Chapter 4

Design and Fabrication of a Microfluidic Platform Using Xurography Method and its Validation in Cytocompatibility Studies

4.1. ABSTRACT

Over the past few decades, rapid advancements among *in vitro* cell culture models have become valuable assets in neurobiological research to understand the complexities of the human brain, disease development, and progression at cellular and molecular levels. However, conventional 2D cell culture methods often fail to provide deeper insights into the complex phenomenon of neural cytoarchitecture. This limitation has led to neural organoids, such as neurospheres, which offer a closer representation of several neuronal cells. Microfluidics-based neuronal culture platforms enhance neurosphere generation by enabling precise spatiotemporal control of physical and chemical cues. Here, we report the design and fabrication of a low-cost novel microfluidic device using a cutting-edge and cost-effective Xurography technique. We further performed primary neuron culture, forming neurospheres and single cells at varying seeding densities inside the microchannels.

Furthermore, to validate the compatibility of the microfluidic device for neuronal disease model generation, we cultured SH-SY5Y cell lines and checked their differentiation inside the chamber. Additionally, we demonstrated the application of the fabricated device as a co-culture model using astrocytes and primary neurons that were used for subsequent drug screening. Therefore, our multi-faceted microfluidic device can open avenues for neuronal culture and co-culture, neurodegenerative disease modelling, and screening of novel neurotherapeutic interventions.

4.2. INTRODUCTION

In the era of rapid scientific and technological advancements, miniaturization plays a crucial role in biology, chemistry, physics, and pharmacology. In line with this, microfluidics and nanofluidics have emerged in recent years, facilitating the manipulation of fluid amount, behaviour, and flow rate at micro and nanoscales [1,2] to recapitulate in vivo-like environments in biological research. The small geometrical dimensions of microfluidic devices (less than 1mm) correspond to biological cells and tissues that facilitate the precise control of cell numbers and density in a regulated environment compared to the traditional flask-based cultures[3]. At this scale, researchers exploit the scaling of several physical laws like rapid diffusion, laminar flows, Dean flow, and rapid thermal transport and use a large surface area compared to volume[4]. This attribute of the microfluidic 3D culture technique proves advantageous over a traditional 2D culture where cells typically grow in a single layer and cellular morphology, differentiation, and the inherent physiological environment are not well characterized and recapitulated. In 2D culture platforms, cells tend to become more prone to apoptosis, which puts their viability in question[5].

There are different microfabrication techniques like laminates, replica molding (soft lithography), injection molding, hot embossing, 3D printing etc. However, these methods often involve complex multi-step processes, specialized equipment, and cleanroom facilities, calling for a significant cost [4]. At this juncture, there is a need to go for alternative approaches that would offer simplicity and flexibility in fabrication and be cost-effective.

To circumvent the challenges associated with pre-existing microfabrication techniques, researchers have developed a cutting-edge rapid prototyping fabrication technique called Xurography, also known as "razor writing". The term Xurography was first introduced in 2005 by Bartholomeusz *et al* [6]. It is a paper-cutting-based technology that uses a cutting plotter to precisely carve microchannel patterns from adhesive polymer films or other suitable materials, which are then assembled to form functional microfluidic platforms.
The working principle of the xurography technique is based on the cutter plotter that utilizes a computer-controlled cutting head to follow the blade's path on a sheet of polymer or paper. The type of blade used usually depends on the cut material. The depth of the cut can be controlled using the force or depth setting in the cutter. A computer-aided design (CAD) program can be used to create the pattern. To prevent any movement during the cutting process, the material is placed on a glue pad, also known as a cutting mat. To create a clean pattern, the cutting parameters such as speed, force, acceleration, and offset—are then optimised. Thereafter, by patterning multiple 2D layers and bonding them using adhesive, vacuum, heat and other techniques, a complete 3D microfluidic setup can be achieved[7].

Even though the Xurography technology cannot match lithography's precise resolution, it can be a useful platform for microfluidic-based biological research and a quick and affordable way to produce organ-on-a-chip devices. Apart from the fabrication of microfluidic devices such as micromixers[8], sensors[9], and fuel cells[10], the Xurography technique has been widely adopted by researchers worldwide to fabricate diffusion-based microdevices that can be used in cell culture studies. For example, Atencia *et al*. in 2012 employed the Xurography method to fabricate a chip to monitor cellular responses towards the concentration gradient of a toxin and perform dynamic cell-based assay under control diffusive chemical gradients[11].

Microfluidic lab-on-chip platforms have become indispensable tools in biological research since they can mimic the inherent physiological environment of the human body and thereby facilitate the development of potential personalized therapeutics. Biological micro-electro-mechanical systems (BioMEMS) together with microfluidics technology have appealed to neuroscientists throughout the globe in recent years[12]. These chip-based 3D platforms are used to replicate and evaluate various biological phenomena of the nervous system, including axon degeneration, neuromuscular junction formation, myelination, and brain organoid development [13]. Neurons are more precisely seeded in these microfluidic chambers and the cellular interactions are also deeply monitored as opposed to the conventional flask-based tissue culture systems. Manipulation of extracellular environments and chemical treatment of brain cells at a single level is also easier[12]. Microfluidic culture platforms are a cosset for neurobiological research since they aid the generation and analysis of region-specific organoids of the human brain, including forebrain, midbrain, and cortical spheroids derived from pluripotent stem cells. These organoids can be used to model human brain development, myriad of brain pathophysiologies and the effects of various therapeutic interventions and stimulations, on neural circuit formation and maturation[14]. Enthralled by the opportunities that microfluidic lab-on-chip devices bring, here in this article we have fabricated a low-cost and efficient microfluidic device using Xurography technology. To the best of our knowledge, very few microfluidic devices have been fabricated by following the Xurography approach to date for neurobiological research. We further used this device for neurosphere generation and

culture. To further check the cytocompatibility of the device, we cultured SH-SY5Y cells and induced their differentiation inside the microchambers. In addition, using our device we generated a co-culture model using primary neurons and astrocytes that was amenable to screening the efficacy of our novel neuroprotective molecule against Alzheimer's disease.

4.3. MATERIALS AND METHODS

4.3.1. Design and fabrication of the microfluidic device

A highly versatile and efficient Xurography approach (setup from BlackHole Lab) was adopted to fabricate the microfluidic device. Initially, the pattern was designed with the help of 'LibreCAD', a 2D designing platform. Then, the final design was saved in '.dxf' format and transferred to the 'Cricut Design Space software of the Cricut Explore Air 2' cutting machine. On the other hand, a glass slide (Corning® 75x50 mm Plain Microscope Slides) was prepared by attaching Kapton tape to it and removing the air bubbles formed while fixing the Kapton tape with gentle care.

Thereafter, the design was optimized (uploaded on the Cricut Design Space software V7.30.128) according to the desired size of the device. The height of the device channels was according to the height of the Kapton tape, which was 75μm. Following this, the glass slide with Kapton tape was placed on the cutting mat according to the position of the design on the software. After that, the design was cut by the Xurography Cutting machine (Cricut, Explore Air 2), the unwanted parts of the Kapton tape were peeled off, and the pattern of the design was left on the slide.

Furthermore. the Kapton tape-glass mold was placed on a Laminator machine (SKY335R6) at 80°C to ensure proper attachment of the Kapton tape pattern on the glass slide, restricting the formation of any air bubbles. The mold was rinsed gently with 100% ethanol and dried in the air.

Now that we had the mold, we proceeded further to fabricate the PDMS chip using the SYLGARDTM 184 Silicone Elastomer kit (DOW, GMID 01673921) following the standard protocol as described by Friend and Yeo in 2010 [15]. Briefly, the PDMS and the curing agent were mixed in a 10:1 ratio properly with a glass rod. The bubbles formed during the mixing process were eliminated by putting the mixture in a desiccator for about an hour. The Kapton-based glass mold was placed on an aluminium plate, and the PDMS-curing agent mixture was poured over it. The mold containing the PDMS mixture was baked in the oven for 2 hours at 80°C for solidification. After the PDMS mixture was solidified, it was peeled off the mold gently. Then, the PDMS chip was cut into the required dimensions with a scalpel, and the inlet and outlet wells were created using a biopsy punch of 6 mm in size. The whole process for the fabrication of the device is described schematically in **Figure 4.1**.

4.3.2 Post fabrication Treatments of the Microfluidic Device

The devices were dipped into 100% ethanol overnight as a step to dissolve uncured PDMS oligomers and platinum. Glass coverslips (Blue Star, 22*40 mm) were treated overnight with Piranha solution (3:2 H2SO4: H2O2) and then washed 3 times in Mili Q water and then dipped into 100% ethanol overnight. Both the coverslips and PDMS devices were then dried inside the cell culture hood. Now all the equipment including coverslips, PDMS devices and Corona treatment machine (ETP, Model- BD-20V) were sterilized under UV light for 15 minutes[16]. The PDMS devices and the coverslips were plasma bonded properly and placed inside a Sterile petri dish. A wet cotton was placed inside the petri dish to prevent quick evaporation of the

media from the device. Within 10 minutes of plasma bonding, devices were coated with 0.1 mg/ml Poly-D-Lysine (PDL) solution (P6407, Sigma). PDL-coated devices were then placed inside the incubator at 37°C temperature, 5% CO2 and humidified condition overnight. After that, devices were washed thrice with double distilled water and filled with appropriate culture media until the cell seeding.

4.3.3. Cell culture studies inside the fabricated device

4.3.3.1. Primary neuron culture

Primary neuron culture was performed by following the protocols of Khan *et al* [17]. All animal studies were conducted with approval from the Institutional Animal Ethics Committee (IAEC) of JNVU, Jodhpur, India, following the guidelines outlined by the Committee for Control & Supervision of Experiments on Animals (CPCSEA), New Delhi, India. In brief, P0 postnatal Weistar pups were sacrificed. Heads were then decapitated and brains were isolated. The hippocampal portion was dissected out of the brain and kept in an ice-cold Hanks' Balanced Salt solution (HBSS) (Thermo Scientific, 14025092) solution. After washing with ice-cold HBSS solution thrice, the hippocampal parts emerged in 9 ml plating media. 1 ml 10X Trypsin-EDTA solution was then added and the entire total mixture was kept at 37°C temperature, 5% CO2 and a humidified incubator for 20 minutes. After the incubation process was complete, the plating media was replaced with fresh 10 ml plating media (MEM Earl's BSS, 10% FBS, 1 mM sodium pyruvate, 1X glutamax, 1X penicillin-streptomycin). Thereafter, all media was aspirated out, 1 ml of fresh plating media was added and the tissues were triturated gently. After trituration, the mixture was strained through a 70 µm cell strainer. Then, the cell number was calculated and cells were seeded on the PDL-coated device through the inlet pore keeping the live cell number 1×10^6 cells. After 4 hours of cell seeding, plating media was replaced with maintenance media (Neurobasal media, 1X B27 supplement, 1X glutamax, 1X penicillin-streptomycin.) and 50 % of the maintenance media was regularly changed every 24 hr.

4.3.3.2. SH-SY5Y Culture

To check the compatibility of the device with cell line culture, SH-SY5Y, a thrice-cloned subline of a neuroblastoma cell line from a metastatic bone tumour, was cultured inside the device following the process as mentioned by Eleonora De Vitis *et al*. in 2021[18]. In brief, SH-SY5Y were cultured in a T-25 flask in DMEM/F-12 (1:1) 10%FBS media with 1% antibiotic-antimycotic. Cell viability studies were conducted using the conventional MTT reduction assay on the SH-SY5Y cell line (S1, Supporting Information). When about 80 % confluency was attained, cells were trypsinized and seeded on the PDL-coated microfluidic device with the cell number 50000 per device. 50% of the media was replaced every 24-hour interval until the desired confluency was obtained inside the device.

4.3.3.3. Cellular Differentiation Studies

Differentiation of SH-SY5Y was done using 10µM RA and 50 ng/ml BDNF treatment [18,19]. In brief, when the cells were almost 80 % confluent i.e. within 48 hr of cell seeding, DMEM/F12 (1:1) 10% FBS media was replaced with DMEM/F12 (1:1) media containing 1% FBS and 10µM RA as a differentiation media for 72 hours. After that, the differentiation media was replaced with DMEM/F12 (1:1) media containing 1% FBS and 50 ng/ml Brain-derived neurotrophic factor (BDNF) to promote neuronal processing for the next 5 days.

4.3.3.4. Co-culture of primary neurons and astrocytes inside the microfluidic chamber

Astrocytes were isolated from dissociated rat brain tissue and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) following the protocol established by Sebastian Schildge *et al* [20]. After 5 days of culture, the astrocyte culture was trypsinized and used for co-culture experiments. We developed a microfluidic setup to culture neurons and astrocytes in a vertical orientation inspired by the work of Mingjian Shi and colleagues [21]. For this, both the PDMS components and the glass surfaces of the microfluidic device were coated with 20 µg/ml poly-L-ornithine and 5 µg/ml laminin to enhance cell adhesion. Initially, the astrocyte cells were inoculated into the microfluidic device, which was placed in a reverse orientation to promote adherence to the PDMS surface. The device was then incubated at 37°C for 24 hours. Following this, neuronal cells were inoculated into the microfluidic device and maintained in an upright position at 37°C for another 24 hours to facilitate adherence of the neuronal cells to the glass surface. 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.

