasma or UV, there is loss of the methyl group to make PDMS hydrophilic. The hydrophilic nature is reversible and to prevent it from reverting back, PDMS should be

kept in contact with polar solvents. Due to it's hydrophobicity, PDMS can be reversibly bond onto glass or irreversible bond after plasma/UV treatment [10].

Microfluidic system made of PDMS are fabricated using soft lithography technique. This includes using rapid prototyping for device design using AutoCAD, followed by replica molding of PDMS [11].

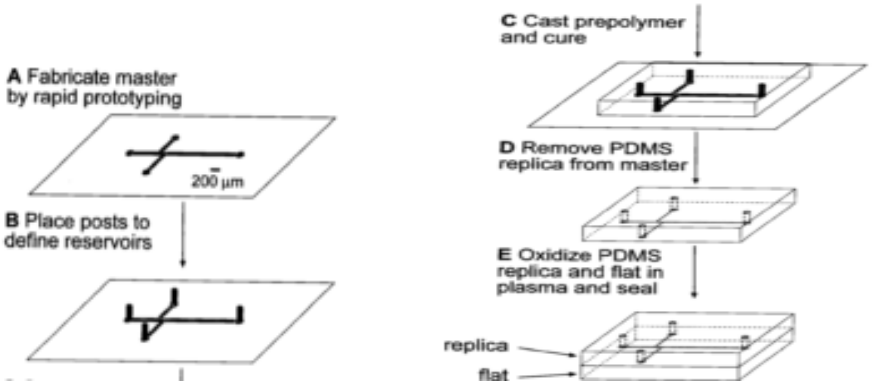

Figure 1.4.b: Fabrication process of microfluidic devices made from PDMS

Due to these various advantages of microfluidic devices made from PDMS, it has several applications for neuroscience. It has been used for neuron cell culture, neuron manipulation, neural stem differentiation, neuropharmacology, neuro electrophysiology and neuron biosensors [12]. In this regard, a multicompartment microfluidic device was designed specifically for neuroscience research. This device is an improvement over the Campenot chambers. The device has been extensively used for the study of Alzheimer's disease. These devices were used to isolate single axons and manipulate different regions of the neurons [13].

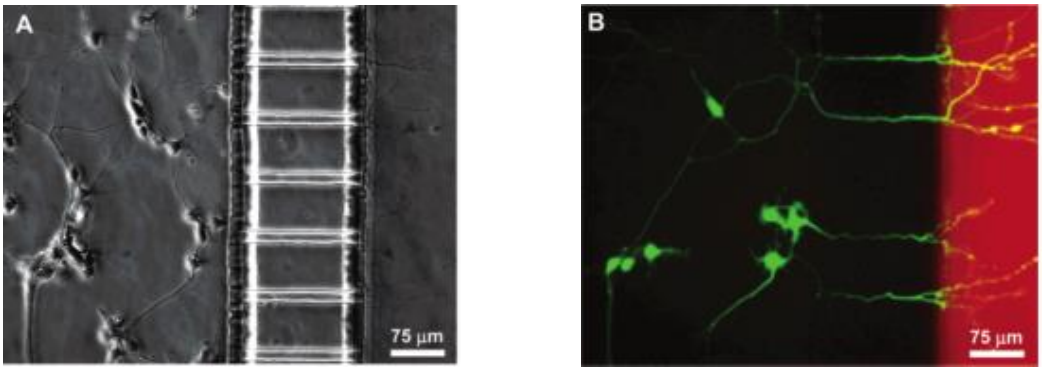

Figure 1.4.c: Images of micro-channels in microfluidic devices cultured with neurons

CHAPTER 2

Validation of click-labelled APP in primary cells

2.1 Experimental procedure

Primary cells- N2a, were used to test the click-labelled APP molecular tool. The cells were transfected with APP-mClover DNA and APP-Amber-mClover DNA. Following transfections, the cells were fixed and stained with antibodies. The cells were then imaged using confocal microscopy and analyzed using Imaris. The analysis was based on colocalization of APP with various subcellular. The total percent of colocalization of click-labelled and mClover tagged APP was compared with wild-type APP-mClover.

2.2 Experimental protocols

2.2.1 Cell passaging and plating-

i. Media is aspirated from the flask and 3ml of PBS is added.
ii. PBS is aspirated and 2ml of 0.05% trypsin is added and incubated for 3 minutes.
iii. 8ml of media is added to stop the trypsin action and the cell count is taken.
iv. The cells are resuspended and 150uL of the cells are added to each well in the quadrant dishes. ~17,000 N2a cells/well is added.

2.2.2 Transfection-

i. Label two tubes per condition, one for DNA and one for lipofectamine
ii. Add opti-MEM to each tube
iii. Add lipofectamine to the lipo tube and incubate for 5 minutes at room temperature.
iv. Add DNA to the DNA tube.
v. Mix DNA with lipofectamine and incubate for 20 minutes at room temperature.
vi. Remove 50ul of media per well in quadrant dishes and add 50uL of DNA-lipofectamine mixture.
vii. Incubate at 37^{o}C for 6-8 hours. Remove transfection reagents.
viii. Add media with unnatural amino acids (CPK+HEPES).
ix. Incubate at 37^{o}C overnight.

2.2.3 Fixing and permeabilizing cells-

i. Media is removed from the wells.
ii. Wash with 1xPBS.
iii. Add cold 4% PFA and incubate for 10 minutes. Wash with 1xPBS, thrice.
iv. Permeabilize cells with 0.3% triton X-100 for 10 minutes at room temperature.
v. Wash with 1x PBS.

