Lab on a Chip

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ARTICLE

Microfluidic devices for imaging neurological response of *Drosophila Melanogaster* larva to auditory stimulus

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Two microfluidic devices (Pneumatic chip and FlexiChip) have been developed for immobilization and live-intact fluorescent functional imaging of *Drosophila* larva's Central Nervous System (CNS) in response to controlled acoustic stimulation. The pneumatic chip is suited for automated loading/unloading and potentially allows high throughput operation for studies with large number of larvae while the FlexiChip provides a simple and quick manual option for animal loading and is suited for smaller studies. Both chips were capable of significantly reducing the endogenous CNS movement while still allowing the study of sound-stimulated CNS activities of *Drosophila* 3rd instar larvae using genetically encoded calcium indicator GCaMP5. Temporal effects of sound frequency (50-5000Hz) and intensity (95-115dB) on CNS activities were investigated and peak neuronal response of 200 Hz was identified. Our lab-on-chip devices will not only aid further study of *Drosophila* larva´s auditory responses but can be also adopted for functional imaging of CNS activities in response to other sensory cues. Auditory stimuli and the corresponding response of the CNS can potentially be used as a tool to study the effect of chemicals on the neurophysiology of this model organism.

1. Introduction

Drosophila Melanogaster is a widely used model organism for studying human biology and diseases at the molecular-genetic level[1-3]. This is due to its many advantages such as molecular-genetic, developmental, cellular/neuronal simplicity, genetic tractability and the increasingly incisive application of advanced optical-methods for live imaging of biological processes. At its larval stages, *Drosophila* contains different types of sensory neurons that are patterned in a segmental configuration. They sense various environmental cues (e.g. mechanical, visual and chemical) and relay information to the Central Nervous System (CNS) to help elicit stereotypic motor behaviors. This simple architecture continues to be exploited for studying numerous developmental-genetic and neurobiological problems primarily through deploying surgical, histological, transgenic and behavioral methods[4, 5].

Studying the behavioral responses in the larval stage of *Drosophila* using neuroimaging methods is challenging because the larva exhibits robust digging and burrowing behavior. This behavior is carried out by a cylindrical body wall that contains segmentally iterated sets of skeletal muscles and a specialized structure at the anterior end called the cephalo-pharyngeal skeleton (CPS). The latter is operated by specialized muscles to enable digging into food substrates [6]. This digging movement is an impediment to temporal imaging of fluorescent activities in the larval sensory neurons and the CNS (see supplemental data S2). Conventional immobilization methodologies involving the use of anesthetic drugs will affect animals' neurophysiological status[7]. Other methods such as the use of tissue glue used to immobilize embryos are irreversible, contain solvents that could affect the physiological state and do not completely immobilize the CNS. Ideally, immobilization has to be in a simple and reversible manner while still allowing sensory stimulus to affect the larva. Miniaturized microfluidic devices are best suited for this purpose.

Microfabricated lab-on-chip devices are increasingly being used in the study of various model organisms such as *Caenorhabditis elegans*[8, 9] and Drosophila[10-12] as they enable automated immobilization of these small organisms. After immobilization, visualization and tracking of cellular and physiological responses in-vivo can be performed through their transparent body wall without motion-artifacts. Microfluidic-based immobilization techniques for *C. elegans* have been developed using chemical (CO_2) or mechanical (tapering microchannels or encapsulation using deflectable PDMS membranes) approaches[13-16]. In the case of *Drosophila*, microfluidic devices have also been recently developed mostly to automate the embryo assays[17, 18] (self-assembly of eggs and morphogenesis[19, 20], developmental studies[21] and injection[19, 22]) and less attention has been given to on-chip larval studies. Immobilization of *Drosophila* larvae is more difficult than *C. elegans* as it exerts stronger force. Complicating matters further, the internal organs of interest such as the CNS capsule that needs to be visualized can loosely move inside the hemolymph-filled body cavity even if the outer body is completely immobilized by encapsulation. Recently, mechanical encapsulation [10] and CO_2 anesthetic exposure [11] approaches have been used to immobilize Drosophila larva. They allow whole-larval body compression inside the chip so that neuronal transport processes [10] and sensory neuron regeneration upon injury [11] can be visualized. Both these devices reduce the movement artifacts as compared to the freely moving larva but do not eliminate them. The use of anesthetic leads to spurious neurobehavioral responses and the use of encapsulation prevents the exposure of the larva to