4.4. RESULTS AND DISCUSSIONS

4.4.1. Design and fabrication of the microfluidic device

The most predominant microfabrication technique has always been the Soft Lithography approach which produces microfluidic chips with excellent resolution. However, the primary disadvantage of this method is the requirement of high-end equipment and clean-room facilities[15]. Therefore, in our research, we adopted a much facile, cost-effective, and less time-consuming approach. Our microfluidic chip was fabricated using the Xurography technique where LibreCAD, a free and open-source tool was used to create the desired design **[Figure 4.1]**. The 2D designing software LibreCAD is easier to use than other complex 3D designing software like AutoCAD and Solid Works. During the early stages of work, we used Scotch tape **[Figure 4.3]** and PVC tape **[Figure 4.4]** as materials for mold formation. However, the PDMS surface developed against the mold pattern became too much uneven. Due to the high baking temperature of around 80°C after pouring of PDMS-curing agent mixture on the mold, an oily substance from the PVC tape was released which made the surface less transparent and that led to poor imaging. The demerit of uneven PDMS surface was also associated with the use of scotch tape. Finally, after all the trial-and-error methods, Kapton tape was selected as a material for suitable mold development as mentioned by Nicholas et. al **[Figure 4.5]** [22]. Kapton tape-based PDMS-driven microfluidic device was found to be suitable for both cell and organoid culture and imaging as well as for disease model generation**.** Apart from this, the Kapton-tape-based mold could be reused after cleaning simply with 70% ethanol which imparted affordability in the context of device fabrication using this mold. **Figure 4.2** gives an overview of the steps involved in µFD fabrication using the Xurography method.

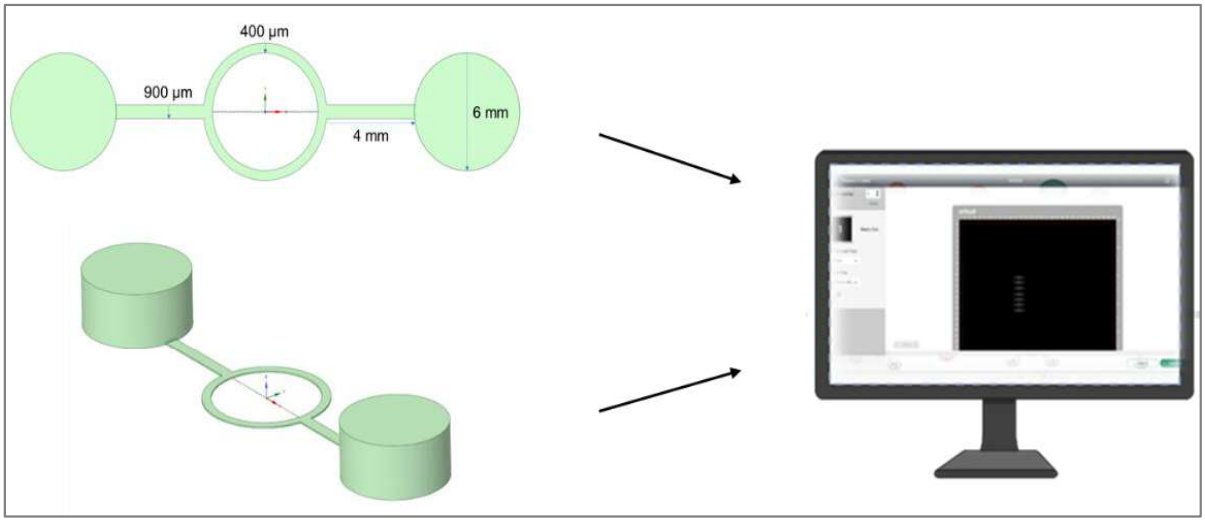


Figure 4.1: 3D Design of the device is transferred to the Cricut Design Space Software prior to fabrication

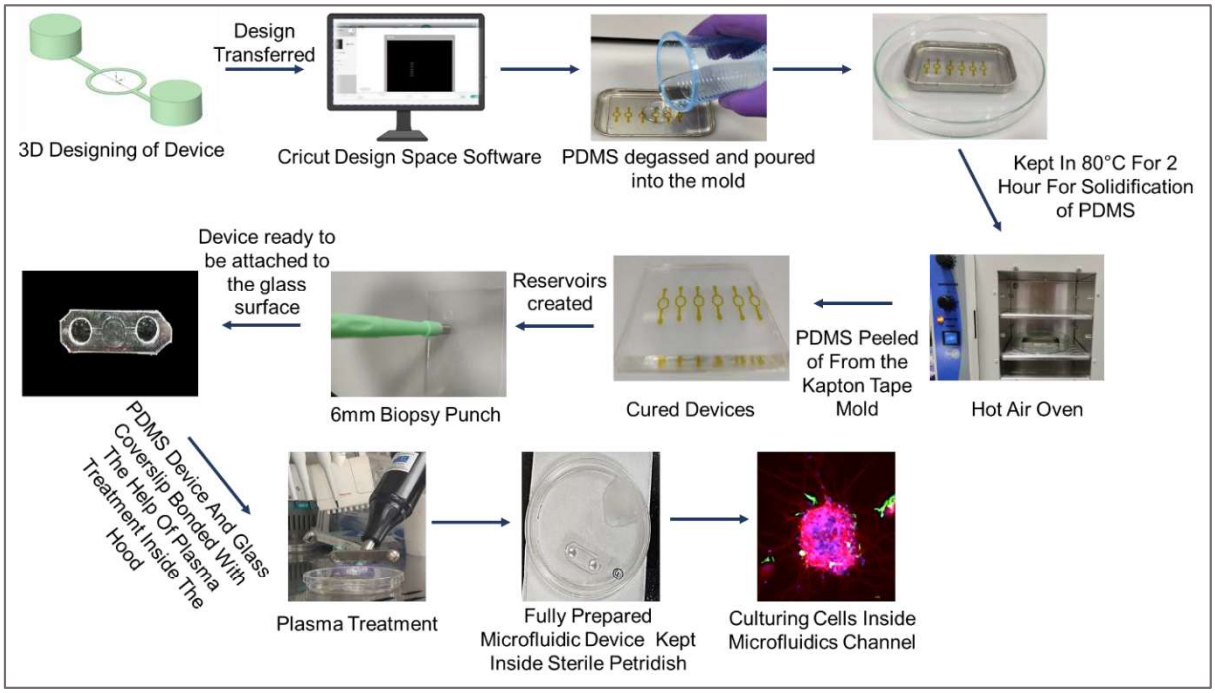


Figure 4.2: Overview of the steps involved in µFD fabrication using the Xurography method

The fabrication process involves a. designing the microfluidic device, transferring the design to Cricut Design Space software in .dxf format, and plotting the mold on Kapton tape. b. Degassed PDMS and curing agent mixture (10:1 ratio) poured over the mold, d. Kept at 80°C for 2 hours for solidification of PDMS, e. Solidified PDMS device and PDMS peeled off from the Kapton tape mold, f. Inlet and outlet created using 6mm biopsy punch, g. Fabricated microfluidic device, h. PDMS device and glass coverslip bonded with the help of plasma treatment inside hood, i. Fully prepared microfluidic device, kept inside sterile Petridis along with wet cotton to minimize evaporation of culture media j. primary neuron cultured inside a microfluidic chamber.

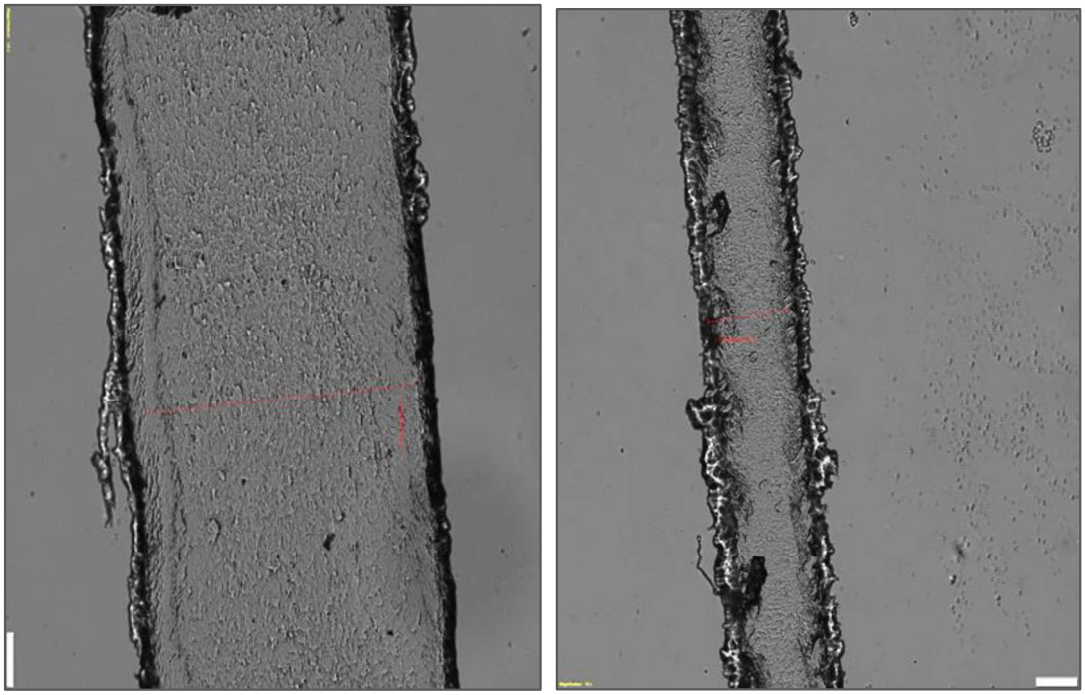


Figure 4.3: DIC images of the PDMS chip (main channel and microchannel) fabricated using Scotch tape as an adhesive. Scale Bar corresponds to 100µm

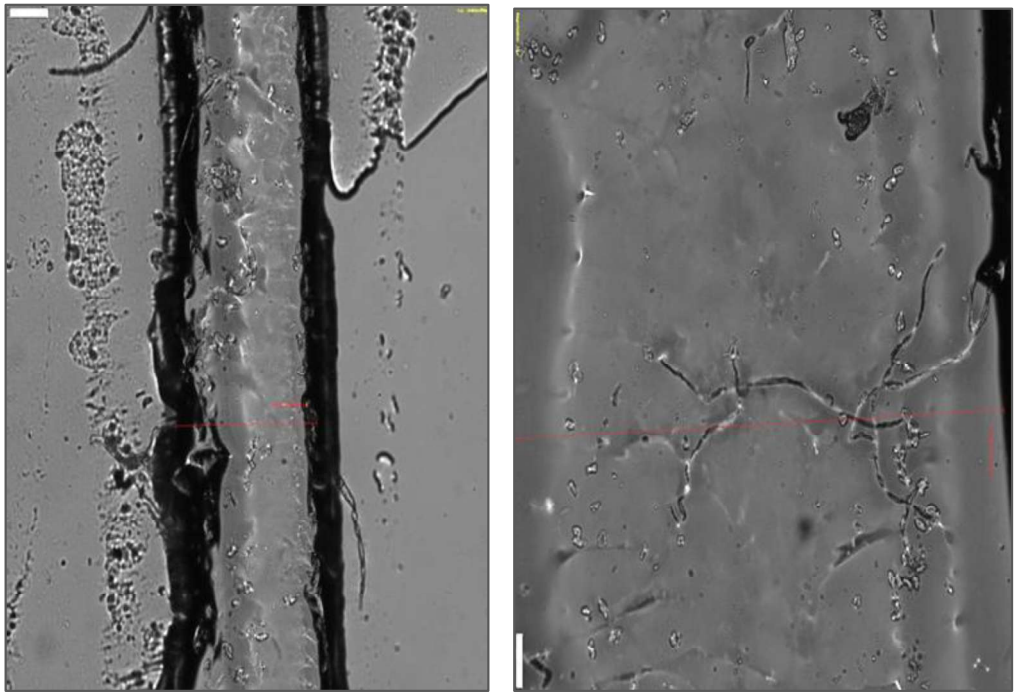


Figure 4.4: DIC images of the PDMS chip (main channel and microchannel) fabricated using PVC tape as an adhesive for the mold. Scale Bar corresponds to 100µm