2.2.4 Antibody staining-

i. Add blocking agent to each well.
ii. Allow blocking for 30 minutes.

iii. For N2a cells plated on cover slips, transfer the coverslips to flat bottom box (place wet tissues on either side to keep the box moist)
iv. Add primary antibody. Leave it for 30-45 minutes. Wash with 1x PBS, thrice.
v. Add secondary antibody. Leave for 30-45 minutes at room temperature.
vi. Wash with 1x PBS, thrice.
vii. For N2a cells plated on coverslips, add 10uL droplets of Gelvitol on a glass slide. Place each cover slip on the droplets, so that the cells are in contact with Gelvitol (place inverted with respect to plating). Allow the coverslip to get fixed on the slide. (About 1 hour).

Blocking agent for N2a- 1x PBS + 1% Saponin + 10% goat serum

2.2.5 Imaging –

The cells were imaged in Nikon A1R confocal microscopy. 100x resolution oil immersion were used to visualize the cells. The wavelength of laser lights differed for different antibodies. The antibodies of APP were colocalized with Golgi, lysosome, and endosomal subcellular markers. A Z-stack image was captured.

2.2.5 Analysis –

The colocalization analysis was carried out using the Imaris software. The colocalization analysis was carried out by picking the overlapping signal from APP and subcellular marker. The percent APP was calculated by the APP intensity in a subcellular site divided by the total APP

intensity in cell. The percent APP was calculated for APP-Amber-mClover N2a and WT APP-mClover N2a.

2.3 Results and discussion

2.3.1 Selection of subcellular antibodies:

The subcellular organelles involved in endocytic pathway of APP processing include early endosomes, Golgi and trans-Golgi network and late endosome/ Lysosomes [3, 14]. Various antibodies were tested to get the best result for colocalization. EEA1 is an early endosomal marker used for the current work. EEA1 is essential for early endosomal membrane fusion and trafficking. Rab7 was used for late endosome and lysosomal marker. It is a Ras-related protein and is important in late endocytic pathway. Golgin97 was used as the subcellular marker against trans-Golgi network. Golgin97 is known to help maintain trans-Golgi integrity and function by localization of TGN resident proteins.

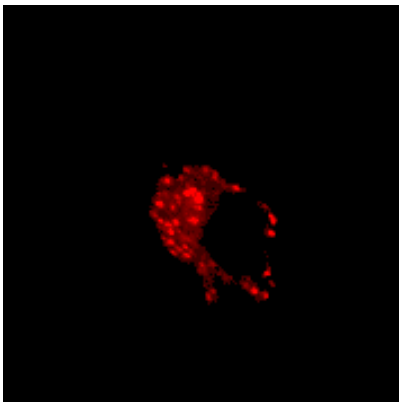

Figure 2.3.1a: EEA1 antibody to identify early endosome

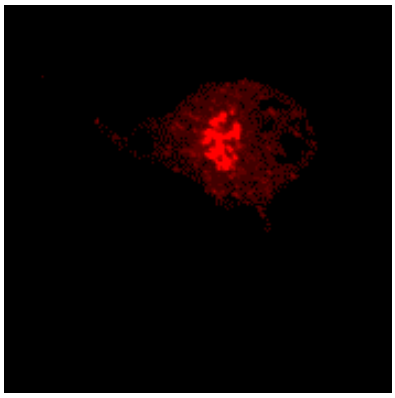

Figure 2.3.1b: Golgin97 antibody to identify trans-Golgi network

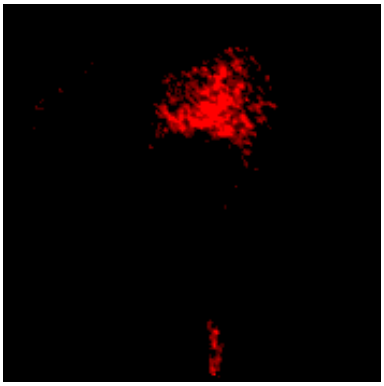

Figure 2.3.1c: Rab7 antibody to identify late endosome / lysosome

2.3.2 Imaging:

The cells were imaged in oil immersion at 100X resolution. The Z-stack of the cells were obtained. The colocalized images were captured.

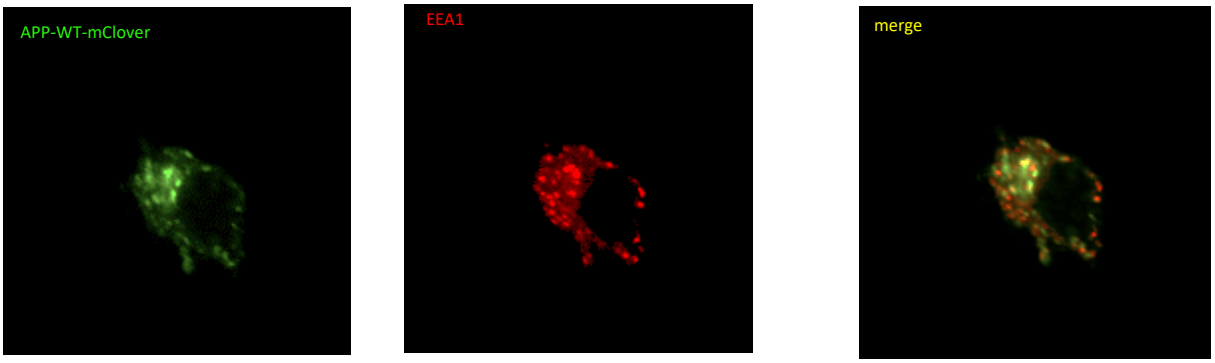

Figure 2.3.2a: N2a cells stained with EEA1 and colocalization of mClover signal with EEA1