external sensory stimulus

Very recently, behavioral responses of *Drosophila* larva in reaction to mechanical stimuli (vibration and sound) have been studied[23,24]. Zhang et al.[23] used Ca^{2+} imaging and electrophysiological methods and found that *Drosophila* larvae's Cho and class IV DA neurons responded optimally to sound waves at 500Hz frequency. In these studies, the immobilization for Ca^{2+} imaging was achieved by compressing the larvae (in saline) between coverslips. This is a manual process and the degree of compression, the access of the larva to the exernal stimuli as well as the orientation of the animal are all variable and dependent on the operator. It is also conceivable that the overt whole-body mechanical compression could be disruptive for the full-range of endogenous sensory-motor activities to take place.

In this paper, we demonstrate two lab-on-chip designs that standardize the immobilization of the larva while still allowing access to external stimuli and for the first time, conduct an on-chip study the CNS activity of *Drosophila* larvae evoked by acoustic signals. Use of acoustic signals enabled fast, automated, remote and tunable stimulation of specimens. Specifically we investigated these responses fluorescently at the ventral cord of the CNS, an anatomical structure where a large majority of sensory afferents synapse with interneurons. We expressed a new genetically encoded Calcium sensor GCaMP5 [25] in all sensory and cholinergic interneurons. Our devices are engineered to stabilize the CNS specifically from ongoing motor movements and the resulting internal hemolymph displacements. We demonstrate that localized anchoring of the larval CPS permits functional imaging of CNS in response to auditory stimuli. Our larval-lab-on-a-chip platforms will also be useful for studying CNS responses to other sensory cues (light, chemosensory, tactile, electric/magnetic fields).

2. Methods

The neurological response of Drosophila larvae to auditory stimuli was studied using two different lab-on-chip designs. Device design and fabrication, experimental procedures, data acquisition and processing as well as animal preparation are described in this section.

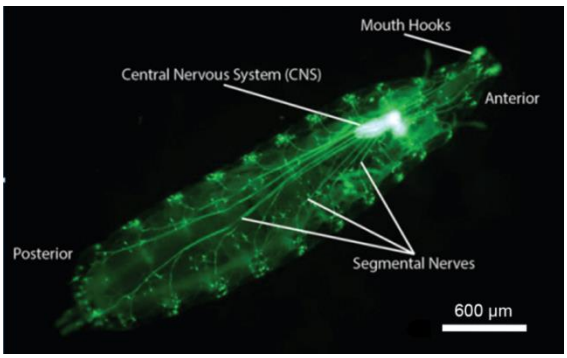

Fig. 1. An epifluorescent image of a 3rd instar larva expressing GFP in all cholinergic neurons as driven by Cha-Gal4, UAS-GFP transgenes. The Central Nervous System (CNS) zone is indicated, its neuronal activity was monitored by expressing a UAS-GCaMP5 transgene.

2.1. Design of the Microfluidic chips

Drosophila larva's burrowing and locomotory behaviors make the CNS capsule move inside the hemolymph-filled body cavity because it is loosely anchored (Fig.1). In order to immobilize the CNS of the larva and to subsequently study their neurological responses to auditory stimulus, the two chips, named the pneumatic chip and the FlexiChip were designed.