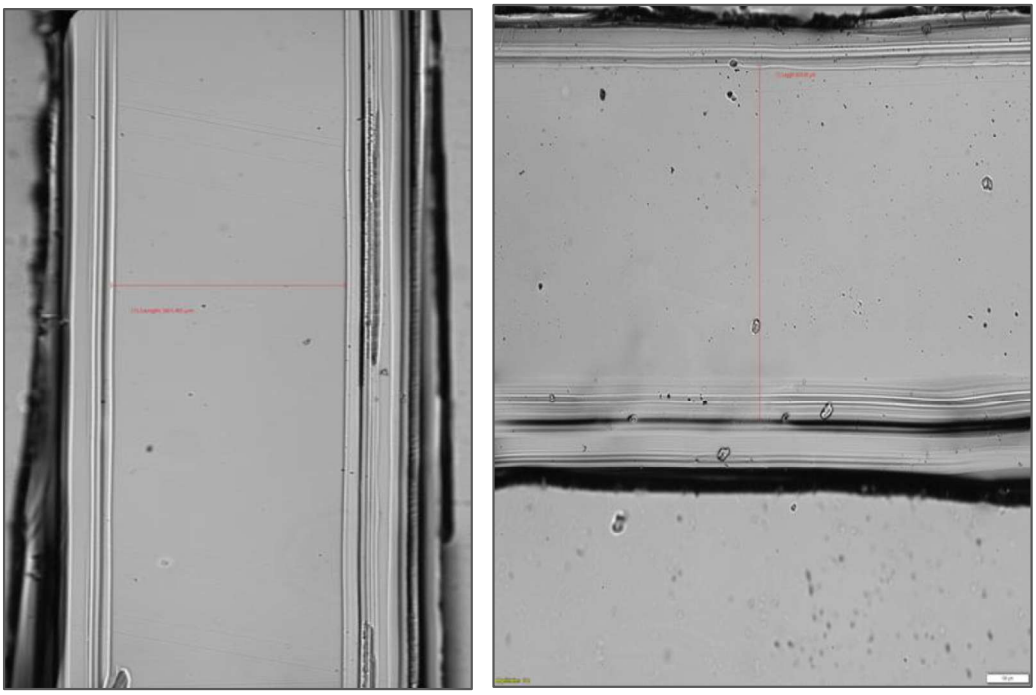


Figure 4.5: DIC images of the PDMS chip (main channel and microchannel) fabricated using Kapton tape as an adhesive for the mold. Scale Bar corresponds to 100µm

4.4.2. Post-fabrication treatment

Consequent to the casting of the PDMS layer on the fabricated mold, biopsy punches of 6 mm diameters are used to punch holes creating the reservoirs. Before proceeding to the bonding step, the mold containing the PDMS layer has to go through ethanol treatment for 24 hours and 15 minutes of UV exposure before plasma bonding with a glass coverslip to minimize the non-polymerized PDMS oligomer and ensure the removal of all specks of dust, and particles, from the surface. The PDMS-based microchannel is then bonded to another substrate like glass to form the complete chip. The bonding of the PDMS mold to other substrates can be either reversible or irreversible. Here, we used the Corona discharge technique **[Figure 4.6]** that creates irreversible bonding through hydroxylation of the PDMS surface[23] that is the Si-CH3 functional group of the PDMS is transformed into a Si-OH function. The corona treatment activates silanol moieties on PDMS surfaces. The portable corona discharge device produces functional OH groups at room temperature. It potentially ionises the air by establishing a high voltage potential between its electrodes, resulting in a localised corona discharge and the formation of a strong and permanent Si-O-Si link between the PDMS mold and the glass substrate[23, 24, 25].
To enhance the hydrophilic properties of PDMS that could result in better cytocompatibility studies, we coated the PDMS device with a charged molecule like Poly-L-Lysine within 10 minutes subsequent to bonding as it is reported that the charged molecule could form electrostatic interaction with the cultured cells[26].

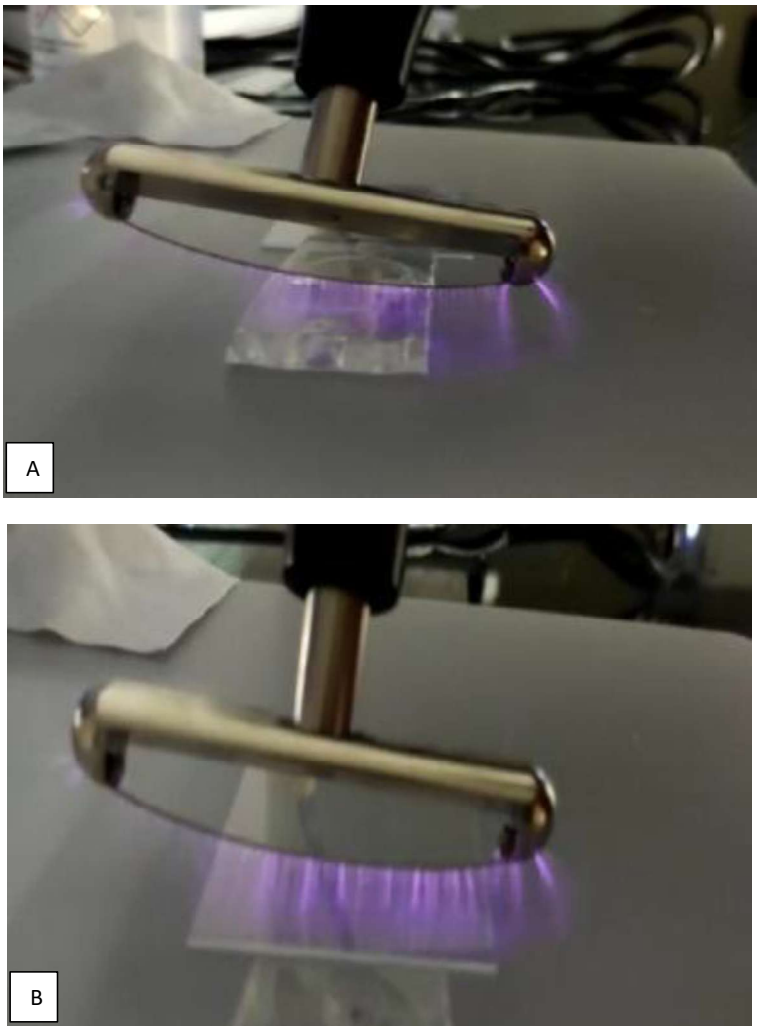
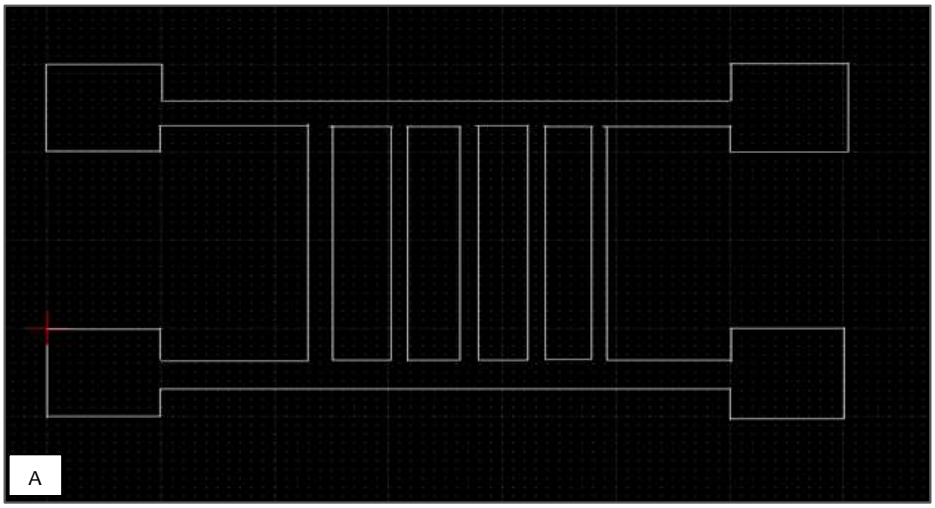


Figure 4.6: Surface Activation by Corona Discharge Technique (A) PDMS surface activation, B) Glass surface activation. To bond the PDMS, the surface needs to be activated, transforming the Si-CH3 function of the PDMS to a Si-OH function. When put in contact with another activated surface showing the same function (e.g. PDMS or glass), a strong and permanent Si-O-Si link is created. To do that, the most efficient tool is a plasma cleaner working with O2 or air. With the equipment provided by the BlackHole Lab, the vacuum level inside the plasma chamber is controlled at around 500mTorr, and a high plasma for 2 min is efficient.

4.4.3. Device characterization

Initially, we fabricated three µFDs with varying designs and dimensions. During the course of our study, we primarily focused on a "static cell culture" without the use of any pump. Thus, before the evaluation of their cytocompatibility, we investigated the flow rate inside the three devices using nanoparticle solutions. After measuring the distances travelled by nanoparticles in the three respective devices at 0.25 seconds, we calculated the average speed (µm/s) of the particles and plotted them using GraphPad Prism **[Figure 4.13]**. From the quantitative graphical analysis, it could be deciphered that in Devices 1(H-shaped) and 2(X-shaped), the flow of nanoparticles was quite rapid compared to Device 3 (C-shaped). Based on the results obtained, we intended to choose only the best dimensions. However, to further confirm the results from the graphical analysis, we performed primary neuron culture in the three devices.

Briefly, after choosing Kapton tape as an adhesive of interest for mold fabrication, we proceeded to fabricate devices of different dimensions. At first, we designed an H-shaped device **[Figure 4.7 A, B, C]** with microchannels of Width*Height=3mm*6mm dimensions. These types of microfluidic devices are mostly used for the generation of concentration gradients across the channels. However, in basic cell culture requirements where there is a flow of only one type of fluid, these devices might not be useful. In the H-shaped device, the flow of the media could not be controlled and cell culture became difficult as the cells were getting washed out from the channels along with the media. **Figure 4.8** shows the distance a particular nanoparticle travels in 0.25 seconds inside the H-shaped device.
Thus, we chose another design for our study: an X-shaped µFD [Dimensions: 3mm W*4mm H] **[Figure 4.9 A, B, C, D].** In this case, although the flow rate of media was relatively slower than the H-shaped one **[Figure 4.10]**, still cell adhesion was difficult resulting in clogging of the microchannels. Therefore, we finally decided to design a very simple and easy-to-fabricate C-channel microdevice **[Figure 4.11 A, B, C, D]**. We fabricated a µFD comprising narrow channels with dimensions around 400 µm that could provide a steady flow of fluid **[Figure 4.12]**. As the low resolution is a basic limitation of Xurography technology, narrow channel mold development requires manual input with a laboratory scalpel otherwise achieving a clean and even channel fabrication is difficult. So, we chose 400 µm as the appropriate channel dimension for culturing primary neurons and mammalian cell cultures like SH-SY5Y. The height of the channels depends upon the thickness of the Kapton tape used, in our case, it was 75 µm.
Here, primary neuron culture and cell line culture were easily achieved with proper cell adhesion as would be discussed in subsequent sections.



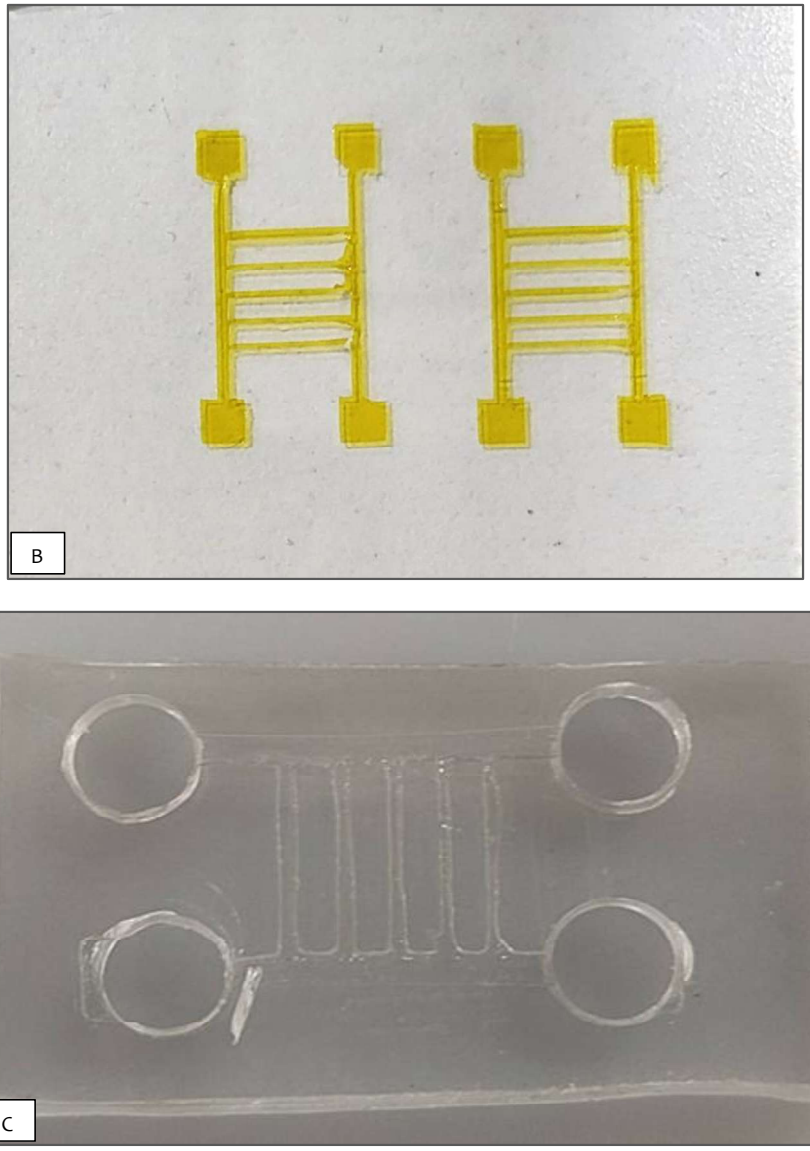


Figure 4.7: H-shaped µFD (A)2D Design of the device on the LibreCAD software; (B) Mold for the device fabricated on a glass side using Kapton Tape as an adhesive; (C) Fabricated PDMS device with H-shaped channels prior to bonding to the coverslip