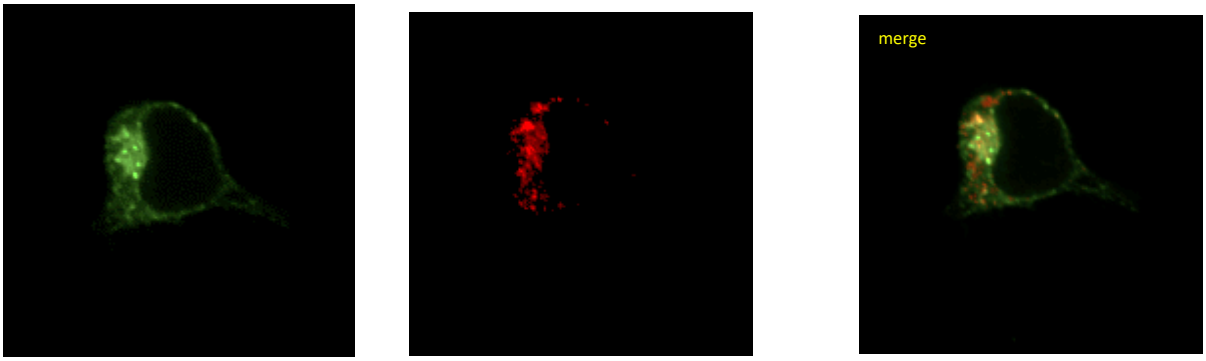

Figure 2.3.2a: N2a cells stained with Rab7 and colocalization of mClover signal with Rab7

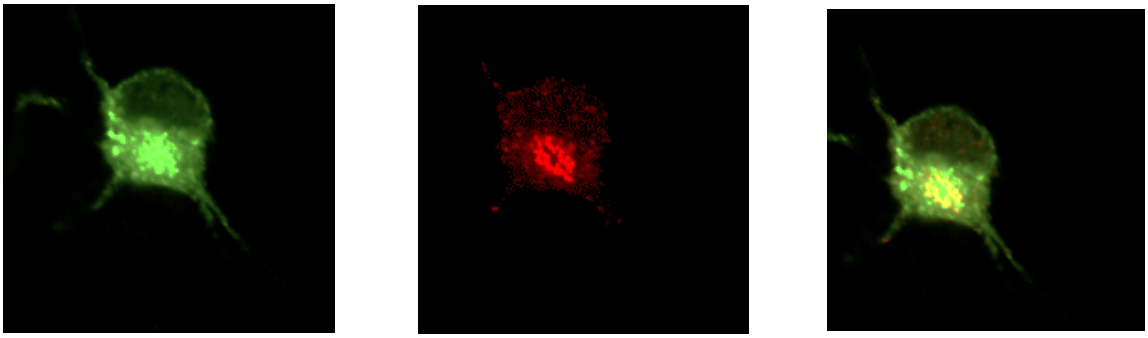

Figure 2.3.2a: N2a cells stained with Golgin97 and colocalization of mClover signal with Golgin97

2.3.3 Analysis:

The percent APP was calculated by the APP intensity in a subcellular site divided by the total APP intensity in cell. The percent APP was calculated for APP-Amber-mClover N2a and WT APP-mClover N2a.

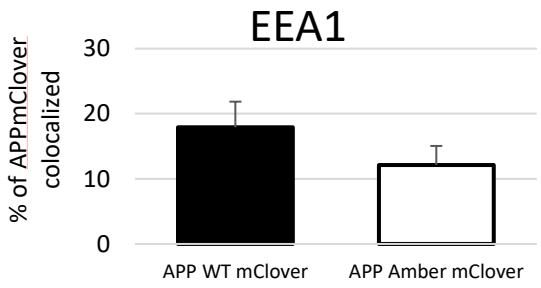

Figure 2.3.3a: Comparison of percent colocalization of APP with EEA1 in click-labelled and WT cells

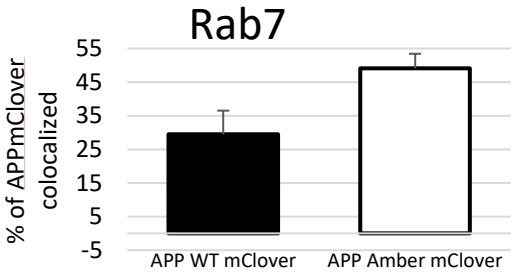

Figure 2.3.3b: Comparison of percent colocalization of APP with Rab7 in click-labelled and WT cells

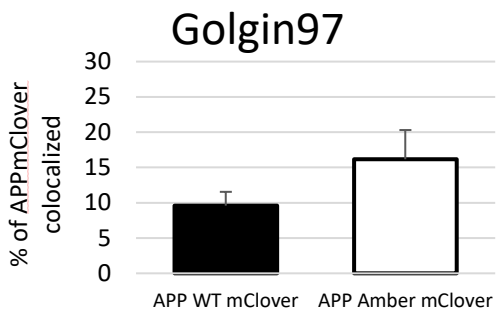

Figure 2.3.3c: Comparison of percent colocalization of APP with Golgin97 in click-labelled and WT cells

The colocalization of click-labelled and WT APP-mClover was carried out and the percent APP was found. We expect a co-occurrence with the sub-cellular markers and not a correlation. The percent APP colocalized in both the cells are not very different indicating the click-labelling doesn't alter the trafficking. This shows that the tool is useful in visualizing APP trafficking.

CHAPTER 3

Fabrication of microfluidic device to study APP trafficking in neurons

The neurons are a complex cell that have various organelles that are highly compartmentalized. It consists of axons, dendrites and soma. The trafficking of proteins in these structures takes place with the help of highly specialized trafficking organelles. Hence the trafficking of APP can be complex in neurons and requires unique devices to study each part of a neuron. Microfluidics can be exploited to study the trafficking of APP in neurons. These devices can fluidically isolate soma and axons. This helps us in understanding the trafficking and processing of APP in axons. This also allows us to manipulate the axons alone. Using this device, it is possible to click-label axons alone or click label the synapses to visualize the APP trafficking.

3.1 Experiment procedure:

Microfluidic device was designed to isolate single axons in order to study APP trafficking. It involves design, fabrication, testing and trouble-shooting of the device. The device is an open channel type and was fabricated using soft lithography technique.

The device fluidically isolates the soma and the axon. The neurons are plated in the somal chamber and the axons extend into the micro-channels and reach the axonal chamber. A volume difference is maintained between the chambers which ensures that the flow is from soma to the axon chamber. When the volume in both the chambers is kept equal, there is no flow between the chambers creating a fluidic isolation.