2.1.1. Pneumatic Chip

The Pneumatic chip was designed for automated loading, immobilization, testing and unloading the animals. The first chip (schematically shown in Fig. 2) consisted of an inlet port for animal loading into the device, a channel (inlet in Fig. 2) for transporting the animal towards the tapered trap that was designed to immobilize the larvae for imaging and an outlet for ejecting the tested animal upon completion of each experiment. The trap consisted of a narrowed channel (770×700 μm^2 cross-section with 500 µm length), primary (200µm width and 450µm depth) and secondary gates (100µm width and 425µm depth) and a stopper (100µm width and 100µm depth). The primary and secondary gates were designed to pin the 3rd instar larvae at two locations on its body while the rest of it was encapsulated in the narrowed channel. We found that without the two-point pinning, the CNS could move longitudinally inside the body despite the complete encapsulation of the larval body in the trap, thereby compromising clear imaging of the neuronal activities in the CNS. The dimension of the secondary gate was designed such that only the nose region of the immobilized 3rd instar larva could protrude through the gate. This gate was used to prevent the larvae from escaping the trap when a small sustained pressure was used on the posterior side for complete immobilization (see section 2.4.1).

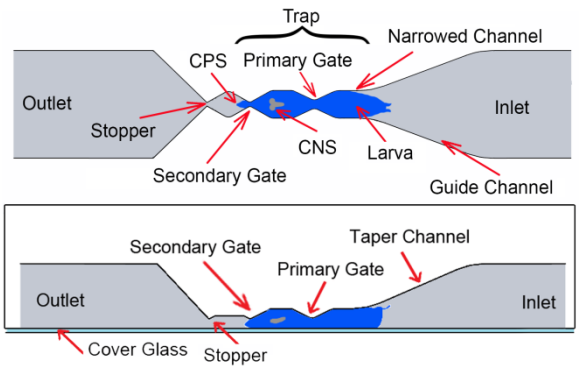

Fig. 2: Schematic design of the pneumatic chip (not to scale) – topview (top image) and side view (bottom). The inlet channel was 25 mm long, 3 mm wide and 2 mm deep with an inlet port for animal loading located at its end. The outlet channel was 8 mm long, 3 mm wide 2 mm deep for ejection of tested animals.

2.1.2. FlexiChip

In order to study the influence of the device design on the auditory response of the larvae, another chip (FlexiChip) with different mechanical and acoustic properties, that facilitated manual animal loading was used. Schematic of the FlexiChip (Fig. 3) summarizes its basic design and operation. The key features of the FlexiChip were a main channel that fits the 3rd instar larva (similar to the pneumatic chip) and a clasping mechanism (clip) that was designed into the PDMS substrate so as to clamp the head of the larvae (Fig. 3). Both features are included into the 3D printed mold. The clip mechanism opens

when the PDMS is flexed and closes shut when the flexion is removed. The clip-gate also contains a 100 µm diameter glass wire on the top to create an enclosed hole or a burrow-like opening that encourages the larva to enter it, the glass wire also stabilized the anchoring upon clip closure. The auxiliary channels are available to keep the preparation moistened, or for the introduction of electrical or mechanical probes for body-wall stimulation. Arrowhead in Fig. 3 indicates the approximate location of the CNS ventral cord (VC) that resides just below the ventral body-wall of the larva so that live-imaging could be carried out, often with almost no extraneous tissue obstruction. FlexiChip allowed the larva to continue breathing while being subjected to various types of sensory stimulations.

