Chapter 5: Long-term growth and high-resolution Imaging of C. elegans in microfluidic device

5.1) Introduction

Advancement in microfabrication technology has led to the development of various microfluidic devices for study in cells (Santra and Tseng, 2013; Tseng and Santra, 2015; Zhu and Yang, 2017), tissues (Yang et al., 2016) and model organisms such as C. elegans (reviewed in Muthaiyan Shanmugam and Subhra Santra, 2016), Drosophila (reviewed in Ghaemi and Selvaganapathy, 2016) and zebrafish (reviewed in Yang et al., 2016). Though microfluidic devices have been extensively used for high throughput cells screening, culturing and imaging, advanced microfluidic devices for organisms are now gaining interest due to their biocompatible environment for the organisms. Additionally, understanding the cell biological, physiological and developmental processes in model organisms is gaining interest due to their resemblance to human pathology and intact metabolism compared to cells in culture.

C. elegans due to its short life cycle, smaller body size, transparency, easy maintenance, 40% homology with the human genome and well-characterized genetics, have gained a special interest in exploring cell biological and developmental processes. It has established itself as a preferred model organism especially for studying neuron development due to its transparency and a low number of neurons which show sufficient complexity and conserved mechanisms of neuron development (Hobert, 2010; Sainath and Gallo, 2015). To understand any developmental phenomenon, observation of the process for a long-time period is required. In microfluidic devices, despite multiple attempts, long-term imaging of C. elegans has been a challenge due to poor efficiency of worm viability. Nonetheless, multiple microfluidic platforms have been designed for C. elegans for high throughput screening (Cáceres I.de et al., 2012; Chung et al., 2008; Chung et al., 2011; Wang et al., 2013; Ai et al., 2014), high-resolution imaging (Chalasani et al., 2007; Mondal et al., 2011; Cáceres Ide et al., 2012; Cornaglia et al., 2015), drug screening (Gosai et al., 2010; Mondal et al., 2016), synaptic development (Allen et al., 2008), long-term imaging for calcium imaging (Chronis et al., 2007; Chalasani et al., 2007) and neuron regeneration (Guo et al., 2008). C. elegans have been immobilized in microenvironment by
micron size beads (Kim et al., 2013), anesthetic gas (Chokshi et al., 2009), temperature sensitive gels (Krajniak and Lu, 2010), lower temperature (Chung et al., 2008), and flexible membranes (Guo et al., 2008).

Exposure to anaesthetics may lead to adverse effects on animal health, metabolism or cellular activities such as neuronal growth, regeneration, and trafficking (Guo et al., 2008; Mondal et al., 2011). In comparison, anesthetic-free immobilization allows the animal to remain viable and healthy for a longer term after release from the immobilization. Inside the microfluidic chamber, the animal can grow in liquid culture with constant bacterial food supply (Krajniak and Lu, 2010; Krajniak et al., 2013; Xian et al., 2013; Lee et al., 2014). Many groups have tried to maintain C. elegans culture in microfluidics for high throughput screening (Rohde et al., 2008; Chung et al., 2008), high-resolution imaging of synapses growth (Allen et al, 2008) and low-resolution imaging to see development and biomarker imaging. A recent study shows long-term imaging of C. elegans in the multi-channel device and captured cellular processes (Keil et al., 2017). However, to best of our knowledge, no study has shown high-resolution long-term imaging of single C. elegans development beyond 5 hours.

In this work, I have developed and established a protocol for using growth and imaging device developed in the laboratory by Sudip Mondal (see method section for fabrication protocol) for long-term imaging and culture of C. elegans to observe various cell biological and developmental processes. The growth and imaging device are fabricated with PDMS elastomer which is a suitable material for microfabrication due to its chemical stability (Yue et al., 2011), biocompatibility (Folch & Toner, 1998; Peterson et al., 2005) and modifiable stiffness (Palchesko et al., 2012; Larsen et al., 2016). Further, transparency of PDMS allows transmission of light thus, ease imaging in the trapped animal.