3.2 Experimental protocols

3.2.1 Microfluidic device fabrication

1. Mask design- The mask was designed using AutoCAD 2017 software [15]. The mask was designed following the mask design rules from Stanford Microfluidic Foundry [16]. The mask design consists of two layers- micro-channels layer and chamber layer.

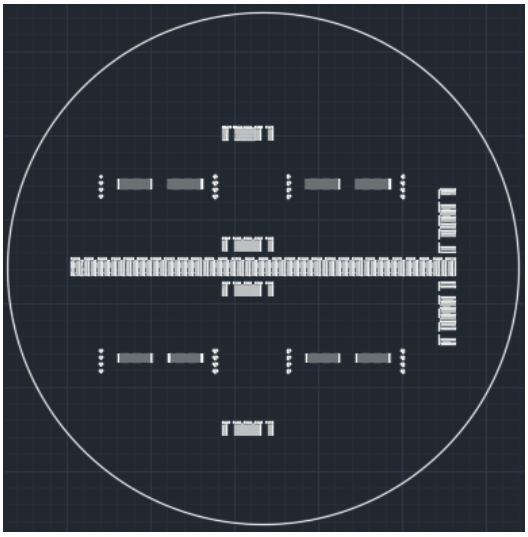

Figure 3.2.1a: First layer of the mask consisting of micro-channels and alignment marks

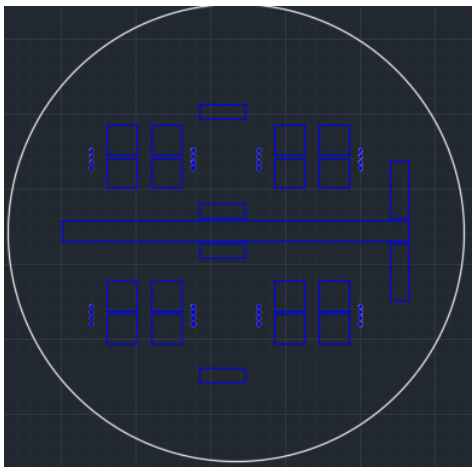

Figure 3.2.1b: Second layer of the mask consisting of main channels

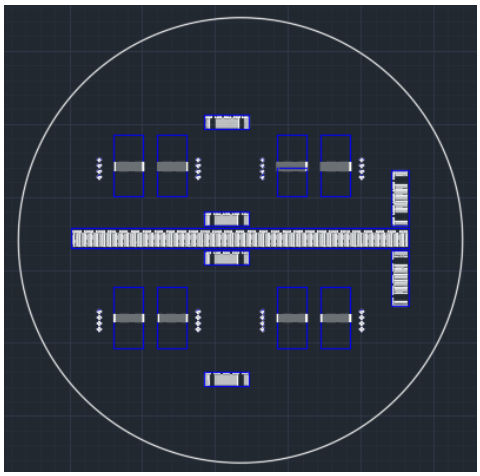

Figure 3.2.1c: Final mask design with both the layers

2. Device specifications- The micro-channels are 500um long, 5um wide, 10um deep and have 10um spacing between channels. The total channel count per device is approximately 300. The current mask design consists of four pair of devices (eight devices in a mask). The space between

two adjacent devices is 2.5 mm. The main channels are 5 mm in length, 5mm in breadth and 100 um in depth [17].

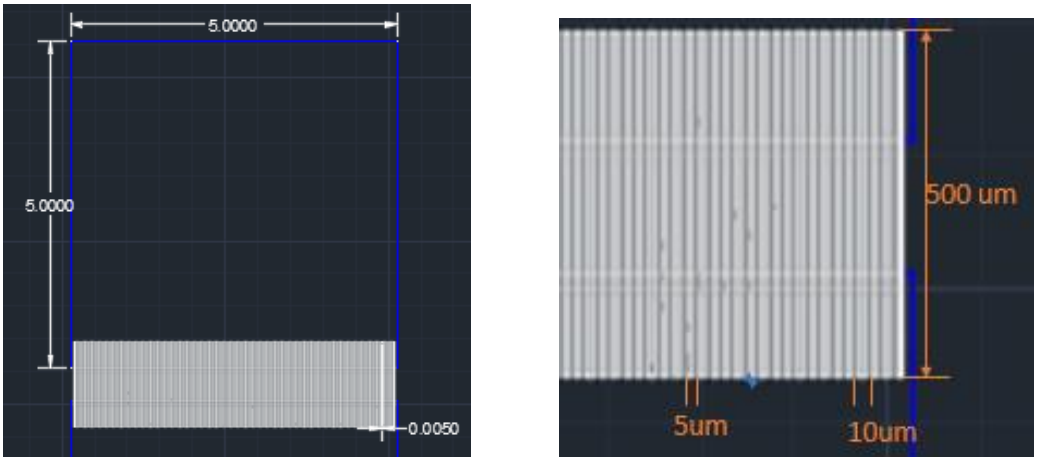

Figure 3.2.2a: Microfluidic device dimensions- main channels and micro-channels

Table 3.1: Microfluidic device dimensions

Micro-channels	Length	500 um
	Width	5 um
	Depth	10 um
	Spacing	10 um
Main channels	Length	5 mm
	Width	5 mm
	Depth	100 um

3. Mold fabrication- The mold was fabricated on a 3-inch silicon wafer. The mold was fabricated in-house. The protocol for fabrication was followed from the paper by Park *et.al.* 2006. Soft lithography technique was used for fabrication [15]. The fabrication steps are as follows [18]:

i. The 3-inch wafer is cleaned with acetone, isopropyl alcohol and sterile water. It is dried with nitrogen.

ii. The wafer is placed in the spin coater. Approximately 1ml of SU8-3010 photoresist is added. It is spun at 3000 rpm for 32s at the rate of 300 rpm/s to obtain a height of 10um.