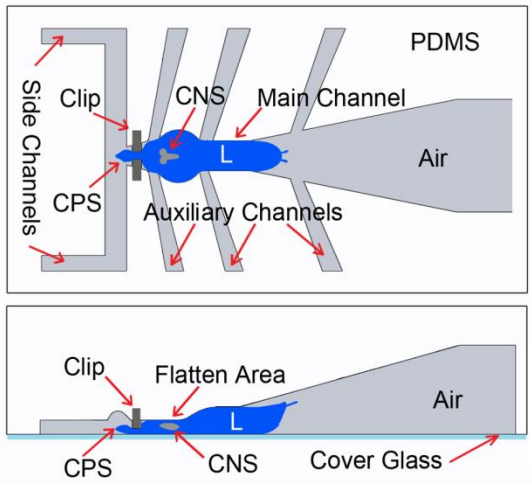

Fig. 3: Schematic design of the FlexiChip (not to scale) – topview (top image) and side view (bottom)

2.2. Device Fabrication

Devices were fabricated by 3D printing of two plastic master molds that dimensionally corresponded to the design discussed in section 2.1 for the pneumatic chip and the FlexiChip. Following master mold fabrication, soft lithography[26] was used for conventional PDMS (10:1 ratio base:agent, Sylgard SYLGARD® 184) casting, curing (70C, 2hr), bonding to glass slides (80s, 50W, plasma oxygen) and installation of inlet/outlet tubes (for pneumatic chip, Silicone Tubing, 3/16"ID x 5/16"OD, Cole-Parmer Canada Inc.). The glass wire in the FlexiChip was placed into the 3D mold at the location of the clip before casting the PDMS into the mold.

2.3. Experimental Setup

The experimental setup (Fig. 4) consisted of a sound-insulated box (custom made to isolate environmental noises using acoustic sound damping foam UL 94, Parts-Express, USA), an acoustic signal generation system (function generator (AFG3022B, Tektronix, CA), amplifier (RAMSA WO-1200, Panasonic, CA) and speaker (Eminence Beta-12CX coaxial 12", Parts-express, USA)), an optical/fluorescent imaging system (Lumascope 500, Single color 488nm Ex. Fluorescence, 40x magnification, Etaluma, CA), the microfluidic device and a software control system (LabVIEW© , flyCapture2© and Imagej© software).

The function generator connected to the amplifier was controlled through a custom LabVIEW© code and was used to generate the desired pure-tone sinusoidal voltage signal inputs to the speaker. The speaker was installed on the roof of the sound insulating box. Various voltage frequency and intensity levels were generated and amplified to the speaker and the corresponding sound frequency (50-5000Hz) and intensity (95-115dB) produced in the box were measured using a mini sound level meter (DT-85A, CEM) This was done to calibrate the speaker and the sound insulating box. Frequency ranges were selected to cover the hearing range of response of *Drosophila*[23] and intensity levels were selected using preliminary experiments that produced a response in the CNS.

The microscope was positioned inside the box right beneath the speaker with a 15cm distance between its focal plane and the speaker. The microscope was controlled by software and used in the optical mode for loading the animal and in the fluorescent mode for imaging GCaMP5 activities in the CNS of an immobilized larva.

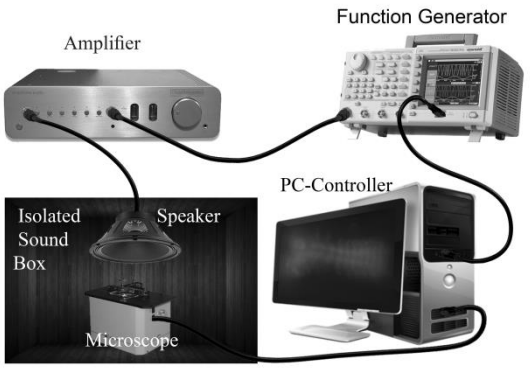

Fig. 4: Experimental Setup used to examine the auditory response of Drosophila larvae. Insulation sound box with internal walls covered with sound damping foams was used to accommodate the microscope right underneath the speaker. The speaker was connected to a function generator (FG) through an amplifier for sound actuation (sinusoidal voltage output from FG). Both the microscope and the FG were connected to a PC for automated control of image acquisition and signal generation (frequency and peak-peak voltage) respectively.