Study related to aging and/or developmental defects requires tracking of animals for a very long time. Mutants are more sensitive to anesthetics concentrations and thus repeated anaesthetization may cause adverse effects on the physiology and growth of such animals. Our device provides a microenvironment that would be useful for observing the morphological, behavioral and
functional defects in animals over a long period of time. Our device is easy to fabricate and can be set up in any laboratory to address long-term developmental questions in *C. elegans* that require intermediate high-resolution imaging.

5.2) Growth and imaging device for long-term culture and imaging of *C. elegans*

To understand any developmental phenomenon, it is required to track the process for a long time period. In few studies, *C. elegans* have been kept inside a microfluidic chamber (Xian et al., 2013) or channel (Krajnik and Lu, 2010) and imaged periodically at low and high resolution respectively. In this work, I have established a protocol to be able to use a microfluidic device for long-term imaging of *C. elegans* with periodic immobilization for high-resolution imaging. This device is modified version of device published (Mondal et al., 2011) with patent number 640/CHE/2011. The modification includes an increased parameter of trapping membrane and addition of isolation membranes. The device has two layers: control layer where the animal is inserted and cultured with constant food supply by inlet and outlet (fig.1a.) and another is trapping layer (fig. 1b) which is used to trap the animal for successive imaging. Control layer provides a microenvironment for the growth of *C. elegans* with constant food supply mixed with liquid medium. In the second pattern, there are isolation membranes that keep the animal in isolation by deflecting the membrane on the application of pressure in the second layer. A Single animal or egg is loaded into the inlet of flow layer and held between isolate membranes in flow layer in liquid culture (fig.1). *C. elegans* allowed to move freely in 300µm wide flow layer between isolation membranes during the inter-imaging time interval.
**Figure 1: Schematic of a microfluidic chip and membrane usage**

Control layer (a) and trapping layer (b) are bonded together to form chip (c), animals is inserted in inlet of flow layer of the device using micro-tip (d), (e), (f) and animal is trapped in center of the membrane (g) by application of N2 gas through tubes (h), (i) as marked by arrows, (i) represents image of the growth and imaging chip with food supply set up. Patent number 640/CHE/2011

The width and height of channels are 300µm and 100µm respectively, with trapping membrane thickness (orange arrow in b) =1mm.
5.3) Established Protocol for using the microfluidic device for growth and imaging of *C. elegans*

For the growth of the animal, constant growth medium and food supply are important. The deficiency of any of these could affect the health of the animal. We established the following protocol for using the growth and imaging device for high-resolution imaging of single *C. elegans*.

5.3.1) Fabrication of microfluidic device

5.3.1.2) SU8 master fabrication:

1) Design patterns I and II using clewin software (clewin 2.90, wei web software, The Netherlands) and print with the help of 65,024 DPI laser plotters with the feature size of 8 µm on circuit board film (Fine Line Imaging, Colorado, USA).

2) Cut silicon wafers (University Wafer, MA, USA) in pieces of 2.5cm X 2.5cm and clean them with 20%KOH for 1minute followed by rinsing in distilled water (deionized). One device corresponds to two pieces for both flow and control layer.

3) Clean dry the pieces with compressed nitrogen gas followed by dehydration on a hot plate at 120 degrees Celsius for four hours. Before proceeding to next step cool these pieces down to room temperature.

4) Take a silicon piece and put on spinner chunk and turn on the vacuum. On silicon piece, put ~20 µL hexamethyldisilazane (HMDS, Sigma-Aldrich, Bangalore, India) and coat them using the SPIN150 spinner at 500 rpm for 5 s followed by 3000 rpm for 30 s.

5) To get uniform photoresist thickness of ~40 µm, coat silicon wafers with ~1.5 mL of SU8-2025 (http://www.microchem.com/Prod-SU82000.htm, Microchem, MA, USA) using SPIN150 spinner (SPS-Europe B.V., The Netherlands) at 500 rpm for 5 s followed by 2000 rpm for 30 s. This thickness is suitable for imaging early larval stages.

6) Alternatively, to get the thickness of ~80 µm, coat silicon wafers with ~1.5 mL of SU8-2050 (http://www.microchem.com/Prod-SU82000.htm, Microchem, MA, USA) using SPIN 150 spinner (SPS-Europe B.V., The Netherlands) at 500 rpm for 5 seconds
followed by 2000 rpm for 30 seconds. This thickness is suitable for late larval stages, such as late L3 to adult.