iii. The wafer is soft baked at 95^{o}C for 3 minutes.

iv. Wafer is exposed through a high-resolution transparency mask containing the first layer with micro-channels at 200 mJ/cm^2.

v. Post-exposure baked the wafer for 60 seconds at 65^{o}C and 2 minutes at 95^{o}C.

vi. Developed with SU8 developer for 3 minutes.

vii. A second layer of photoresist- SU8-2050 was spun at 1750 rpm for 32 seconds at the rate of 300 rpm/s to get a height of 100um.

viii. Soft baked at 65^{o}C for 5 minutes and at 95^{o}C for 20 minutes.

ix. It was exposed through a high-resolution transparency mask containing the second layer with man channels at 240 mJ/cm^2.

x. Post-exposure baked at 65^{o}C for 2 minutes and at 95^{o}C for 10 minutes.

xi. Developed with SU8 developer for 10 minutes.

xii. Wafer was cleaned with isopropyl alcohol and water, followed by drying with nitrogen gas. The cleaned mold dimensions were measured using the profilometer.

Figure 3.2.3: Microfluidic device mold fabricated on Si wafer using soft lithography

4. PDMS device fabrication- The PDMS device was made by replica molding technique. The following steps were carried out:

i. PDMS and its activator are weighed out in a 10:1 ratio in a disposable container and mixed thoroughly. Approximately 25g of PDMS was used for the devices to achieve a thickness of about 5-7 mm.
ii. The PDMS is poured onto the mold. It is placed in vacuum desiccator for 30 minutes to remove any air bubbles. Any remaining bubbles are removed by using pressurized air/ razor blade.
iii. The PDMS is cured in an oven at 70^{o}C for more than 3 hours.
iv. The PDMS is removed from the mold and cut into individual devices.
v. 6 mm holes were punched into main channels.

5. Cleaning the devices- The devices are dynamically washed with 70% ethanol thrice, followed by washing with sterile water. Any additional particles stuck on the PDMS surface was removed using scotch tape.

6. Bonding- PDMS can be bond to glass slide reversibly or irreversibly [18]. The devices were bond reversibly by placing the device with the channels facing the glass and pressing it gently. The device is bond to the glass by Van der Waal's forces [11]. It is bond irreversibly by UV exposure. The PDMS device (the channels facing up) and glass slide are exposed to UV for 5 minutes and bond together[18].

7. Coating- The bonded devices are coated with poly-D-lysine (PDL) and Laminin [19]. 1ml of 0.1mg/ml of PDL is mixed with 35ug of Laminin. This mixture is added to the main channels and incubated at 37°C overnight. The solution is removed and washed with sterile water.

3.2.2 Cell passaging and plating-

Primary hippocampal cells are obtained from rat brain. [Courtesy of Dr. Steven Mennerick]

i. 1:1 dilution of neurons in media is made.
ii. 200uL of diluted cells is added to dishes coated with PDL+Laminin and incubated at 37°C overnight.
iii. In the microfluidic device, 120uL of media is added to one well (axonal chamber) and 150uL of diluted cells is added to the other well. This maintains a flow of media so that neurons move close to the micro-channels.
iv. The devices are incubated at 37°C overnight.

3.2.3 Transfection-

i. Label two tubes, one for DNA and one for lipofectamine
ii. Add Lipofectamine and MEM+ to lipofectamine tube and mix. Incubate for 5 minutes at room temperature.
iii. Add DNA and MEM+ to DNA tube and mix.
iv. Mix DNA and lipofectamine tube and incubate for 20min at room temperature.
v. Warm remaining MEM+ in incubator
vi. Remove media from neurons with 10mL pipette and save for CPK addition

vii. Add 100ul prewarmed MEM+ to each dish

viii. Add 100ul transfection reagents dropwise to each dish. Incubate for 6 hours

ix. Aspirate transfection reagents + media

x. Add conditioned media + CPK + HEPES (Conditioned media:CPK=100:1; CPK:HEPES = 5:1)

xi. Incubate at 37^{o}C overnight.

MEM+ (1ml) = 1ml MEM+ 5uL APV + 2uL NBQX

3.2.4 Fixing and permeabilizing cells-

i. Media is removed from the wells.

ii. Wash with 1xPBS.

iii. Add cold 4% PFA + 4% sucrose and incubate for 10 minutes. Wash with 1xPBS, thrice.

iv. Permeabilize cells with 0.3% triton X-100 for 10 minutes at room temperature.

v. Wash with 1x PBS.

3.2.5 Antibody staining-

i. Add blocking agent to each well.

ii. Allow blocking for 30 minutes.

iii. Add primary antibody and incubate at 4^{o}C overnight.

iv. Wash with 1x PBS, thrice.

v. Add secondary antibody. Leave for an hour at room temperature.

vi. Wash with 1x PBS, thrice.

vii. 1x PBS is added.

Blocking agent for neurons - 1x PBS + 0.3% triton X-100 + 3% Bovine serum albumin

3.2.6 Imaging-

The device with neurons were imaged using confocal microscopy. The Nikon A1Rsi, a scanning confocal Microscopy at the Washington University Center for Cellular Imaging, was used for imaging the samples. The images were viewed through 10x and 20x objective in oil immersion.

3.3 Results and discussion:

3.3.1 Devices used:

Four different devices were tested to see which device was compatible. We tested two commercial devices and two lab made devices.

1. Devices from Xona Microfluidics - The microfluidic device is manufactured by Xona Microfluidics [18]. The device consists of four chamber wells, two main channels and micro-channels. It is a closed type. The device can be segregated into axonal and somal chamber. The dimensions of the device are as follows-
    i. Chamber wells are 5mm x 5mm, 8mm punched holes
    ii. Main channels are7 mm in length, 1.5 mm in width and 100uM in depth
    iii. Micro-channels are 500um in length, 5um in width, 3um in depth and 5um in spacing.