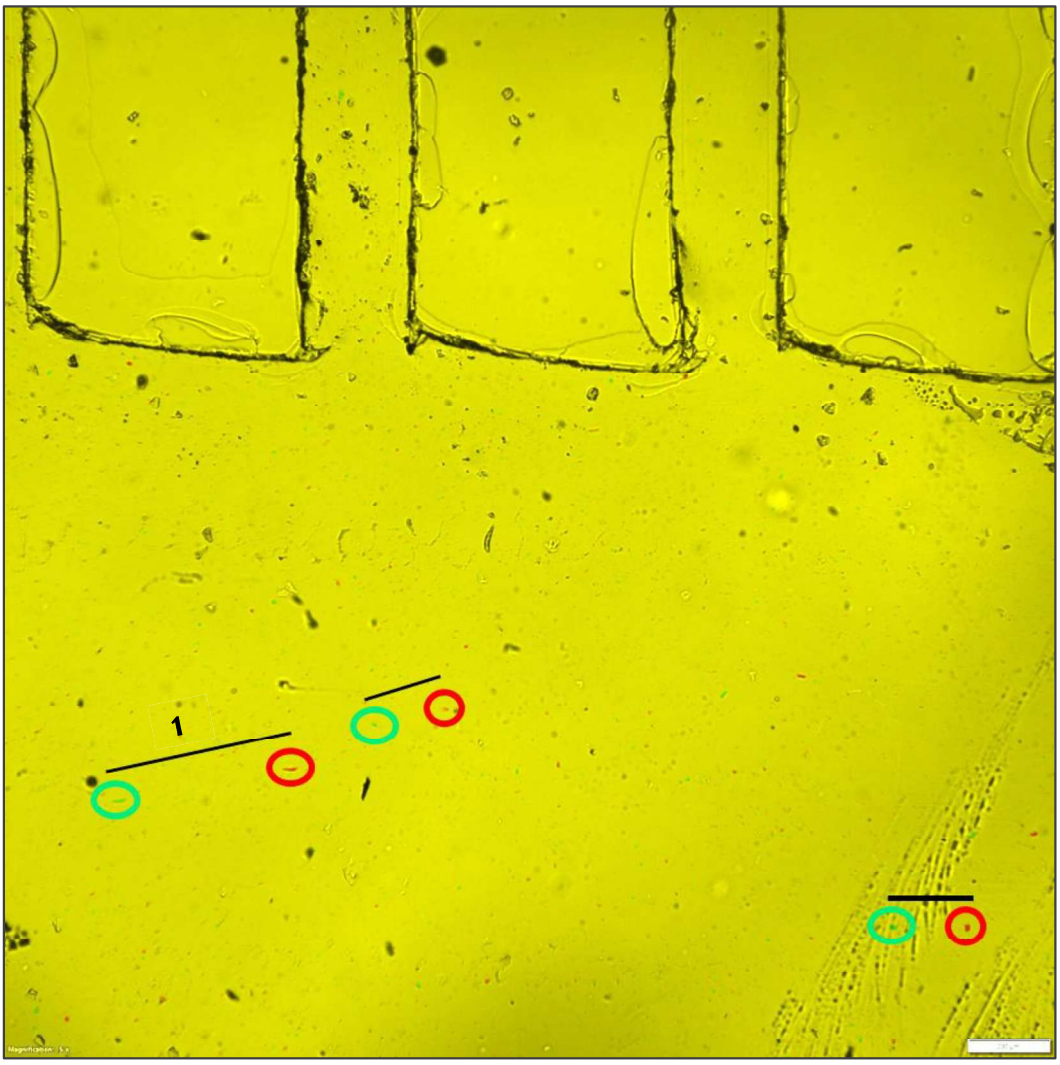
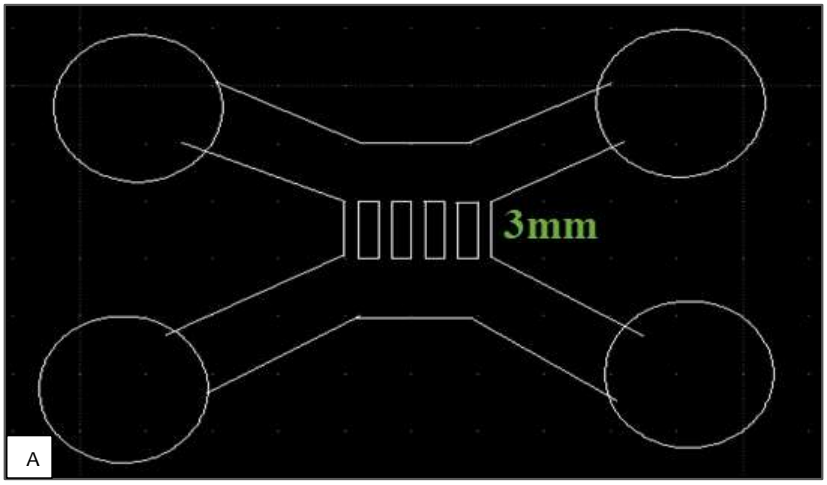
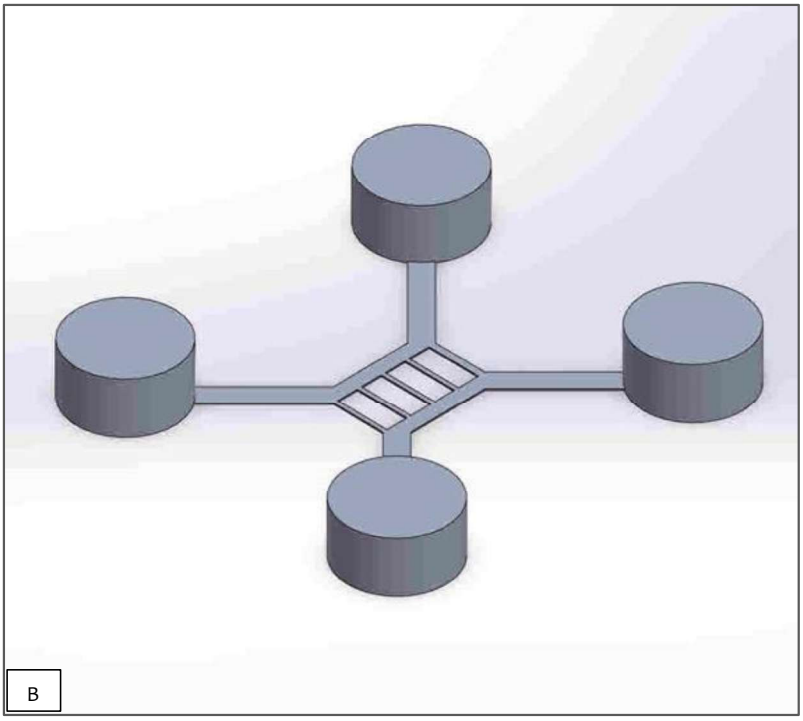


Figure 4.8: Flow rate of nanoparticle solution inside the H-shaped µFD as obtained by time-lapse imaging at four frames per second. (The green circle represents the rear position and the red circle represents the front position of a nanoparticle; the black lines represent the distance travelled by the nanoparticle at a particular time point). For instance, here nanoparticle 1 has moved a distance of 453.5µm in 0.25 seconds as measured during time-lapse imaging by CellSens software. The scale bar corresponds to 200µm.


3mm
A


B

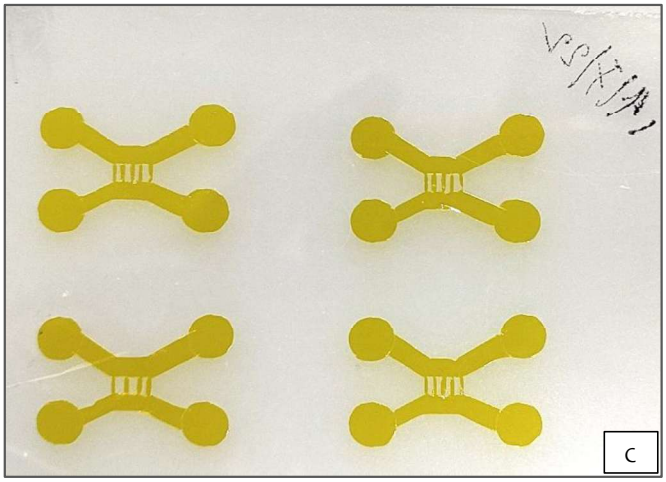




Figure 4.9: X-shaped µFD (A), (B) 2D Design of the device on the LibreCAD software; (C)Mold for the device fabricated on a glass side using Kapton Tape as an adhesive; (D) Fabricated PDMS device with X-shaped channels prior to bonding to the coverslip

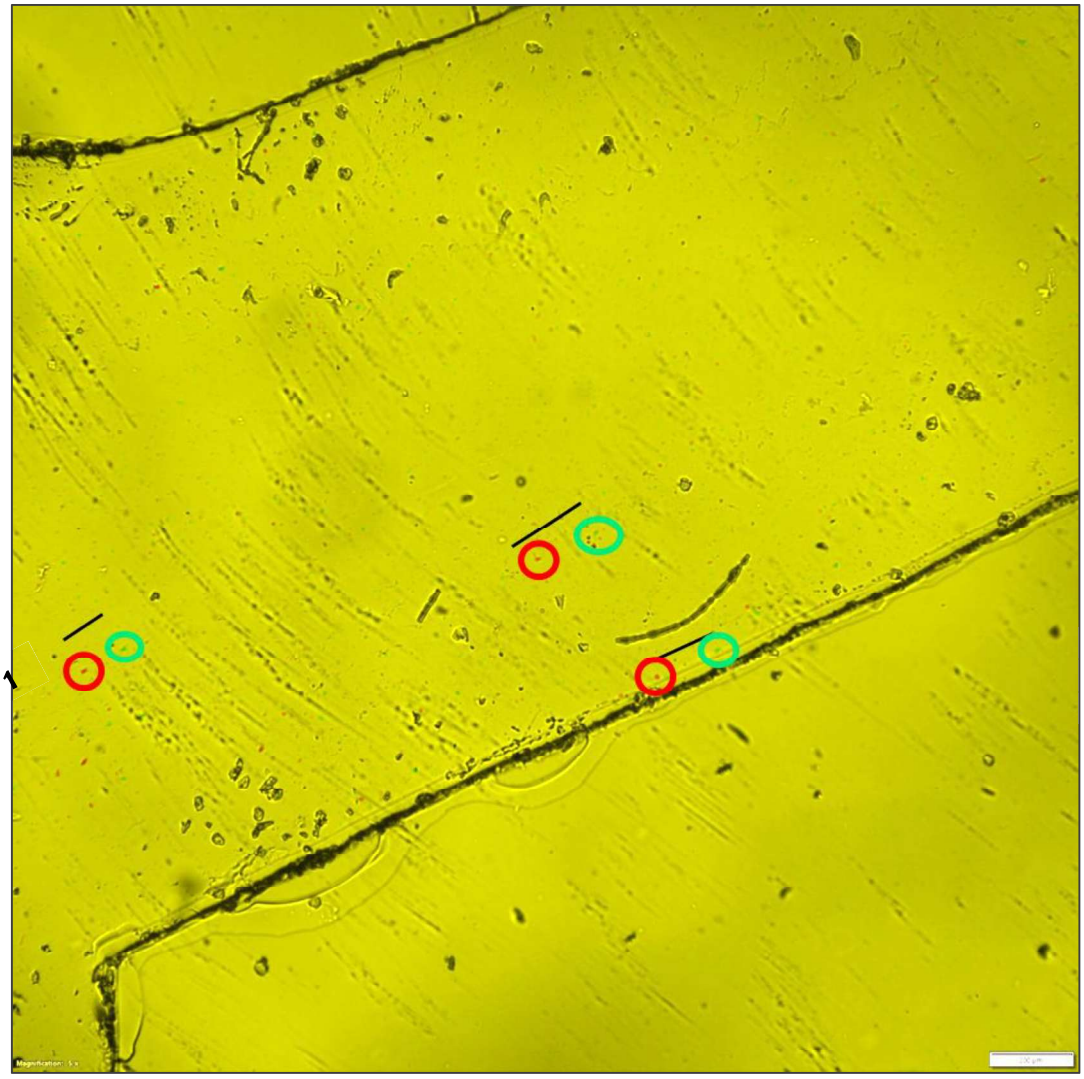
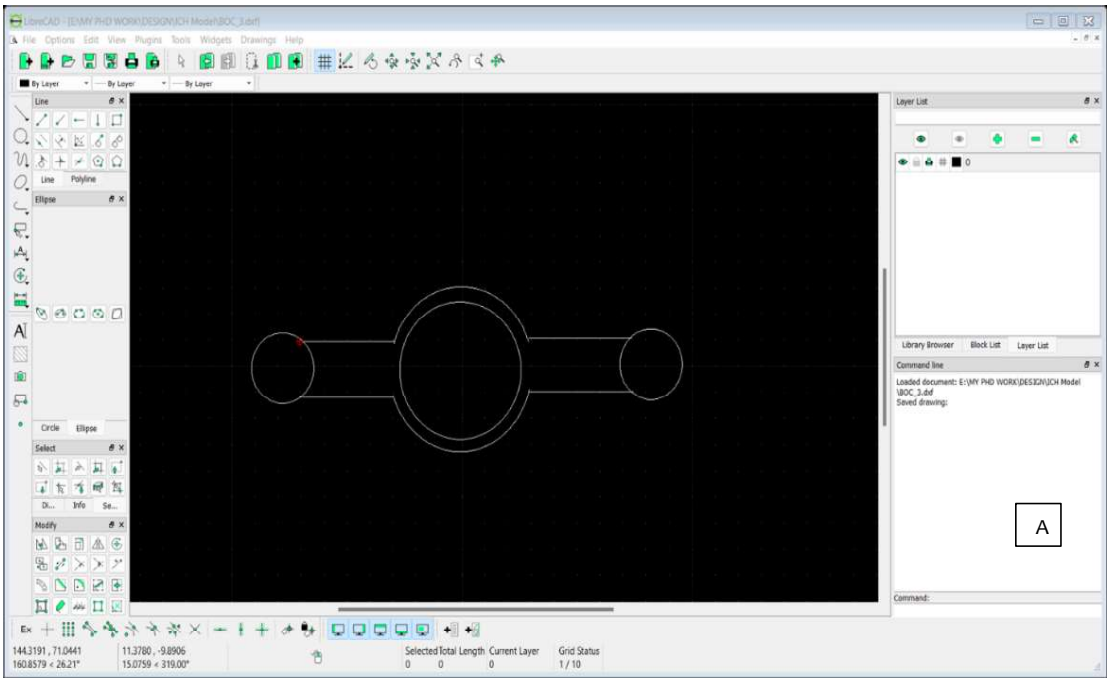
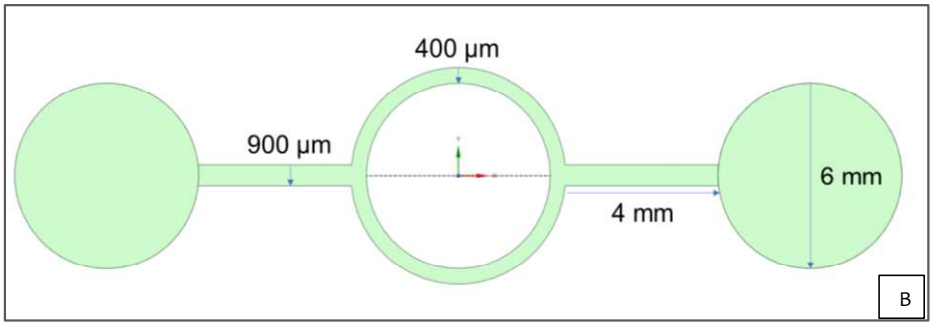