7) Soft bake the silicon pieces with the SU8 coating on a hot plate at 65°C followed by 95°C for 10 minutes. Let the pieces cool down before proceeding to next stage.

8) Put soft baked silicon pieces on exposure stage of UV illuminator with SU8 coated surface facing up towards UV lamp (Oriel Instruments, Bangalore, India). Expose SU8 layer to UV lamp through a photomask with pattern I and II separately to get flow and control layers respectively to build the single device. This UV exposure time is 15 seconds with 200-Watt lamp.

9) Bake the exposed silicon pieces with coated layer facing upside, at 65 °C followed by 95°C for 1 and 10 minutes respectively. Cool down the pieces at room temperature.

10) Develop the patterns by keeping silicon pieces in SU8 developer (Microchem, MA, USA) solution for 20 minutes. Once the pattern is visible, rinse the pieces in Iso-Propyl Alcohol (IPA) and gently blow dry with nitrogen gas (14psi).

11) Keep the silicon pieces in a desiccator with coated surface facing top and along with 50 µL of trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (Sigma-Aldrich, Bangalore, India) vapor in a desiccator for 2 hours.

5.3.1.2) PDMS mold fabrication:

1) Make PDMS in a plastic cup by mixing Sylgard 184 base with the curing agent in the ratio 10:1 (Dow Corning Corporation, MI, USA). Mix well the contents by stirring constantly for 3 minutes to get air bubbles.

2) Degas this mixture by keeping in a desiccator to remove all air bubbles.

3) Keep silicon wafers for control layer (pattern II) in a petri dish. Pour gently 5 mm thick PDMS mixture on silicon pieces. Degas by keeping the petri dish in desiccator at low vacuum

4) Keep silicon wafer with the pattern I, flow layer, on spinner chuck with the help of vacuum pressure, enough to hold the wafer. Pour silicon wafer with ~1 mL PDMS and coat it using SPIN150 spinner at 500 rpm for 5 s followed by 1000 rpm for 30 s.
5) Bake these PDMS coated silicon wafers of both patterns I and II at 50 °C in hot air convection oven for 6 hours. After baking, wait to cool down pieces at room temperature.

6) From petri dish with wafers of pattern II, cut out the PDMS piece using a sharp blade and punch a hole of ~1 mm diameter using Harris puncher at the reservoir which connects main trap to the gas lining.

7) Place silicon wafer with the design I with PDMS coating facing up on a plastic tray. Also, keep punched PDMS block with design II on the tray with molded side facing up. Keep the plastic tray inside plasma cleaner (Harrick Plasma, NY, USA) and expose the two blocks to 18-Watt air plasma for 2 minutes under low vacuum. Reduce the pressure till the chamber turns bright violet.

8) Take out the two plasma treated blocks and gently bond by pressing plasma treated surfaces of patterns I and II together. Bake this bonded pattern at 50 °C for 2 hours in hot air convection oven.

9) Cut the bonded device out of silicon wafer with the pattern I and punch holes in inlet and outlet reservoirs of flow layer.

10) Place this bonded PDMS block with flow layer facing up on a plastic tray. In addition, keep clean coverslip on the same tray and expose to 18-Watt air plasma for 2 min. Adjust pressure to see violet chamber.

11) Bond the plasma exposed layer of PDMS and coverslip and keep in an oven at 50 °C for 2 hours.

12) For future use, keep the device at a clean place.

5.3.2) Using microfluidic device membrane:

1) Connect micro flex tube (Inner diameter ~ 5mm, outer diameter ~8 mm) to a compressed nitrogen gas line with the 3-way connector on the other side. Further, connect micro flex tube with inner diameter ~1.6 mm, outer diameter ~5 mm to one of the outlet port of 3-way stopcocks with
another end connected to 8 mm 18 gauge needle.

2). With the help of micropipette, fill the flow layer with buffer.