Figure 3.3.1a: Microfluidic device from Xona

2. Devices from Ananda devices- These microfluidic devices were manufactured from Ananda devices [20]. It is a closed type of device and consists of somal and axonal chambers. The device consists of four chamber wells, two main channels and micro-channels. The dimensions of the device are as follows-

   i. Chamber wells are 5mm x 5mm, 4mm punch holes

   ii. Main channels are 5mm in length, 1.5 mm in width and 100uM in depth

   iii. Micro-channels are 500um in length, 5um in width, 30um in depth and 30um in spacing.

Figure 3.3.1b: Microfluidic device from Ananda

3. Lab made devices- Two sets of devices were fabricated in the Institute of Material Science and Engineering at Washington University in St Louis. The device is an open type device [17]. The device consists of two wells- somal and axonal wells, that are connected by micro-channels. The dimensions of the device are as follows-
   i. Chamber wells are 5mm x 5mm, 6mm punch holes
   ii. Micro-channels are 500um in length, 5 um in width, 10um in spacing and 15/ 10 um in depth.

Figure 3.3.1b: Lab made microfluidic devices

3.3.2 Device comparison:

1. Xona devices- The devices were easy to bond to the glass slides. The large punch holes gave an easy access to angle the pipette. The reversibly bonded devices were found to leak and the devices were UV bonded. The bonded devices were used to plate the neurons. It was seen that neuron survival was low and no neurons were found near the micro-channels. The devices could only be used a few times as it was difficult to

remove the cell debris in the main channels. Due to these disadvantages the device were not chosen to carry out any further studies.

2. Ananda devices- The devices easily bond to glass slides and were not UV bonded as they were tightly sealed. The small punch holes made it difficult to angle the pipette tip close to micro-channels. There was an improved survival of the neurons in the somal chambers compared to that of Xona devices but very few neurons were found in the main channels and none were close to the micro-channels to extend axons. The devices were found to harden after a few uses and difficult to bond. Due to these disadvantages the device was not chosen to carry out any further studies.
3. Lab-made devices (15um)- The lab made devices easily bond to the glass slides and the large punch holes made for an easy access to angle the micropipette close to micro-channels. There was a significant increase in the neurons that survived in the wells. The neurons were growing close to the micro-channels and possible axon extension into the channels were seen. But the micro-channel walls were not straight and was difficult to segregate them. Due to these disadvantages the device was not chosen to carry out any further studies.
4. Lab-made devices (10um)- The lab made devices easily bond to the glass slides and the large punch holes made for an easy access to angle the micropipette close to micro-channels. There was a significant increase in the neurons that survived in the wells. The neurons were growing close to the micro-channels and axons were extending into the channels. This device was selected.

3.3.3 Trouble-shooting:

1. Micro-channels- The 15um devices had channels that were not straight and was difficult to segregate. To overcome this problem, the height of the micro-channels was reduced to 10um. This height reduced the strain on the walls and hence were straight.

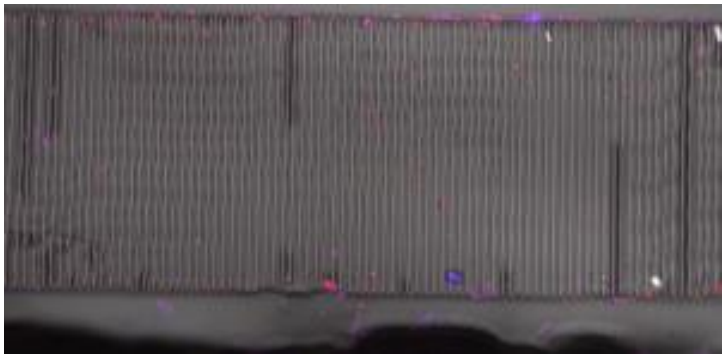

Figure 3.3.3a: Micro-channels from 15um device with crooked walls

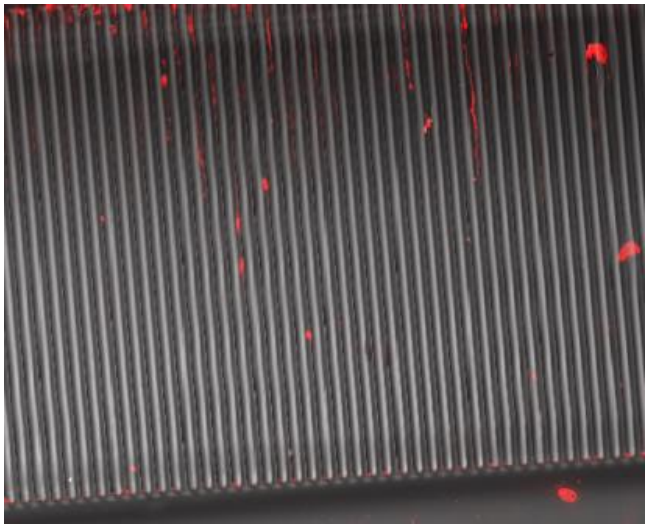

Figure 3.3.3b: Micro-channels from 10um device with straight walls

2. Cell debris- The micro-channels of 15um devices was blocked by cell debris. This prevented the axons from extending into the micro-channels. Addition of AraC with the media reduced the

cell debris. The angling of the micropipette slightly away from the micro-channels also reduces the cell debris clogging the channels.