Figure 4.10: Flow rate of nanoparticle solution inside the X-shaped µFD as obtained by time-lapse imaging at four frames per second. (The red circle represents the rear position and the green circle represents the front position of a nanoparticle; the black lines represent the distance travelled by the nanoparticle at a particular time point). For instance, here nanoparticle 1 has moved a distance of 91.50 µm in 0.25 seconds as measured during time-lapse imaging by CellSens software. The scale bar corresponds to 200µm.


A


400 µm
900 µm
6 mm
4 mm
B

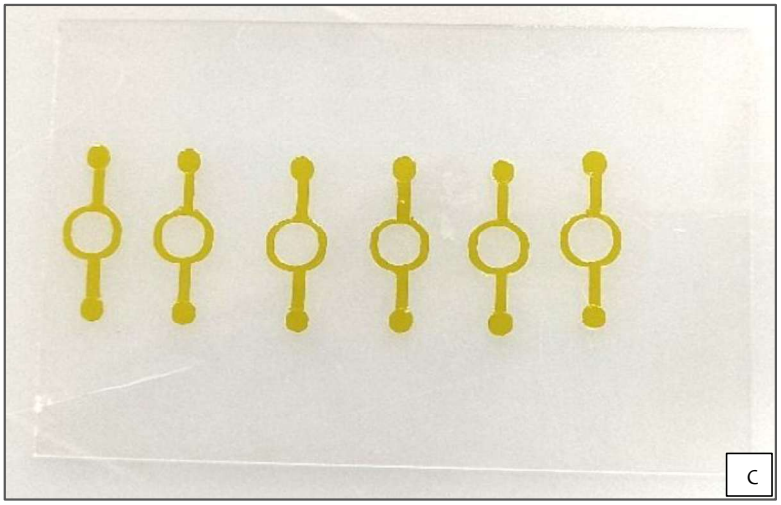

C



Figure 4.11: C-shaped µFD with a straight channel (A), (B) 2D Design of the device on the LibreCAD software; (C)Mold for the device fabricated on a glass side using Kapton Tape as an adhesive; (D) C-shaped Fabricated PDMS device with straight channels prior to bonding to the coverslip

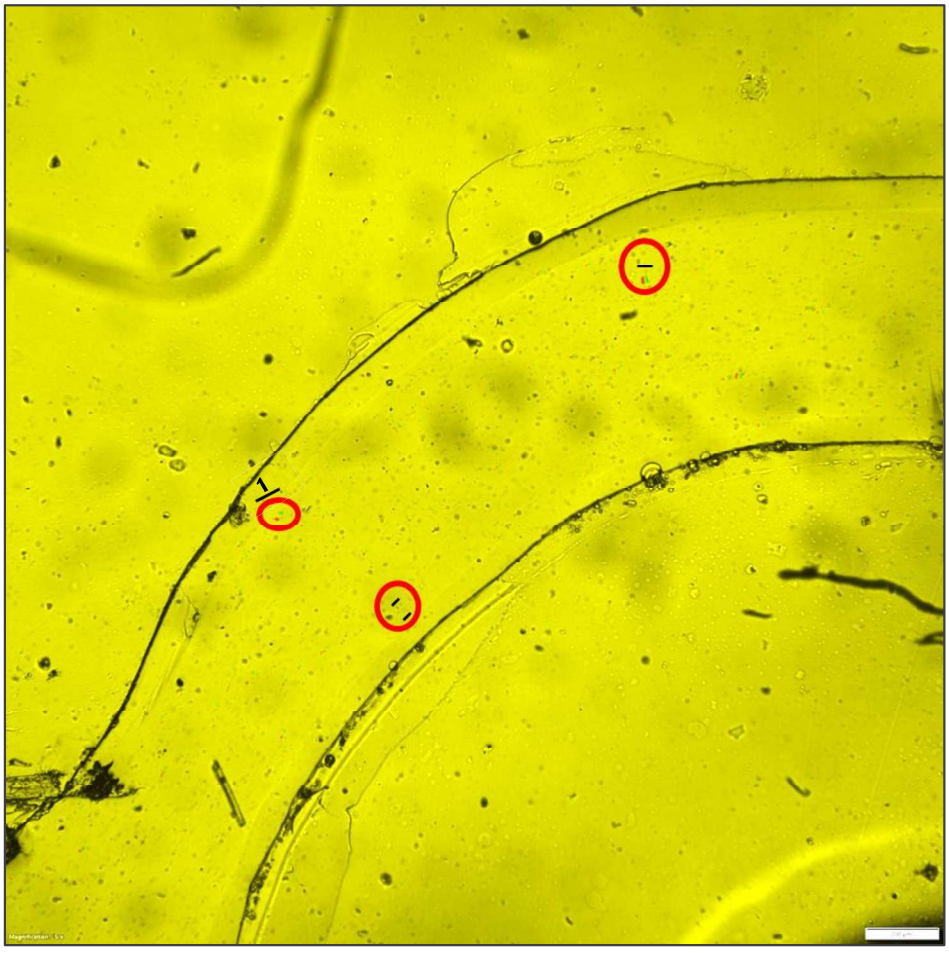


Figure 4.12: Flow rate of nanoparticle solution inside the C-shaped µFD as obtained by time-lapse imaging at four frames per second. (The green circle represents the rear position and the red circle represents the front position of a nanoparticle; the black lines represent the distance travelled by the nanoparticle at a particular time point). For instance, here nanoparticle 1 has moved a distance of 11.7 µm in 0.25 seconds as measured during time-lapse imaging by CellSens software. The scale bar corresponds to 200µm.

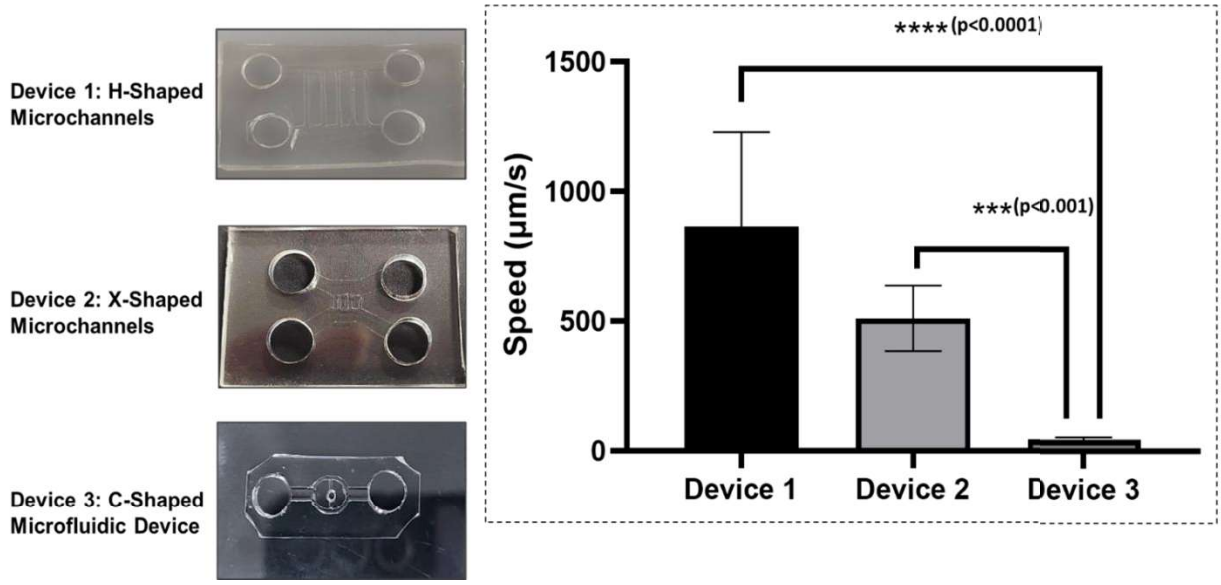


Figure 4.13: Quantitative Graphical Analysis of the speed (µm/s) of nanoparticles inside the three µFDs: Device 1 – H-shaped microchannels; Device 2: X-shaped microchannels; Device 3: C-shaped with straight microchannels. The graph was plotted using GraphPad Prism. One-way ANOVA was applied which reflected a high significance between Device 1 and Device 3 (****p<0.0001) and Device 2 and Device 3 (***p<0.001)

4.4.4. Cytocompatibility Studies of the Microfluidic Device

4.4.4.1. Primary neuron culture and neurosphere generation

Primary neuron culture bears a closer resemblance to the in vivo environment of the human brain. It provides insights into the fields of neural differentiation, synapse formation as well as neuronal communication. However, it still lacks in reconstituting the intrinsic spatial integrity which calls for a 3D culture system in the form of organoids. Neurospheres, which are the free-floating cultures of Neural Progenitor Cells (NPCs) have the potential to represent spatial cellular environment in a better way[17]. So, to develop a more *in vivo*-like environment, primary neurons from postnatal Wistar pups were cultured inside the three fabricated µFD of different designs. As mentioned earlier in sub-section 4.4.3, based on the quantitative graphical analysis data, primary neuron culture was performed in all three devices (H-shaped, X-shaped and C-shaped) **[Figure 4.14]**. Out of this, in the C-shaped device, after 3 days of culture, it was observed that the primary neurons were able to form neurospheres inside the narrow channel in high density prominently **[Figure 4.15 A, B; Figure 4.16 A, B; Figure 4.17 A, B]** and they can also form a monolayer in low-density seeding [**Figure 4.7 C**]. The neurospheres were able to be maintained for 10 DIV inside the microfluidic device. The formation of neurospheres was confirmed by the presence of the neuronal marker - beta-III Tubulin (TUJ1) (Anti-β-Tubulin III Antibody-MAB1637, Sigma-Aldrich, Mouse Monoclonal, diluted at 1:500), followed by incubation with anti-mouse IgG H&L Alexa Fluor 594 (Abcam, ab150116). The neurosphere culture could be used for detailed analysis of complex signalling events taking place between different neuronal stem cells (NSC) when present in immediate contact with other supporting cells like astrocytes, and oligodendrocytes. The 3D structure of the neurosphere creates a niche that allows the modelling of different environments by modifying them with different chemical and physical cues[27,28]. A combination of neurosphere

and microfluidics-based culture platforms can give more in vivo mimicking platforms for different disease model studies[29].