3). Fill both tubes with de-ionized water through end connected to a needle. Allow this water to fill the top surface of pattern II through the punched holes, connecting isolating and trapping membrane.

4). For filling water, open the nitrogen gas regulator at 14psi to push the water forward. Wait till water fills the channel without the presence of any air droplet in both channels.

5). Once the channels are filled with water, release the pressure by a 3-way stopcock.

6). Next, prepare S. medium under sterile conditions and autoclave before use.

7). Using a single colony of *E. coli* OP50 inoculate a rich L Broth [2.5 g Bacto-tryptone, 1.25 g Bacto-yeast, 1.25 g NaCl, H$_2$O to 250mL] and grow the inoculated culture overnight at 37°C.

8). Inoculate 500 µl of the OP50 solution into multiple microcentrifuge tubes. Eppendorfs with bacterial culture may be stored for 2 weeks at 4°C.

9). Pellet down OP50 by centrifuge at 3000rpm for 5 minutes. Dilute the pellet with 1 ml of S medium so diluted by a factor of 1:2. This can be stored at room temperature for 3-4 days. Use this diluted OP50 to feed *C. elegans* inside the microfluidic devices.
5.3.3) Growing *C. elegans* inside the microfluidic device

1) Mount a growth and imaging microfluidic device on an inverted microscope and observe the control channel at low magnification. Assure that the channels are maintained in a clean environment without dust or particulate matter that may result in blocks.

2) Fill the control layer with S. medium 10 minutes prior to the experiment.

3) Pick a single animal of the required developmental stage from NGM plate using a micropipette containing 10 µl S medium and push the animal through growth/flow layer inlet with subsequent monitoring under lower objective. Keep pushing the animal till it reaches between isolation membranes in the flow layer.

4) Open the 3-way stopcock of isolation channel to apply pressure of 14psi to push the membrane down which restricts the animal between the isolation membranes. Ensure continuous flow of food even when the pressure is on and the animal should be able to move freely between isolation membranes.

5) Leave a drop of S Medium on top of the inlet and outlet reservoirs in order to avoid evaporation of solution and to keep the inlet or outlet port always filled with solution.

6) Take diluted OP50 solution in a 10µl micropipette. Remove the micro tip filled with the OP50 solution from the pipette and press the solution till the tip end by closing the inlet of the tip. It will ensure continuity of food solution without any air gap.

7) Now press the tip with food in the inlet reservoir. Repeat the step to put another tip with 10 µl food solution in the outlet reservoir.

8) Fill additional 20-30µl of food solution in both tips. Add or remove food solution to and from the micro-tip to adjust the gradient to push the animal according to requirement.

9) Monitor the continuous flow of bacteria in growth channel in bright field.
5.3.4) Immobilization and Imaging of *C. elegans*

1) Immobilization:

1.1) Locate the animal in growth channel at lower magnification. Adjust the food solution so as to push animal in the required direction to bring the animal in isolation from the reservoirs.

1.2) Position the animal at the center of the trapping membrane and monitor the animal behavior using low magnification objective (4x). Turn the 3-way stopcock to increase the pressure in trap channel slowly. Immobilize the animal under the trapping membrane in a straight posture along the growth channel boundary wall.

2) Imaging:

2.1) Use the inverted microscope at desired settings for high-resolution bright field or fluorescence imaging. Acquire single or time-lapse fluorescence images at the predefined frame rate at 60X or 100X as standardized.

2.2) After image acquisition, release the trap pressure and monitor the locomotion of the animal at low magnification. Keep the animal restricted within the region defined by isolating membranes.

2.3) Adjust the volume of food solution in the two micro tips to continue a slow food flow in the growth channel. The flow can be visualized in the bright field from the flow pattern of the bacteria in the growth channel.

2.4) Repeat steps 2.1-2.3 after the predetermined time interval to acquire fluorescence/ DIC/ Bright field images of the same individual at multiple time points.

2.5) After the data acquisition for multiple time points from the same individual is done, release the isolate and trap pressure. Flush out the animal in M9 buffer by pushing through the inlet channel with a syringe. Keep the animal in a plate for further health monitoring.