2.4. Animal loading

2.4.1. Pneumatic Chip

Drosophila larva (3rd instar) was picked from the food medium using a soft brush, washed with DI water and loaded into the chip at the inlet. Then, the larva was pneumatically inserted into the entrance region of the trap (Fig.5a) in 10s via the inlet channel. The larvae often oriented themselves and crawled voluntarily with no external pressure half-way into the trap up to the primary gate (Fig.5b-d) which helped in proper orientation and imaging of the CNS. This could take up to 30s but robustly produced desired orientations after immobilization. The animal was then pneumatically pushed further inside the trap (using a 0.8bar continuous pressure) and stopped automatically when the head of the larvae reached the secondary gate in less than 3s (Fig.5e). After animal loading and immobilization, a continuous 0.3 bar pressure was applied and maintained at the inlet port to inhibit any further CNS longitudinal movements and to prevent the larva from crawling back and moving out of the trap. The animal was viably kept inside the aqueous environment for the entire duration

of the experiment (215s, see section 2.5). Using the shown configuration, we successfully immobilized *Drosophila* larva with minimal internal CNS movements for its subsequent live neuronal imaging under various acoustic wave conditions inside the insulating box.

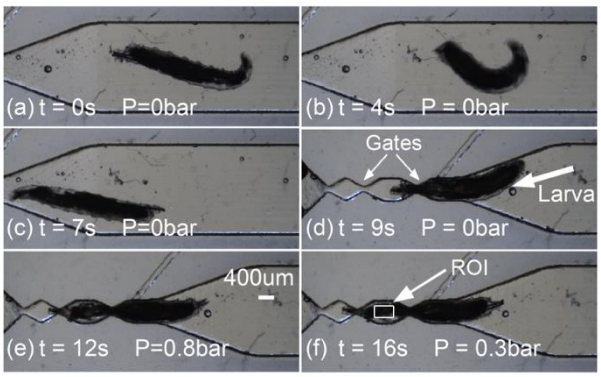

Fig. 5: Steps to load the larva using the pneumatic chip. (a-d) larva swam freely into the trap, (e-f) larva was pneumatically moved into the trap and immobilized. Time-lapsed fluorescent imaging was then conducted on the CNS located inside the Region of Interest (ROI). Scale bar=400 µm for all figures is shown in Fig. 5e.

2.4.2. Flexichip

The loading of the larva into FlexiChip was performed by bending the chip laterally (Fig.6a the bending opened the clip (double-headed arrow) (Fig.6b) so that the CPS area could be inserted into the gap (arrow in Fig.6c) with larva's ventral side facing upwards. When a drop of water was placed at the clamp area the larva automatically attempted to burrow into the clamp area. Release of the bending facilitated the anchoring of the anterior segments of the larva that contains the burrowing apparatus. In addition, a glass wire was used at the top of this clamping area to restrict the movement of larva in this direction and prevent its escape from the clamp for a longer time.

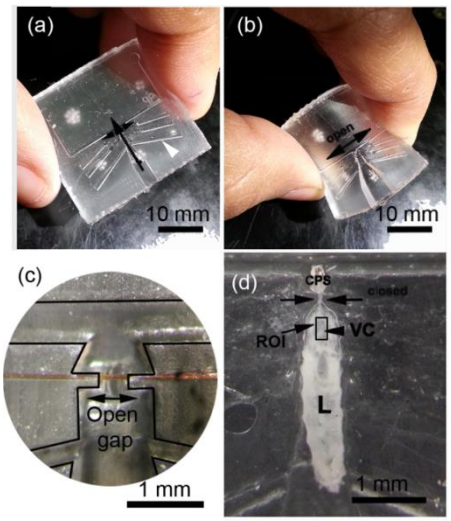

Fig. 6: Steps to load the larva using the FlexiChip. The chip is bent (b) so that the clip (c) opens. Then, the animal is inserted into the gap and the chip is released and sealed by a coverslip (d). Time-lapsed fluorescent imaging was then conducted on the CNS located inside the Region of Interest (ROI)[31]

Afterwards, a cover-glass was placed on top of the larva (Fig.6d) before visualization of fluorescent activities in the ventral cord aspect of the CNS (see supplemental information S4) where a large majority of afferent sensory inputs from the body wall arrive. The larval posterior-end protruded into a funnel shaped outer chamber that was open to ambient air. This allowed respiration to continue through posterior spiracles during live imaging. The procedure for loading the larva into the chip takes approximately 5min.