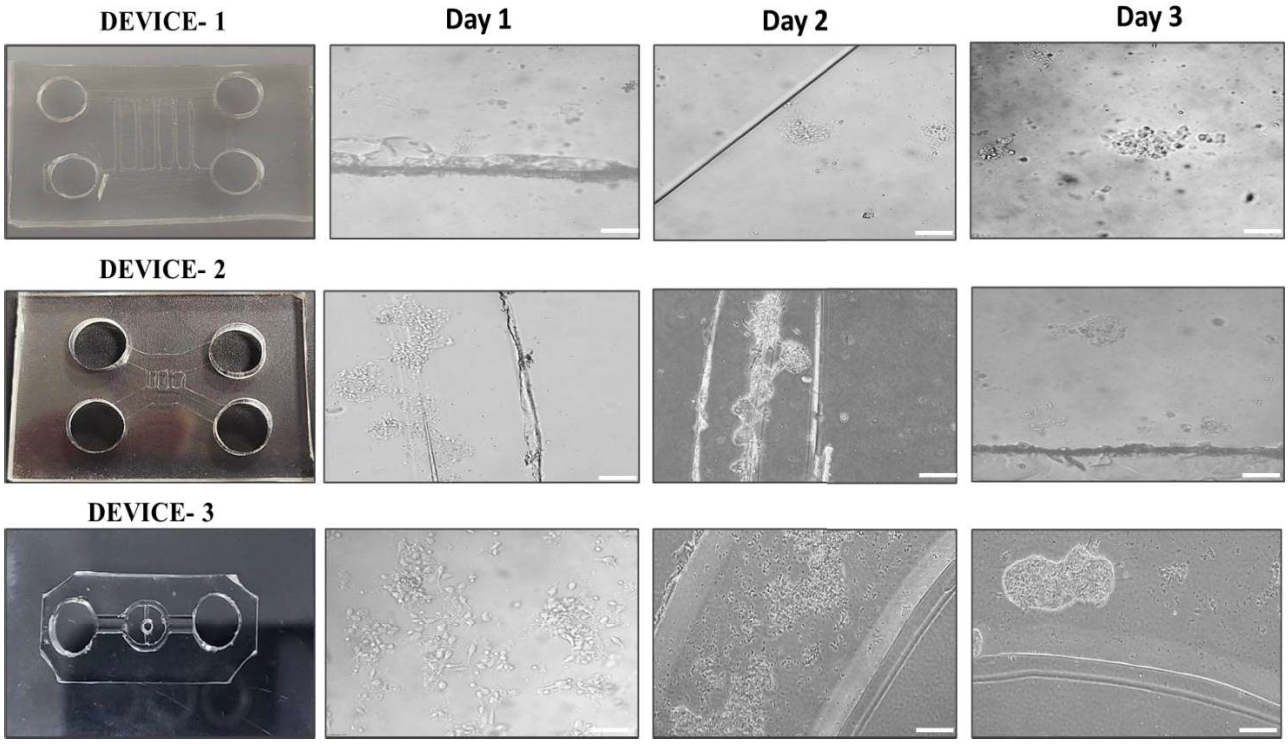
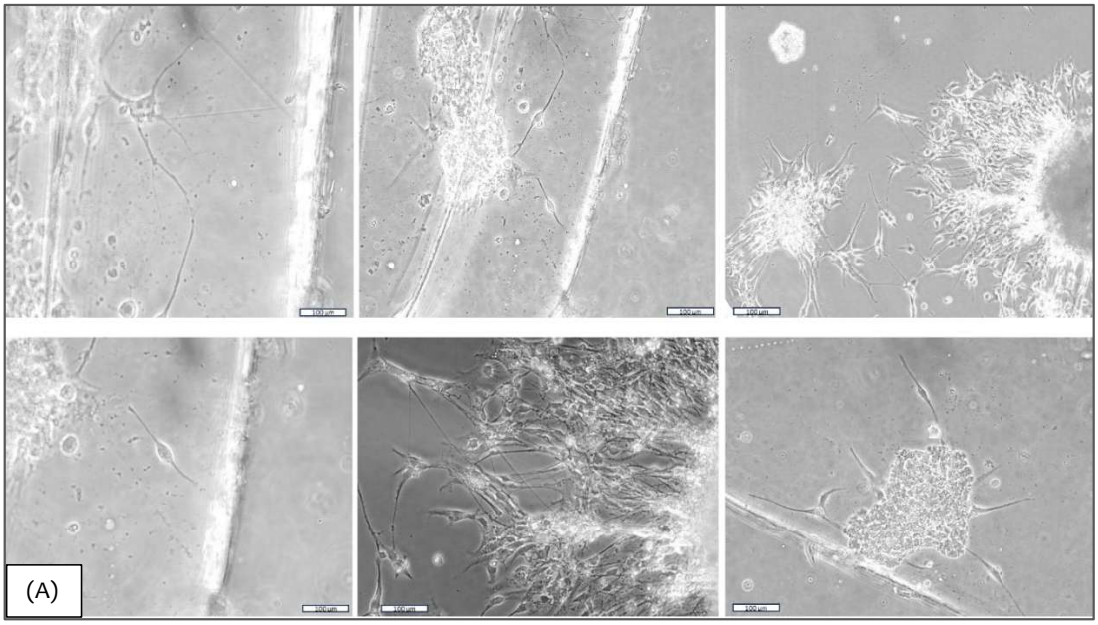


Figure 4.14: DIC images of primary neuron culture inside the microfluidic device with 3 different designs; Scale Bar corresponds to 50µm. In Device-1 and Device-2, cells were either getting washed out or clogging the channels respectively. However, in Device-C, after day 2 of cell seeding, primary neurons started to form prominent neurospheres.



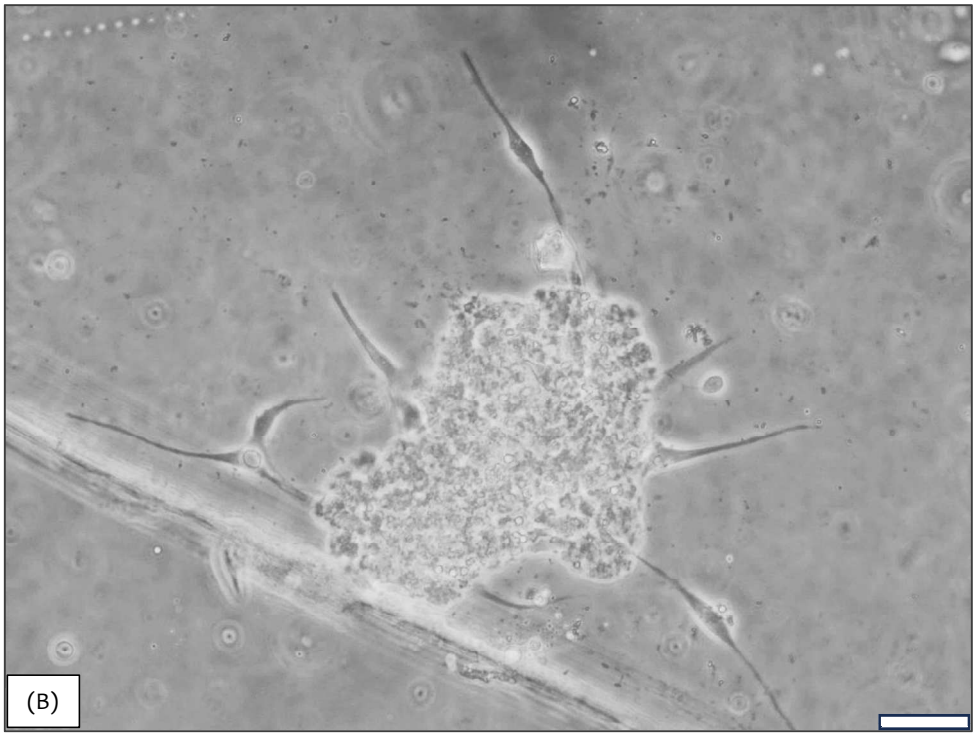


Figure 4.15: DIC images of primary neuron culture inside the C-shaped µFD (A) views at different regions inside the device, scale bar =100µm; (B) Prominent single neurosphere formed after 3 days of primary neuron culture. Viewed at 40X magnification, scale bar=200µm

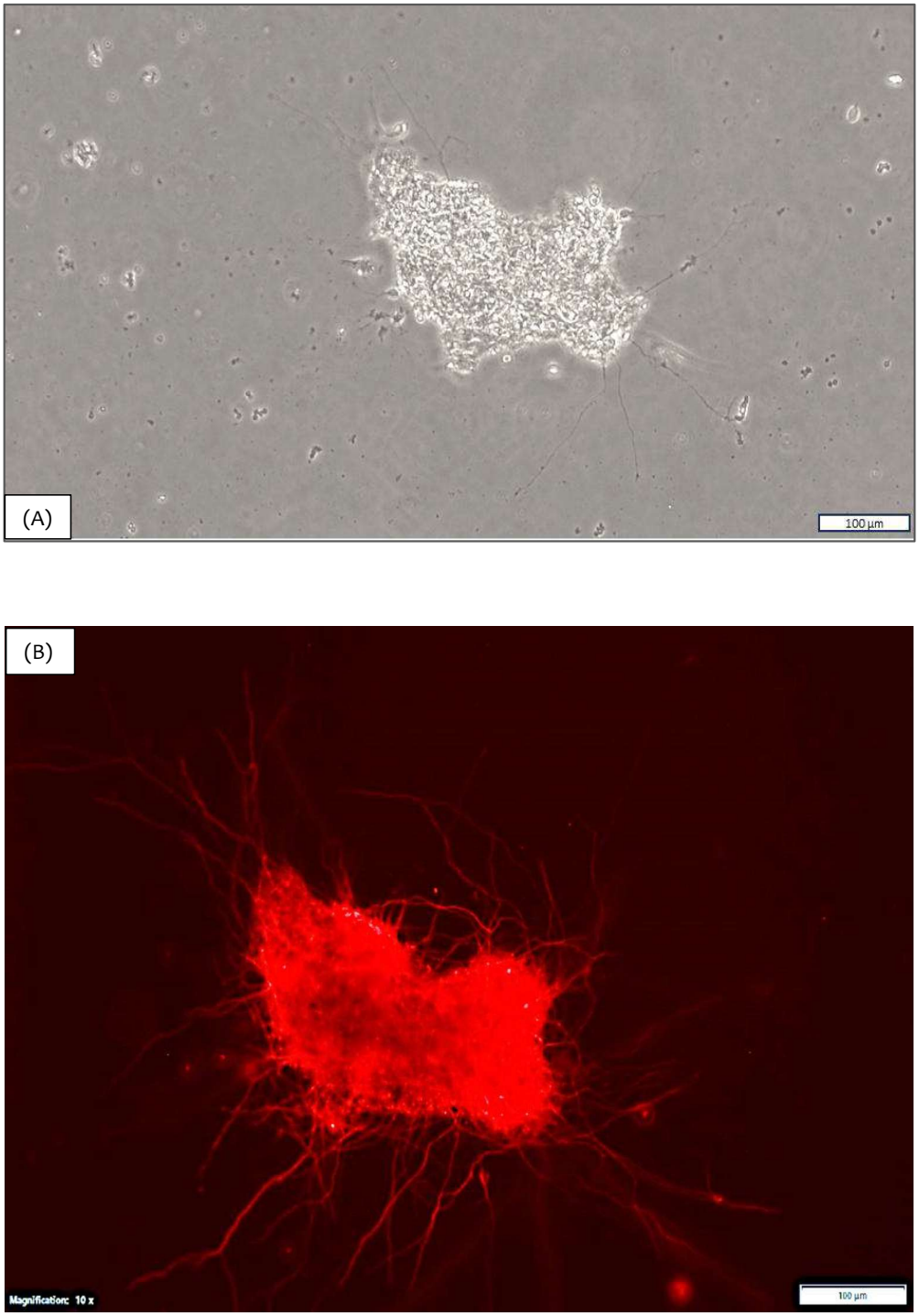


Figure 4.16: Neurosphere formed inside C-shaped µFD from primary neuron culture (A) DIC image of the neurosphere fixed at day 3 inside the C-shaped µFD, (B) Immunostaining of the neurosphere with Tuj1 neuronal marker inside the C-shaped µFD. scale bar=100µm