2.6) Flush the channel few times to remove food/bacteria. Rinse the channel one with alcohol. Dry the channels by pushing air using a syringe. The device can be stored for reuse in future.
5.4) Microfluidic devices do not affect the animal health

To ensure that growing animals in microfluidic devices do not affect the health of the animal compared to the animals grown on NGM media plates, freely moving animal was imaged at low magnification with bright field and their physical parameters were quantified such as body length and body width (fig.2). The calculated parameters showed that the health of the animal is not affected though there is a delay in growth as reported in literature when animals are grown in liquid culture (Lenaerts et al., 2008).

![Images of C. elegans growing inside the microfluidic device at 0, 12, 24, 36, 48, 60, 72, and 84 hours after hatching. The animal is fed with OP50 bacteria and kept in isolation inside the flow channel. Scale bars are 50µm except for 100µm at 72 and 84 time-points. Body length (b) and body width (c) of the animals grown in microfluidic compared with the animals grown on NGM food plate. Strain used=jsIs609. Scale=10µm. Data represented as Mean ±SEM, n=25 animals. (Initial experiment was done by Sudip Mondal)](image-url)
5.5) High-resolution imaging results and discussion

To perform high-resolution imaging, animals are trapped in straight posture by slowly increasing the pressure (fig 1g, h). We used the device along with high-resolution imaging to monitor synaptic growth in the TRN neurons and to observe neuronal process development in the PVD and TRN neurons. The microfluidic device, being transparent, could also easily be used to track cell lineage markers. As an example, I examined the migration of vulval marker with development.

5.5.1) Synaptic cluster increase in size with development

To capture high-resolution images of synaptic growth during the development of *C. elegans*, I used jsIs821 (GFP:: RAB-3) to visualize the changes in the size of GFP::RAB-3 accumulation at the ventral synapses. RAB-3 marks presynaptic vesicles that are crucial organelles for the development, synapse formation and its maintenance. Synaptic vesicles carry neurotransmitters to synapses and recycled back to cell body (Matteoli M et al. 2004). Thus, Synaptic vesicle markers can be seen accumulated at the synapses and the size of accumulation change with the development. For this imaging, L2 animal was put in the device and the ventral synapses of PLMs were imaged at 6 hrs intervals for 56 hrs. The animal was trapped slowly in an orientation to bring both PLM synapses into focus. We could see the synapse development as an increase in the size of GFP:: RAB-3 puncta from L2, L3, L4, through to the adult stage of the animal (fig.3a). Images were captured as a time lapse in Z-axis at the synapses. High-resolution time-lapse images were analyzed for synapse size which shows a gradual increase from 16 hours after hatching (L2) to 72 hours after hatching (fig.3b) in consistent with a previous study (Mondal et al., 2011). Synapse size annotation (fig.4) showed an increase in size for both the animals in microfluidic device and animals grown on NGM plates and imaged at L2, L3, and L4 larval stages.
Figure 3: Synaptic size growth from L2 to adult

a) Schematic of ventral synapses observed in the animals. b) Animals (jsIs821) kept in the device at 14 hours after hatching (HAH) and grown till adult and observation at ~16, 36, 57, 69 hours after hatching showed an increase in the size of GFP-RAB-3 accumulation at the ventral synapses. Scale =10µm
Figure 4: Graphical representation of synapse growth

Annotation of average synapse size in pixels in the animals grown in microfluidic device (jsIs821) (a) compared to that grown on NGM plates and imaged in 30mM Sodium azide (b) at L2, L3, L4, and adult stage. n>7 for animals in device and n>15 for animals grown in NGM. Data represented as ±SD.

5.5.2) High-resolution imaging of vulval cell marker shows Vulval development:

The microfluidic device platform allows the observation of processes such as cell division, cell migration, and cell lineage tracking by using a cell-specific marker. Vulval development is well-studied and multiple markers are known for tracking the cell lineage and thus development. EM reconstruction shows seven types of vulval cells vulA, vulB1, vulB2, vulC, vulD, vulE, and vulF. For tracking the vulval development, we used zmp-1::GFP in PS3239 strain (Inoue et al., 2002). Zmp-1 codes for zinc metalloproteases and expresses in anchor cell in L3 and early L4, and in vulA, vulD, and vulE. vulD and vulE in L4, however, the expression in vulA starts in the adult animal. This variation in expression represents an example of temporal gene expression where the same gene is expressed in different cells in different time windows of development.