2.5. Automated animal testing

After the animal was properly loaded into the trap and immobilized as shown in the Figs. 5 and 6, the auditory response of the larva was examined at the abdominal ganglia region of the ventral cord. A custom-made LabVIEW© code controlling the function generator was used to generate a step-like periodic series of acoustic waves (5s On and 5s off) while the animal's CNS fluorescent signal activities were recorded in a movie format using the microscope. Each on-portion cycle of the applied wave corresponded to one frequency (50, 100, 200, 500, 1000, 2000 or 5000 Hz) and one intensity (95, 105 or 115 dB) level. The experiment was continued automatically until the entire 21 frequency-intensity combinations were tested. The animal was then washed off the chip and another one was loaded to repeat the experiments. The movies were then analysed as discussed below for quantification of neuronal activities.

2.6. Data acquisition and processing

Movies recorded for each animal were analyzed by ImageJ© software (National Institutes of Health, USA) to quantify the fluorescence intensity variations in the CNS in response to the applied acoustic signals. The RGB image sequences for each video were converted to 8-bit black and white images. After subtracting the background with rolling ball radius of 100 Pixels, a Region of Interest (ROI in Fig. 5f and 6d) covering the CNS was selected and the mean grey value for the entire image stack was measured inside the ROI and recorded in an Excel file. The intensity variation in each condition was calculated by taking a ratio of the increase in the mean gray value in the ROI during the stimuli to the mean gray value 2 seconds before sound being applied in each experiment.

It is important to note that the inherent movement of the animal also results in an increase in CNS activity that may lead to elevated baseline reading. Movement was measured as the change in the center of mass of the CNS and experiments that had high CNS movement were not included in the analysis.

2.7. Animal Preparation

Larvae of the genotype w, Cha-Gal4/CyO; UAS-GCaMP5/TM3, Sb were used for imaging CNS activity in response to auditory stimulations. Hetereozygotes and homozygotes were not separated before testing. Expression of the GCaMP5 GECI was conducted using the Gal4/UAS system[27]. Through standard fly crosses, a stable fly stock was created containing two transgenes. 1. Cha-Gal4: A promoter sequence of CholineAcetyltransferase (Cha) driving the expression of the Gal4 transcription factor[28]. 2. UAS-GCaMP5 transgene contains the binding sites for the Gal4 transcription factor[25]. Thus, in the Cha-Gal4/CyO; UAS-GCaMP5/TM3 strain, all sensory and central neurons that express

the CholineAcetyltransferase gene express the GCaMP5 calcium sensor. The GCaMP calcium sensor is circularly permuted protein containing the Green Fluorescent Protein (GFP), Calcium binding protein called Calmodulin, and the M13 (Calmodulin binding) peptide[29]. Influx of Ca++ during neuronal activity triggers a conformational change of GCaMP so that solvent access to the chromophore is prevented and thus resulting in higher level of fluorescence[30]. GCaMP5 is recently developed high signal-to-noise ratio calcium sensor[25]. This genotype was generated through a standard genetic crossing scheme. 3rd instar stage larvae were isolated using a fine brush and washed with distilled water and dried on a tissue paper before loading into the chips.