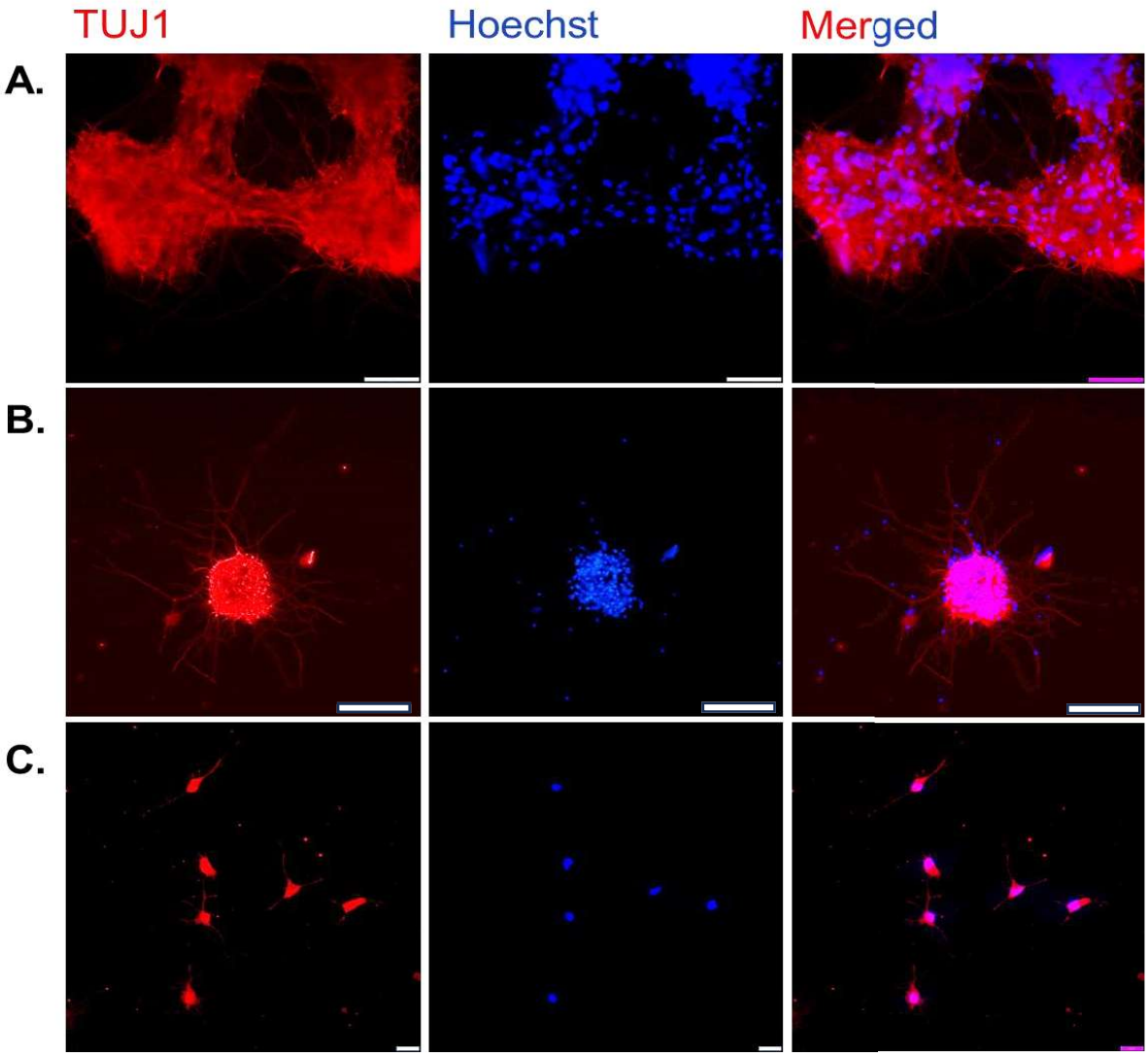


Figure 4.17. Primary Neuron Culture inside the microfluidic device A. TUJ1 immunostained neurosphere forming a connection inside the microfluidic channel. B. TUJ1 immunostained a single neurosphere in the microfluidic channel. C. Monolayer primary neuron culture was immunostained with TUJ1 inside the microfluidic device. The scale bar corresponds to 50 µm.

4.4.4.2. SH-SY5Y culture and its differentiation in the microfluidic device

Neuronal disease models are widely studied with different mammalian cell lines, including SH-SY5Y, a human neuroblastoma cell line. Depending upon the research interest, SH-SY5Y cells are used either after neuronal differentiation or without inducing differentiation. As SH-SY5Y cells resemble neuroblasts, they can be differentiated using a variety of differentiation agents, such as neurotrophins (such as brain-derived neurotrophic factor, or BDNF), and retinoic acid (RA), into a more mature neuronal phenotype. Besides 2D cultures, microfluidic cell culture devices have been found suitable for SH-SY5Y culture and differentiation for neuronal studies. During seeding, 50000 cells were seeded in each device, and it was found that within 4 hours of seeding, cells started to attach to the surface **[Figure 4.18A]** and started to show their typical morphology**.** Within 2 DIV, the cells reached confluency inside the device **[Figure 4.18B]**. Differentiation was attempted first with 5µM R.A and 50 ng/ml BDNF **[Figure 4.19]**. However, due to less successful differentiation, the concentration of R.A. was increased, and differentiation was achieved with the application of 10µM R.A and 50 ng/ml BDNF **[Figure 4.20],** and the neuronal differentiation was validated with the presence of a TUJ1 neuronal marker **[Figure 4.21C]**. Live-dead assay of differentiated SH-SY5Y cells was

confirmed both in low and high seeding densities inside the device using Calcein-AM-Propidium Iodide (PI) imaging that gives green fluorescence upon binding with live cells **[Figure 21: A, B]**. Overall, these results demonstrate the potential of our microfluidic device as a tool for on-chip differentiation experiments, without requiring the replacement of medium or a significant drop in the concentration of nutrients.

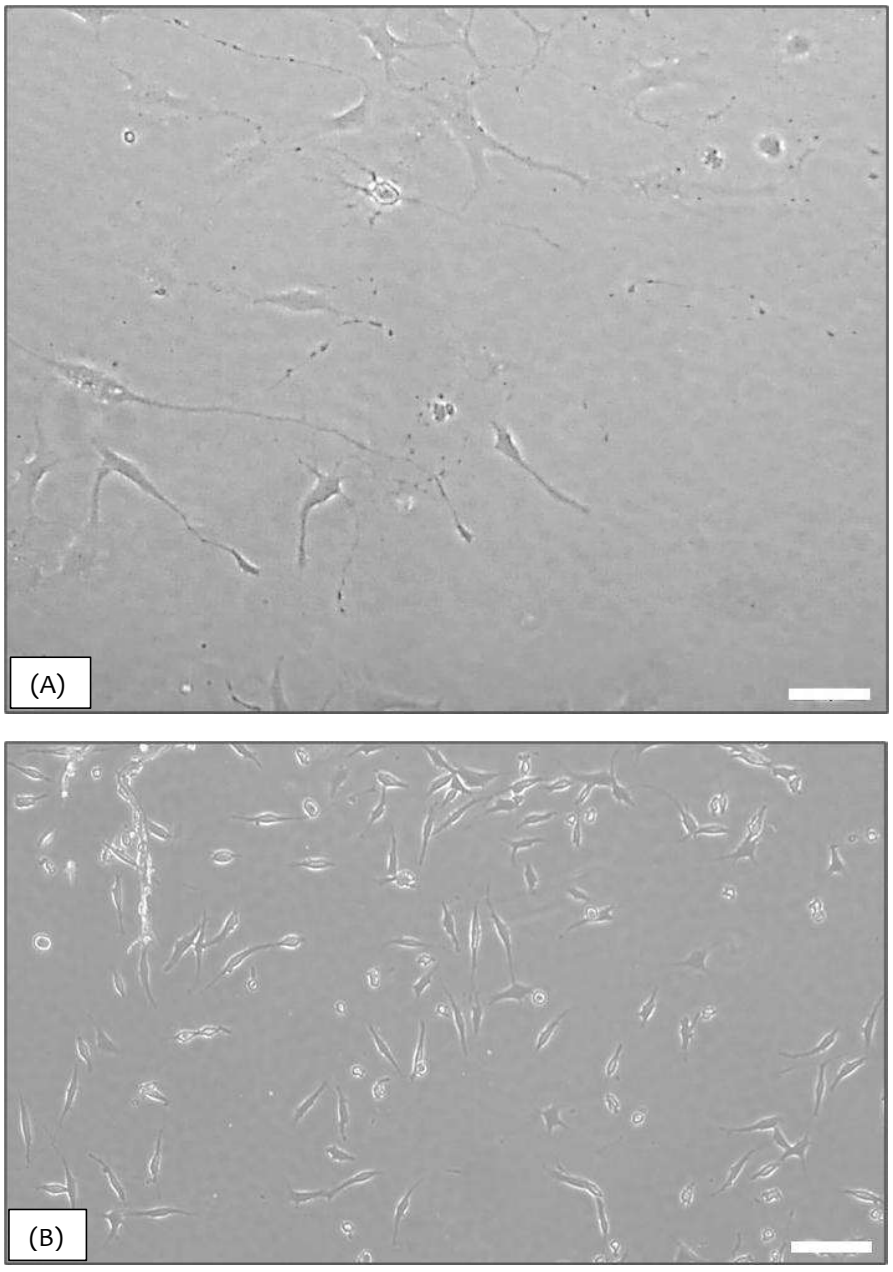


Figure 4.18: SH-SY5Y Culture inside the μFD (A) within 4 hours of cell seeding (B) after 48 hours of cell seeding. Scale Bar corresponds to 100 μm.

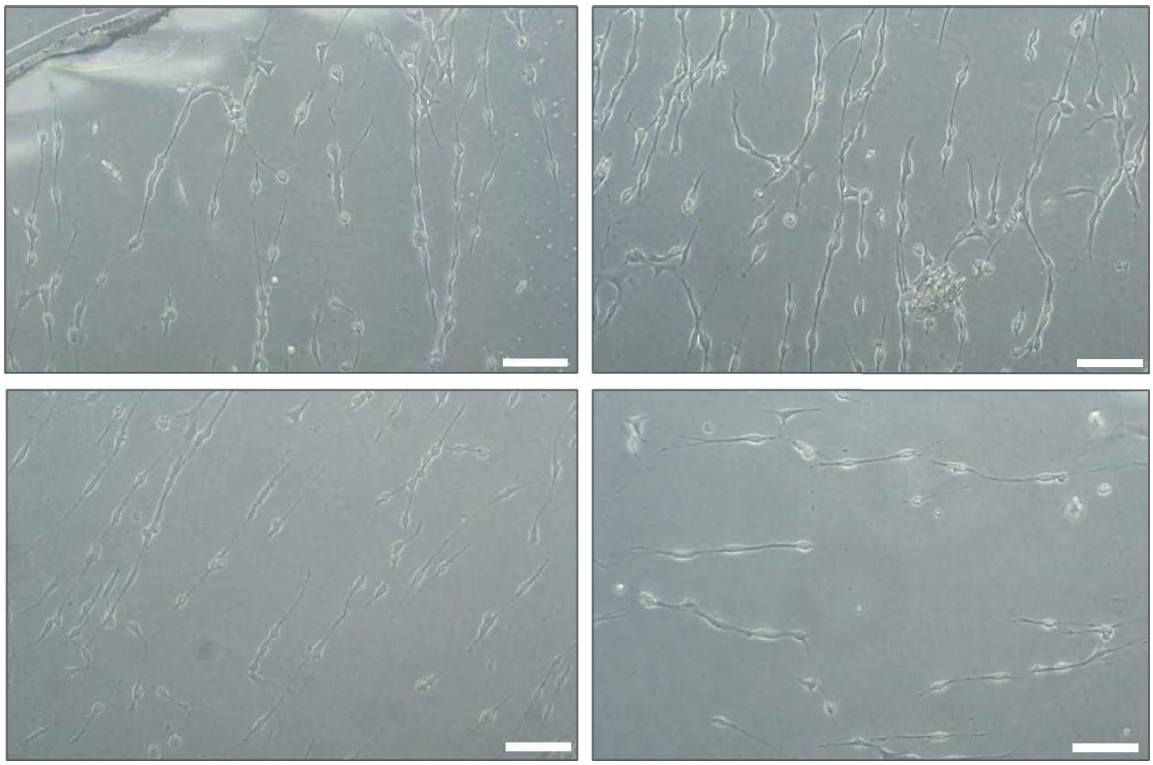


Figure 4.19: SH-SY5Y differentiation using 5 µM R.A. and 50 ng/ml BDNF. Scale bar = 100 µm

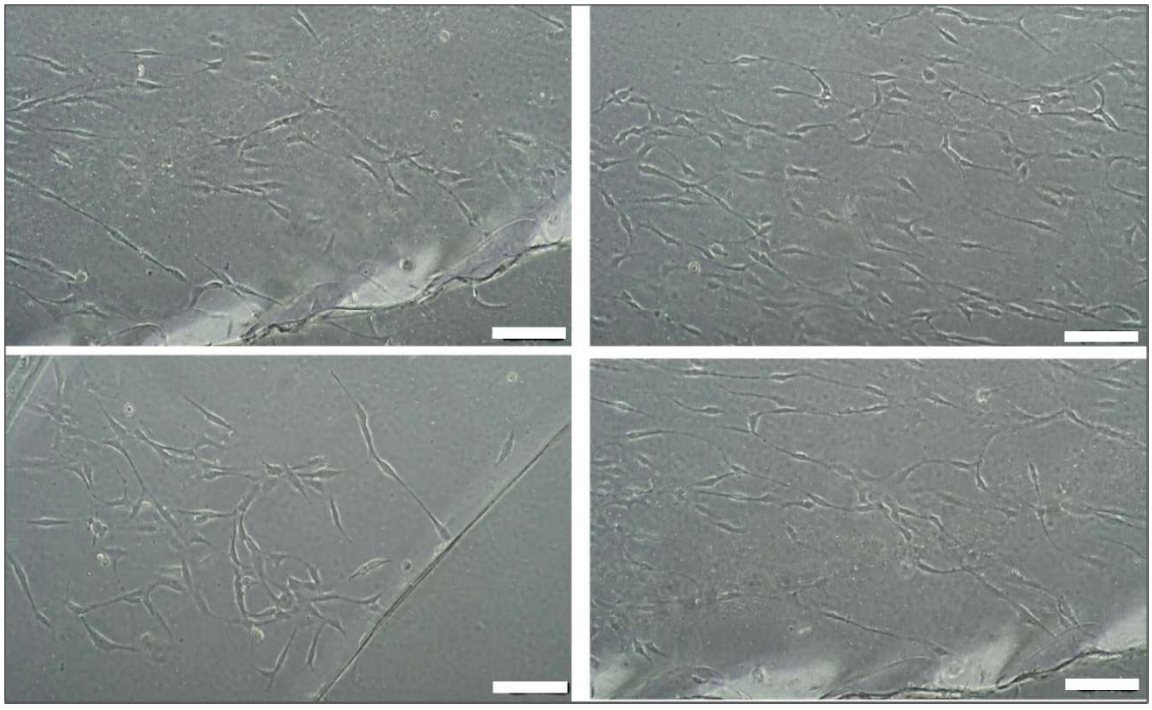


Figure 4.20: SH-SY5Y differentiation using 10 µM R.A. and 50 ng/ml BDNF. Scale bar = 100 µm