To observe cell lineage under long-term high-resolution conditions, I immobilized the same animal marked with vulval cell marker (PS3239) repeatedly after every 8-10 hours
inside the microfluidic device during L3 to the D1 adult stage. The animals were monitored for their health and growth. High-resolution imaging of vulval cells shows the migration of \textit{zmp-1::GFP} from anchor cells to other vulval cells. This migration directly corresponds to the development of the animal. The animals show a small increase in body auto fluorescence due to repeated trapping and imaging inside the microfluidic device when imaged at later stages of adulthood (fig.5). The vulval cells appeared in different z-planes in confocal images due to their expanded localization and were easy to trace in z-projection images.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Expression of the vulval (\textit{zmp::gfp}) markers in the lineage}
\end{figure}

Schematic shows expression of the vulval marker (\textit{Zmp::gfp}) with the development. It appears in anchor cell in L3 (a) and in later developmental stages appears in Vul D, Vul E (b) during L4 and Vul A cells (c) at the adult stage as marked by a circle. Scale= 10µm
5.5.3) High-resolution imaging of neuron development:

To capture neuronal process development, I monitored the development of the TRNs and PVDs mechanosensory neurons. *C. elegans* responds to external stimuli by various mechanosensory neurons circuits (Chalfie and Sulston, 1981; Chatzigeroigiou et al., 2010b, Geffeney et al., 2011 and Hall and Treinin, 2011). TRNs respond to gentle touch, while the response to harsh touch and stimuli is mediated through PVDL and PVDR which have branched dendrites (Way and Chalfie, 1989). We used NC1686 strain, the transgene *wdIs51 [F49H12.4:: GFP, unc-119(+)]* which is expressed in the two PVD neuronal processes (Watson et al., 2008). PVDL and PVDR are generated from V5 ectodermal blast cell during early larval stage L2. The blast cells give rise to the cell body and axon that joins ventral nerve cord (White et al., 1986). The dendrites emerge at late L2 or early L3. PVD cell body is present in posterior-lateral sensory organ including V5 derived cells (Sulston and Horvitz, 1977). The primary processes give rise to secondary and tertiary which in turn give rise to quaternary dendritic branches that innervate the membrane. Secondary, tertiary and quaternary processes appear in late L2, L3, and L4 respectively. Late L3 and L4 shows high arborization (Smith et al., 2010, 2013). The branched dendrites envelop the animal in dorsoventral and anteroposterior axis, thus forming a network along body wall to sense the stimuli.

I observed PVD development and counted the increase in dendritic branching that marks different developmental stages. I could track L2, L3 and L4 stages by the appearance of an increase in secondary, tertiary and quaternary branches. The extent of branching increased with the development (fig.5). Throughout the imaging process, the animals remained healthy and could lay eggs after release. I observed approximately 10 secondaries (SP) but no quaternary processes (QP) at the L2 stage of development, when measured in the device, (fig. 6b, c). The values were not significantly different when measured on animals growing on NGM plates. By the stage of L3, a small amount of QP starts developing and continues to grow in density with development. I measured
approximately 67 QP in the D1 adult stage. The growth of SP is stabilized by this stage. The number of SP and QP were counted at different time points as measured from the immobilized animal in the device while it was growing inside the channel with a constant supply of bacteria for more than 50 hrs (fig. 6d). A similar trend of branching was present in animals grown on NGM plates and imaged in levamisole (fig.7). Since I could image only one of the PVD processes, thus, the QP and SP were counted from one of the symmetric neurons. The numbers matched with values represented in previous work (Smith et al., 2010). I also annotated the distance between PVC cell body in the tail and PVD cell body, that move apart as the animal grows (fig.6c). With development, although the number of secondary, tertiary and quaternary processes increase, they show much variation in the numbers which could be the mechanism of self-avoidance. Self-avoidance refers to the distinction between self and nonself processes that dendrites or neurites follow during patterning (Grueber and Sagasti, 2010). Since the complexity of the dendritic arborization increases with age, an altered structure at an earlier stage could affect later developmental stages. Hence it is important to monitor the same animal all throughout its developmental stage to understand the effect of such mutation over longer time.