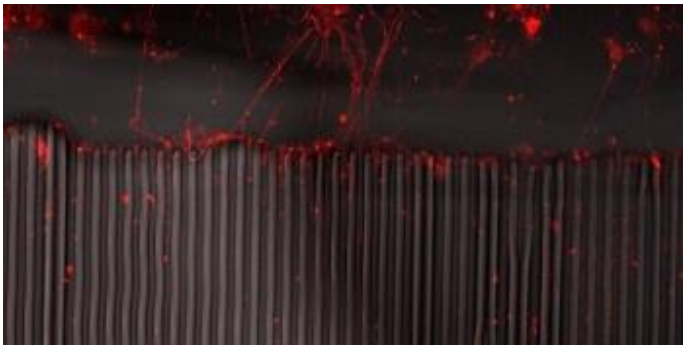

Figure 3.3.3c: Micro-channels blocked with cell debris

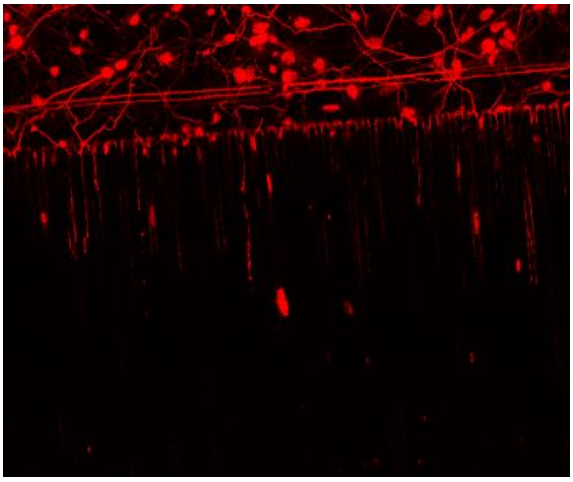

Figure 3.3.3d: Micro-channels with reduced cell debris

3. Neuron survival- The devices coated with only PDL did not have any neurons surviving. The devices were coated with PDL and laminin, incubated at 37ºC for 2 hours. These had very low survival rate. When the devices coated with PDL and laminin were incubated at 37ºC overnight the neurons survived. The neurons survive in the devices for 14 days.

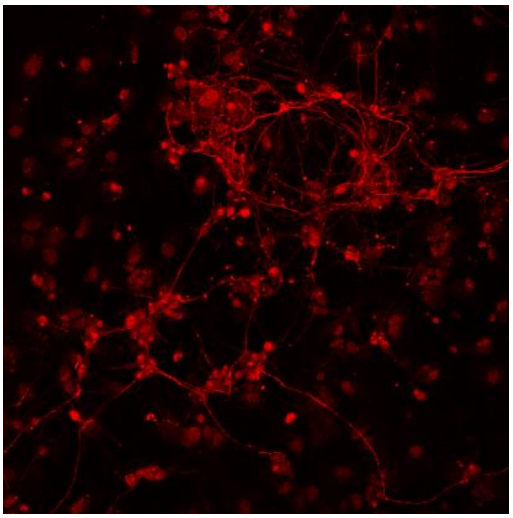

Figure 3.3.3e: Wells with low neuron count

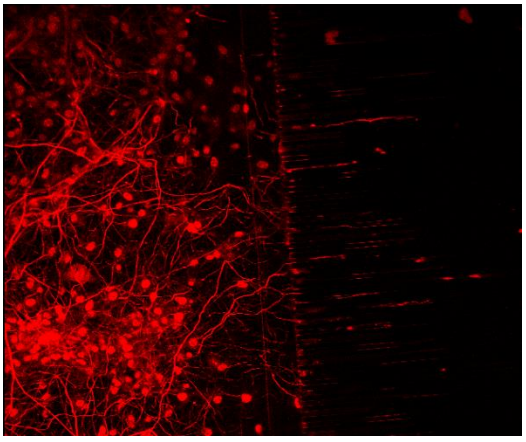

Figure 3.3.3e: Wells with high neuron count

4. Neurons growing underneath the channels- The axons were found to extend the neurons underneath the channels in reversibly bonded devices. When the devices were UV bonded to glass the axons were seen to grow only inside the channels.

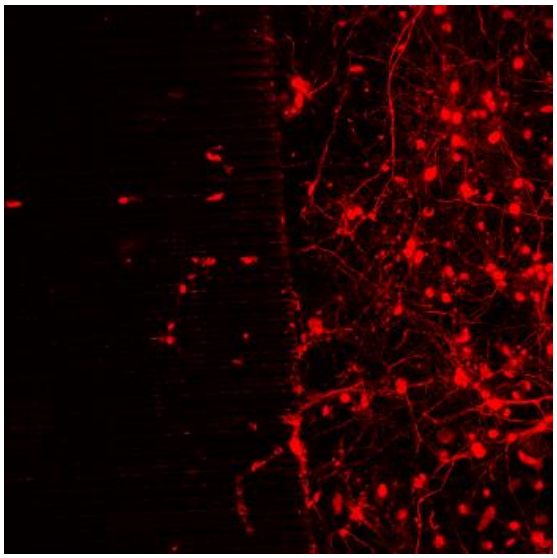

Figure 3.3.3f: Axons growing underneath the channels

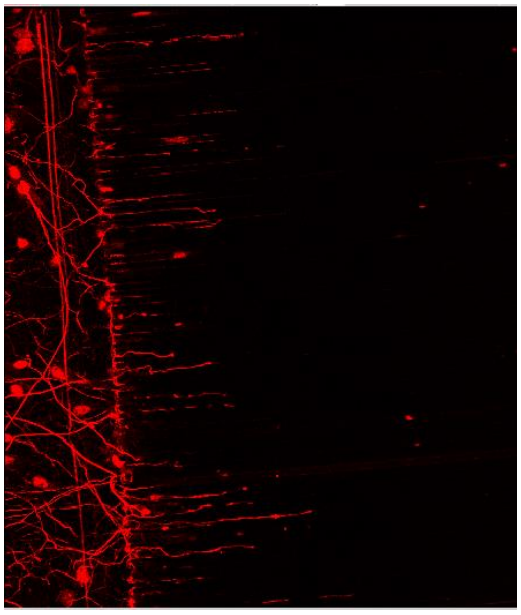

Figure 3.3.3g: Axons growing only in the channels

3.3.4 Imaging:

The neurons plated in the devices were healthy and can survive up to 14 days in vitro. The neurons that were 7 DIV were fixed, stained, and imaged. The neurons were stained with SMI-312 marker known to stain axons. It is directed against highly phosphorylated axonal epitopes of neurofilaments Heavy and Medium subunits. MAP2 marker was used to stain the soma. A healthy number of neurons were seen in the somal chambers. The axonal chamber was free of debris establishing fluidic separation from the somal chamber. Axons were seen extending into the micro-channels.