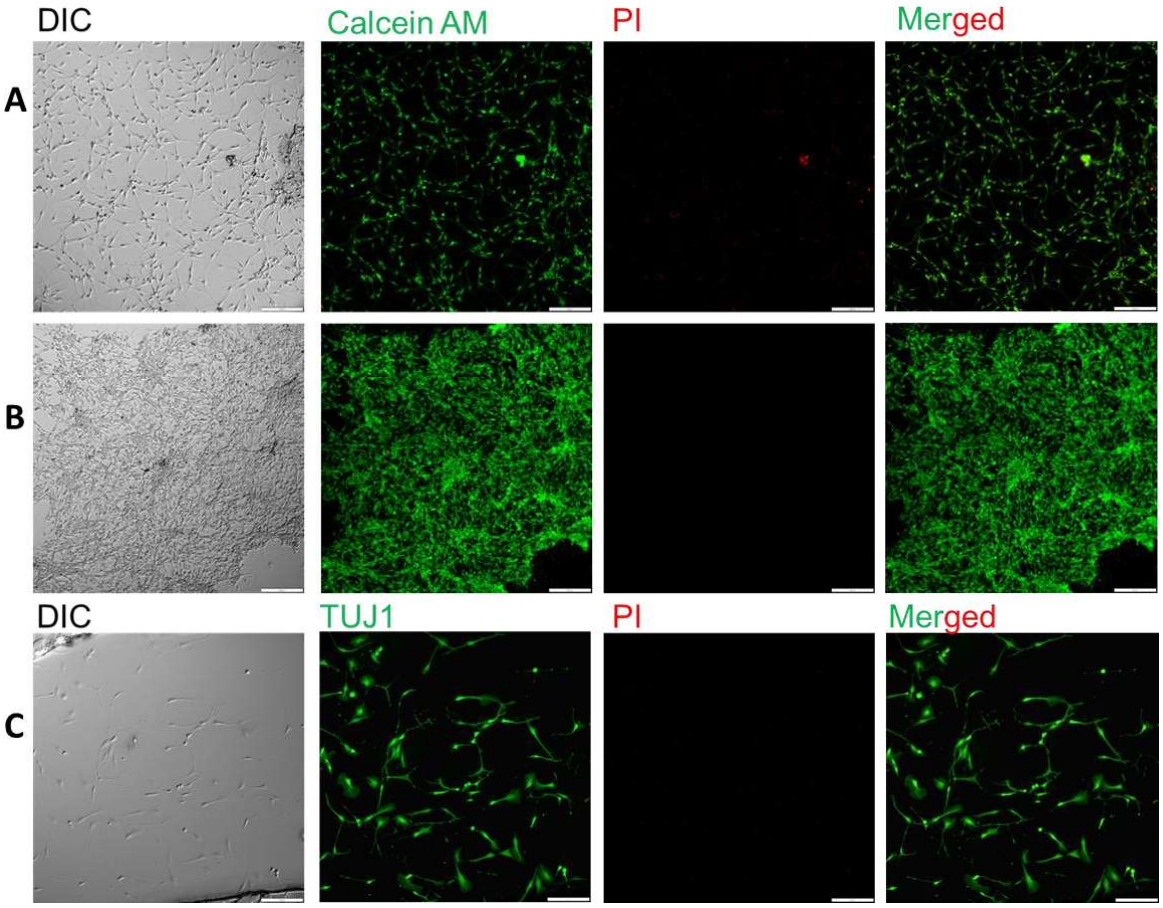


Figure 4.21: Validation of the device as a suitable platform for cell-based studies. A. Suitability of the device for differentiated SH-SY5Y cell lines seeded in low density, their subsequent DIC images and Live/Dead analysis using calcein AM (green) and PI (red) staining. B. Suitability of the device for differentiated SH-SY5Y cell lines seeded at high seeding density, their subsequent DIC images and Live/Dead analysis using calcein AM (green) and PI (red) staining. C. TUJ1 immunostained Differentiated of SH-SY5Y using 10µM Retinoic Acid and 50 ng/ml BDNF. The scale bar corresponds to 200 µm.

4.4.4.3. Co-culture of primary neurons and astrocytes

Non-neuronal cells like glia play an active role along with the neurons in forming the brain's complex cytoarchitecture. It is reported that glia plays a role in establishing synapses because astrocytes, a type of glial cells, parallels the formation of synapses. Furthermore, it has been observed that soluble substances released by astrocytes, such as thrombospondins, activity-dependent neurotrophic factors, and tumour necrosis factor-a, affect synaptogenesis. Therefore, to decipher the role of glia in neural development and communication, additional studies are vital. There exist a few described microfluidic systems that allow neurons and glia to be co-cultured. So, here we report a microfluidic chip that could potentially give rise to a co-culture platform for primary rat neurons and astrocytes. Co-culture of astrocytes and neurons was done in the microfluidic device in vertical manner following the protocol of Mingjian Shi and Devi Majumdar *et al* [30] [**Figure 4.22**]. Astrocytes were grown onto a PDMS surface and neurons were grown onto a glass surface. The co-culture is confirmed by the expression of astrocyte and neuron-specific marker GFAP (Anti-GFAP primary antibody-ab7260 Abcam, Rabbit Polyclonal antibody; diluted at 1:2000) and TUJ1 (Anti-β-Tubulin III Antibody-MAB1637, Sigma-Aldrich, Mouse Monoclonal, diluted at 1:500), respectively

followed by incubation with Alexa Fluor™ 488 [anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody- A11008, Invitrogen, diluted at 1:1000] for GFAP and Anti-mouse IgG H&L Alexa Fluor 594 (ab150116, Abcam, diluted at 1: 1000) for Tuj1 **[Figure 4.23 C]** and Z-slice fluorescent imaging **[Figure 4.23 A, B]**. Our co-culture model was further used for the screening of a neuroprotective agent against Aβ42 toxicity mimicking Alzheimer's disease.

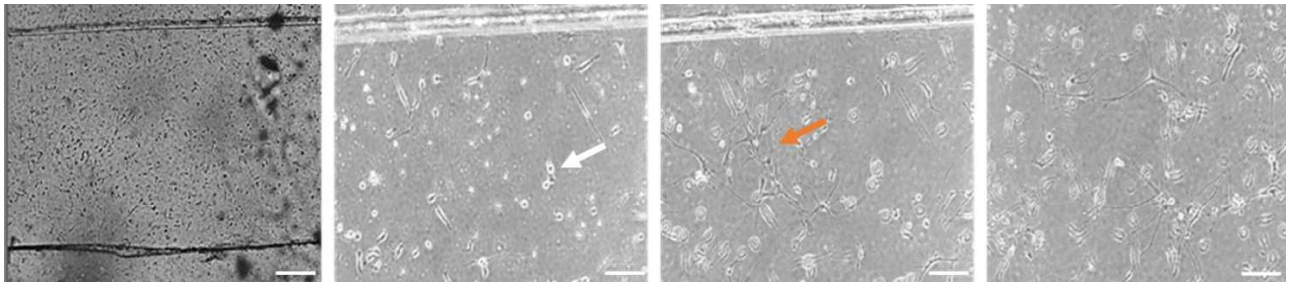


Figure 4.22: DIC images of astrocytes and neurons co-cultured inside the microfluidic channel. Astrocytes were grown onto a PDMS surface (white arrow), and neurons were grown onto a glass surface (orange arrow). As we shifted the focus from PDMS to the glass surface, astrocytes and neurons were visible respectively. The scale bar represents 100 µm.

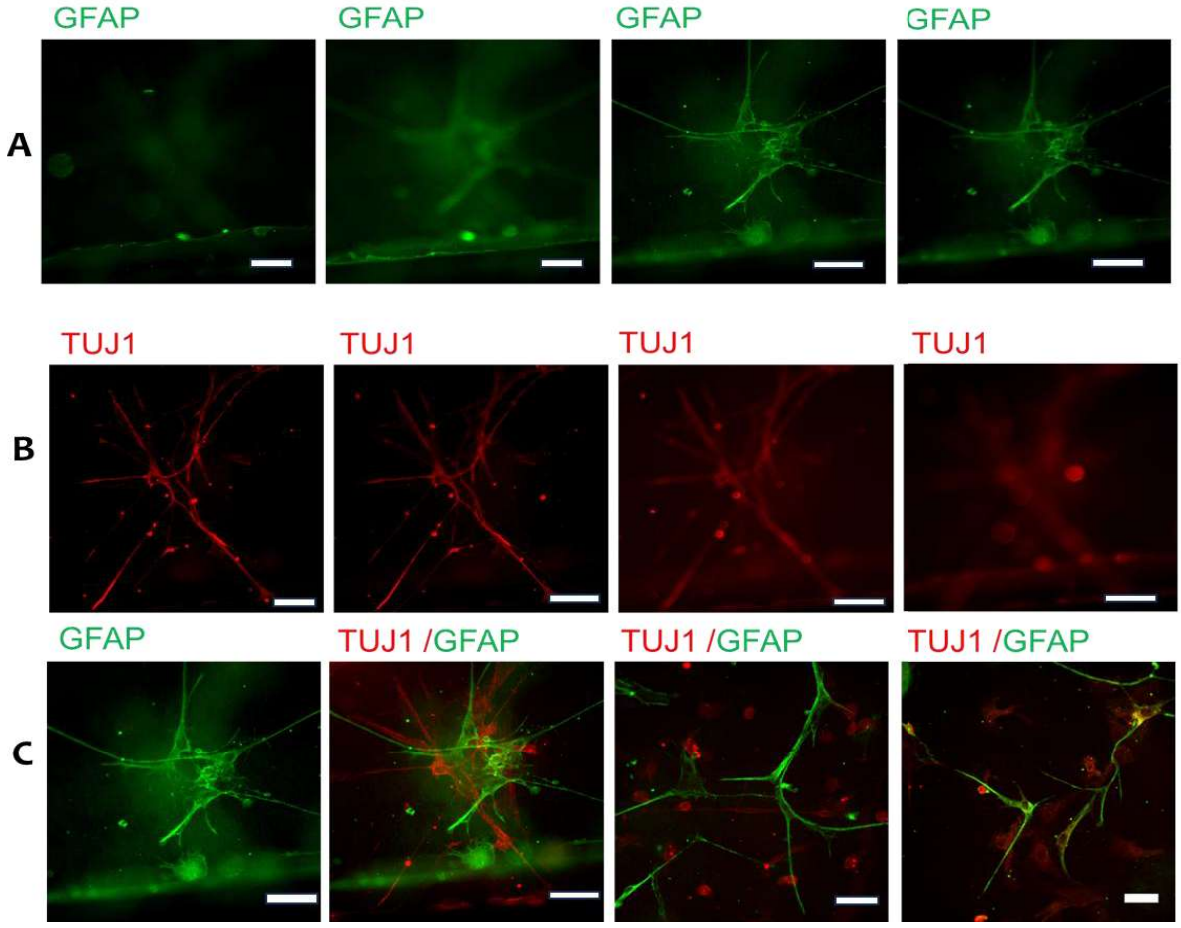


Figure 4.23: Co-culture of Astrocyte and primary Neuron. (A, B) Z slice imaging to confirm vertical co-culture. Images were captured in 0.31 µm Z-slice. Green cells represent GFAP-positive astrocyte cells, and red cells represent TUJ1-positive neurons (C) Merged Z-slice images captured in red and green channels. The scale bar represents 20 µm.

4.5. CONCLUSION

Microfluidic devices have become indispensable tools in various scientific fields, particularly in biological and biomedical research, where precise control over fluid manipulation at the microscale is achievable. Unlike 2D cultures, where cells grow in monolayers, 3D microfluidic devices enable 3D cell growth by creating constrained microenvironments for cells to grow in multiple layers thereby, closely mimicking in vivo tissue architecture and behaviour. Nowadays, different methods have been used so far to develop microfluidic devices with desired features predominantly using the soft lithography technique. Although lithographic techniques offer lab-on-chip devices with better resolution, they often come with the requirement of expensive raw materials like silicon wafers, negative photoresist material, developer solutions and high-end equipment like mask aligners. This calls for a fabrication method that is less cumbersome, and cost-effective yet highly versatile and efficient. Xurography is one such rapid prototyping method that allows for precise patterning of microchannels, facilitating effective fluid control and a highly controlled cell culture environment. In this work, we have opted for this low-cost Xurography approach to fabricate our desired 3D microfluidic chip that can potentially solve the bottlenecks of conventional 2D petri plate culture systems. Our microfluidic device proved to be multifaceted in replicating in vivo-like conditions, ensuring neuronal cytocompatibility and cellular viability. Through the primary neuron culture forming neurospheres at high and single cells at low seeding densities, it was evident that with the use of microfluidic culture platforms, it is possible to develop conditions that can potentially replicate in vivo brain development. The ability of this device to support a co-culture model for drug screening with minimal reagent consumption and high reproducibility highlights its utility as a versatile tool in biomedical research in future. While microfluidic lab-on-chip platforms present transformative capabilities for a multitude of applications, further research is still required to establish standardized protocols for materials selection, and on-chip sample preparation and deal with technical issues like sample loss, cross-contamination and clogging.

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