In addition to PVD, I observed the development of TRNs from L3 to adult (fig.8) in jsIs609(pmec-7::mito:gfp), where mitoGFP has been targeted to TRNs mitochondria. Montage of the whole neuronal process with successive time points at an interval of 3 hours shows an increase in neuronal process length with shifting synaptic branch mitochondrial position. Thus, microfluidic growth and imaging device can be used to track sub-cellular events such as cargo trafficking in the neurons and over longer time durations.
Figure 6: PVD development in microfluidic device

Schematic shows PVD pattern in L2, late L2, L3 and L4. PVD cell body (shown by arrow) is present post desired. F49H12.4::GFP marker show emergence of primary processes (PP) at L2 (a,
b) from both anterior and posterior side of the PVD cell body, secondary processes (SP) appear during late L2 (b) followed by the emergence of tertiary processes (TP) in L3 (c). Quartiary processes (QP) can be seen during late L3 or early L4 (d) developmental stages which increase in number and size in late L4 stage (e) and adult stages (f). Quantitation of n=8 animal shows an increase in secondary and quaternary processes from 16 hours after hatching to 51 hours after hatching. Scale= 10µm. Data represented as ±SEM

Figure 7: PVD development in wdIs51 grown on NGM plates
Animals were grown on NGM food plates and anesthetized in 3mM levamisole for imaging at L2, L3, L4 developmental stages. (a) PVD branching and arborization increase from L2 to late L4. (b) Number of secondary and quaternary processes from L2 to L4. (c) Average distance in microns between PVD (marked in L2 and L3 with orange arrow) and PVC cell body (marked in L2 and L3 with blue arrow). Data represented as ± SEM.

**Figure 8: Growth of TRN in an individual animal**

Animal (*jsIs609*) grown in the microfluidic device from Late L2 to adult and trapped and imaged at an interval of 3 hours. Montage of images for the whole neuron (PLM) in the animal for 24-25 hours after hatching (HAH) (a), 33-34 HAH (b), 39-40 HAH (c) and 45-46 HAH (d). The development of TRN tracked by an increase in neuronal process length and an increase in the distance between cell body (marked with an orange arrow) and synaptic branch point (marked with a yellow arrow). Scale bar=10µm
5.5.4) **Mutant study: TRN branching tracking:**

The growth and imaging device could also be used for tracking development defects in mutants.

As an example of a mutant study, I tracked the development of mutant isolated in our lab *tb118; tbi3222(mec4p::mcherry)*, where touch receptor neurons are targeted with soluble mcherry. The mutant *tb118* shows branching in TRNs (chapter 3). We could track the growth of branching pattern in PLM from 14 hours after hatching to 36 hours after hatching that corresponds to early L2 to the early L4 larval stage (fig.9). The extent and complexity of branching enhanced with the development. It shows that branching appears at the early developmental stage and increase with the development.

![Image of branching](image)

Figure 9: High-resolution imaging of mutant *tb118; tbi3222(p mec4: mcherry)* shows branching

Row a) represents 1st, 2nd and 3rd time point images for animal 1, and row b) represents images of 1st and 3rd time-point for the second animal. 1st time point is represented twice in a different focal plane to observe branches. 1st TP represents 14 hours after hatching, 2nd TP represents 26 hours after hatching and 3rd TP represents 36 hours after hatching. Scale bar=10µm.
5.6) Conclusion and Discussion:

Studies in the field of aging and developmental defects require tracking the animals for a very long time. Mutants are more sensitive to anesthetics concentrations thus repeated anaesthetization may cause adverse effects on the physiology and growth. Our device provides the microenvironment that would be useful for such studies to observe development and morphological, behavioral and functional defects in the mutants. Our device is easy to fabricate and set up in any laboratory to address long-term developmental questions in *C. elegans* which require intermediate high-resolution imaging.