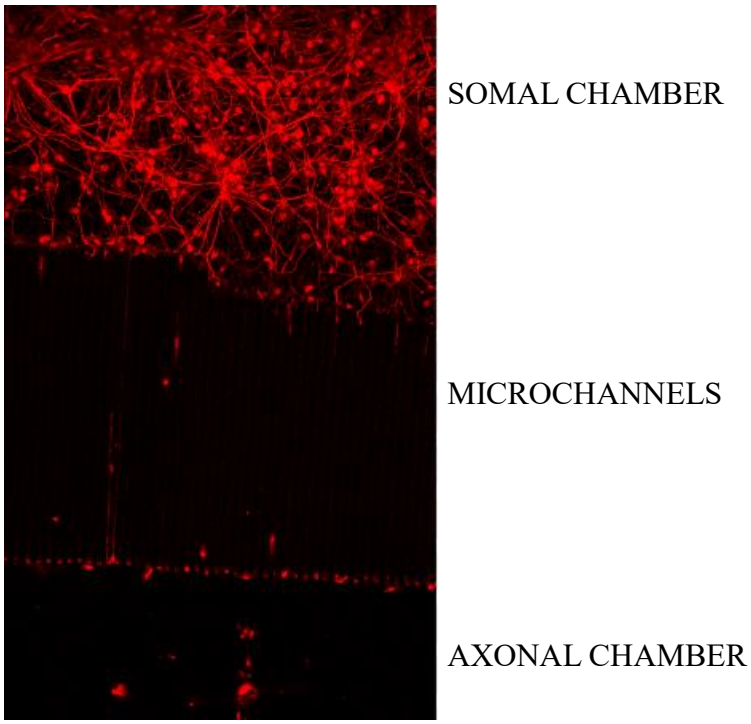

Figure 3.3.4a: Parts of the microfluidic device

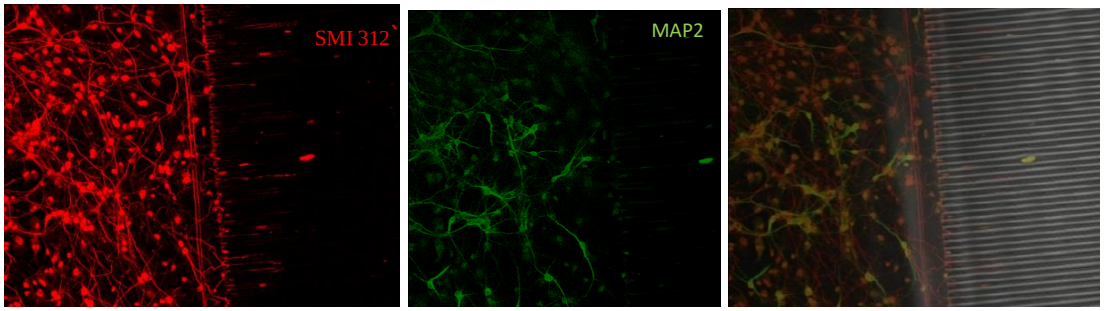

Figure 3.3.4b: Neurons in the microfluidic device stained with neuronal markers

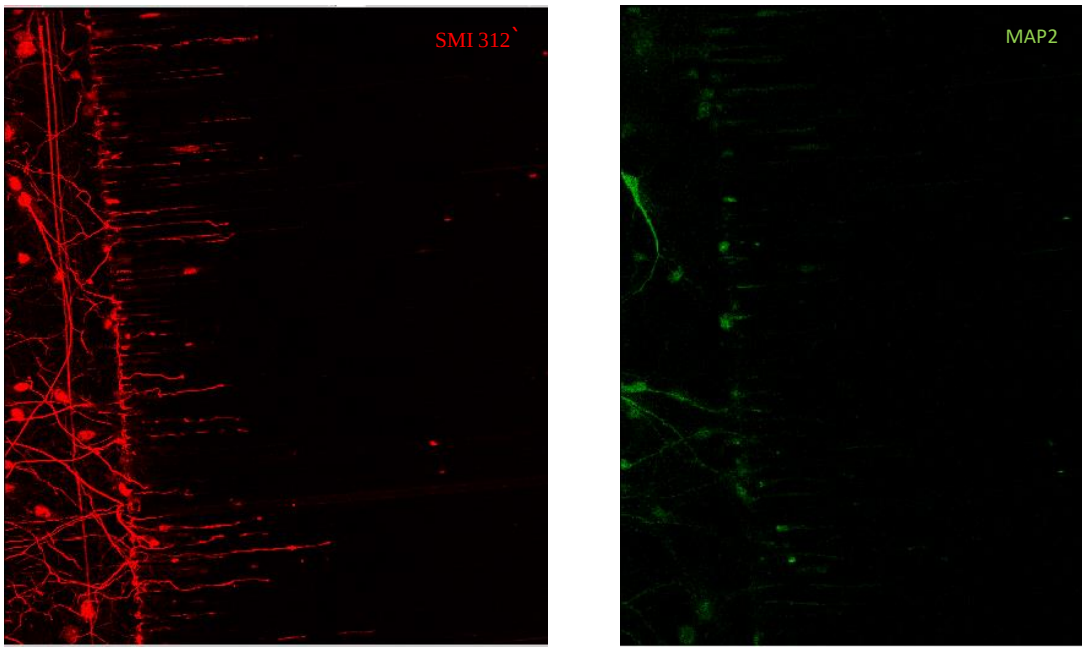

Figure 3.3.4b: Axons seen extending into the micro-channels and absence of dendrites

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