3. Results and Discussion

3.1 Acoustic response of *Drosophila* 3rd instar larva

After immobilizing the larva (3rd instar) inside each of the PDMS devices as shown in Fig. 5 and 6, neuronal activities in the CNS in response to a sound wave (5s duration, 200Hz frequency and 105dB intensity) were measured using the experimental setup described before (Fig.4). We measured the frequency and intensity of the sound inside both of the devices and found them to be the same as that outside. As shown in Fig. 7 (response of immobilized larva in the pneumatic chip), animal's CNS activity increased by 21% (reported by an increase in average fluorescent intensity) upon exposure to the sound signal and returned to its original state within 0.5s after the signal was turned off (image not shown due to similarity to Fig.7a). This type of response was observed consistently for 5 animals tested at the same acoustic conditions and each time it was tested.

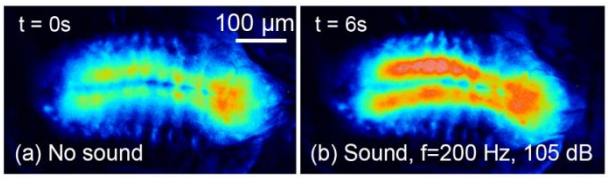

Fig 7: Snapshots of the fluorescent activities in the CNS of a larva (a) before and (b) while it was exposed to a 5s duration sound wave (200Hz and 105dB) in the Pneumatic Chip

3.2 Investigation of the effect of sound frequency and intensity on Drosophila larvae CNS activities

The interesting observation of a significant CNS activity in response to a sound signal (Fig. 7) encouraged us to investigate this phenomenon further in detail. Hence, we recorded the CNS activities of Drosophila 3rd instar larvae in response to sound signals of various intensity (95-115dB) and frequency (50-5000Hz) levels using both the pneumatic chip and the FlexiChip (Fig. 8, averaged for n=5 animals).

As shown in Fig. 8, animals tested in both chips demonstrated a statistically significant increase in CNS activities when the frequency of the sound signal was increased from 50Hz to 200Hz. Further increase in frequency resulted in reduced CNS activities as compared to 200Hz condition. In addition to a peak in response at 200Hz, a secondary but less significant peak in CNS response was also observed at 2000Hz and only inside the pneumatic chip (Fig.8a). This peak was more significantly pronounced at higher intensity levels (105 and 115 dB). In contrast, at 95 dB the difference between the peak frequency and the rest can be observed but is not statistically significant due to lower signal to noise ratio (see supplemental information S3).

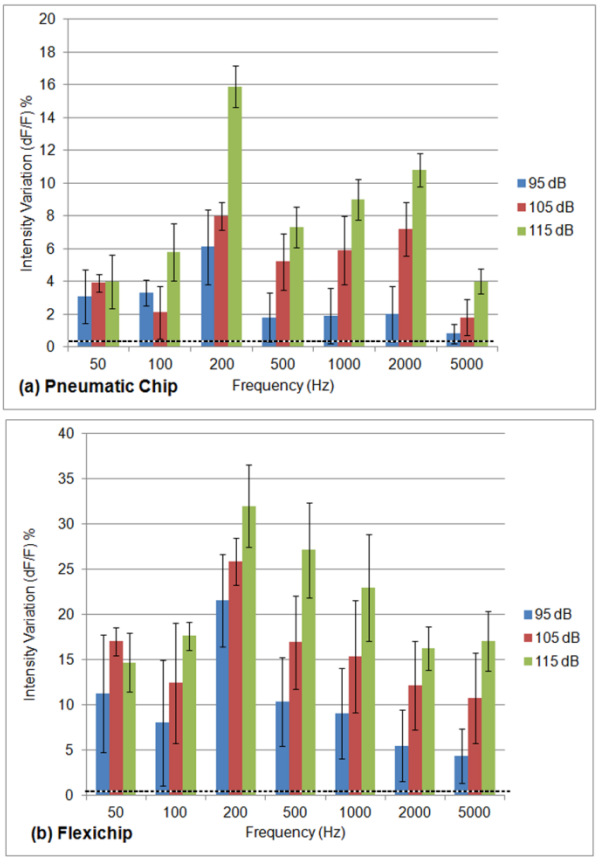

Fig. 8: CNS response of fly larvae (n=5) to various sound frequency and intensity levels tested inside (a) Pneumatic Chip and (b) FlexiChip. A peak in response at 200Hz was observed in both chips with reduction in CNS activities when the frequency of signal was decreased below or increased above 200Hz. Increase in sound intensity resulted in increase in CNS activities. The average response in no sound condition was about 0.2% and 0.1% in the pneumatic and FlexiChip, respectively. The error bars are one standard deviation from mean.

The level of mechanical vibrations induced by the sound waves at frequencies less than 50Hz on the chip did not allow clear imaging of the CNS. The CNS response continued to decrease further beyond 5000Hz (data not shown). Increase in sound intensity from 95dB to 115dB, resulted in corresponding increase in CNS activity. The increase in the sound level resulted in the reduction in the signal to noise ratio indicating that that the auditory response of the fly at higher sound level was clearer.

In order to compare this method with other immobilization methods such as anesthetization, the response of 3rd instar larvae to pure tone sounds were measured before and after exposure to ether. The results indicate that the response to auditory stimulus was quite noticeably reduced in anesthetized larvae compared to control sample as shown in supplemental section (S1).

The peak observed in the CNS response at 200Hz sound waves is in contrast to the recently reported observation that the optimal neuronal response to auditory stimulus in the larva occurs at 500

Hz. We noted two major differences between our experimental design and that used by Zhang et al.[23]. First, in Zhang et al.[23], the behavioral, calcium imaging and electrophysiological measurements involved the placement of the animals (or semi-intact preparations) directly on top of a speaker that delivered the auditory stimulus which may have coupled some of the vibrations to the larva as tactile stimulations. The use of whole body compression provides sufficient contact of the substrate to activate Cho sensory neurons that are spread throughout the body. Second, our devices have physical separation of the speaker from the device ensuring that it is the sound waves that cause the response while also providing a better simulation of sound cues that occurs in nature. Since the larva is a burrowing animal, it is likely that the sensory neurons in the posterior abdominal segments are better tuned to a sound frequency that matches the wing beat frequency of the predatory wasp. We also test two different designs to separate out any possible tactile stimulation effects.

4. Conclusions

With the availability of a new generation of reagents for monitoring neuronal activity through imaging and electrophysiological methods, it is necessary to develop custom engineered microdevices to facilitate experimental manipulations in intact-living specimens. We designed and evaluated two devices to anchor the *Drosophila* larval CNS so that stable optical recording of its neuronal activities could be conducted. The reduction in CNS movement was achieved through an on-chip mechanism that isolated the larval segments within which the CNS capsule is suspended. These microfluidic chips allowed us to stabilize the CNS specifically from ongoing motor movements and the resulting internal hemolymph displacements while the immobilization technique did not use any anaesthetic drugs which would affect animals' neurophysiological status. The Pneumatic Chip allowed automated animal loading, immobilization and unloading and it held larva under positive fluid pressure to reduce the CNS movement entirely. However, since this is a closed-configuration chip, access of the larva to sound stimulation was indirect. The FlexiChip allowed for manual loading, unloading and immobilization. The posterior end of the larva inside the FlexiChip was open, thus allowing the larva to respire, and also for the acoustic vibrations to reach the larval body directly. The stability of the CNS inside both chips enabled the visualization of neuronal activities using a Genetically Encoded Calcium Indicator (GECI) probe, called GCaMP5, in response to auditory stimuli. Both chip designs allowed the stable recording of GCaMP5 fluorescence activity in the CNS. We report an optimal GCaMP5 response at 200 Hz. In conclusion, our customized larval lab-on-chip platforms allow the integration of functional imaging with a sensory-motor response. We anticipate that our intact larva-on-a-chip will also be useful for other studies that involve Calcium imaging, optogenetic and electrophysiological approaches.

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Notes and references

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