Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

S.1 Effects of anesthesia on the neuronal response to auditory stimuli

In this experiment, the effect of anaesthesia on the neural response of the larva to auditory stimulus was examined. A control experiment was performed by loading unanaesthetized 3rd instar larva in the FlexiChip and exposing it to 200 Hz and 500 Hz sound at three different sound intensity (95, 105 and 115 dB). The same experiment was repeated for each condition after exposing the same larva has been to ether solvent for 2 minutes through its posterior end. The results, shown in Fig. S.1, indicate that the neural response to sound as measured by the intensity of the fluorophore (GCaMP5) was quite significantly reduced in anesthetized larvae compared to unanesthetized condition. In addition to the reduction in signal, the differences between various frequencies and intensities is not significant.

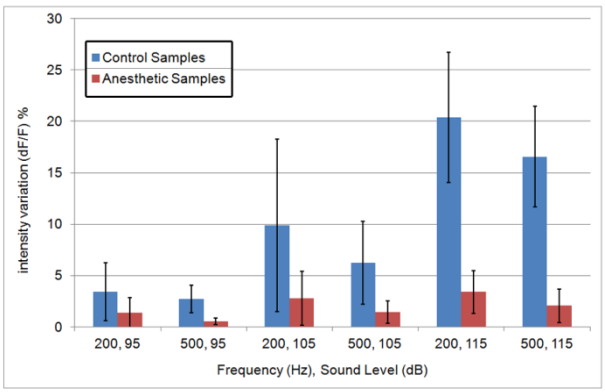


Fig. S.1: The neurological response of Drosophila larva to auditory stimulus while the larvae nerves were blocked (Blue) in compare with control sample

S.2 Characterization of immobilization mechanism

This digging movement is an impediment to temporal imaging of fluorescent activities in the larval sensory neurons and the CNS. A comparison between images obtained from a freely moving larva and an immobilized larva is shown in Fig. S.2. The features of the CNS in the immobilized larva can be discerned clearly while those in the freely moving larva seems to be blurred.
This comparison reveals that the CNS movement reduces the resolution of the fluorescent imaging in compare with an immobilized CNS.

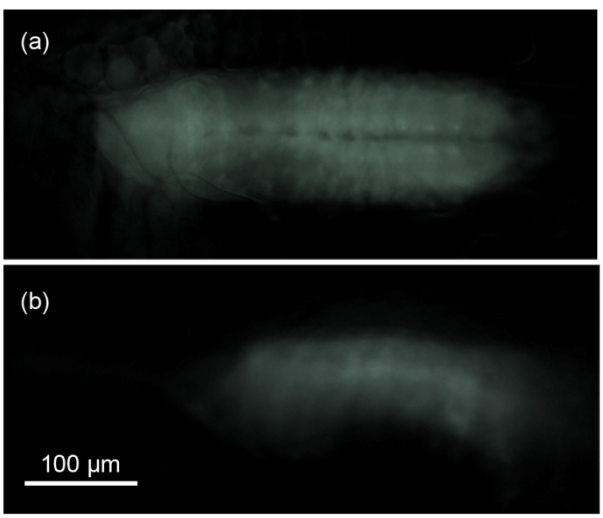


Fig. S.2: An imaging comparison between (a) an immobilized larva and (b) a freely moving larva. The immobilization mechanism reduced the CNS movement for high resolution imaging. The CNS movement in free mobile larva blurs the image obtained.

The immobilization mechanism was also characterized and its influence on the reduction of endogenous CNS movements quantified by the following experiment. First, the movement of the CNS of an immobilized (in a pneumatic chip) and non-immobilized larva were recorded for 120s without any stimulation. The CNS of the larva was visible due to its activity and the corresponding fluorescence of

GCaMP5. Next, the position of the center of CNS in each frame was calculated and the intensity weighted centroid of the image. The movement of the center of CNS is plotted in Fig. S.3. They show that the movement of the CNS of the immobilized larva is smaller than that of the freely moving larva. In this particular experiment, the CNS was ~400x150 μm^2 in dimension and the field of the view of the camera was 620x400 μm^2. Therefore, movement of CNS by more than 110 µm would put it outside the field of view of the microscope. The CNS of the freely moving larva frequently went out of the field of view (partially) of the microscope while that of the immobilized larva was always in view. These movements will be exacerbated when external stimuli is applied further illustrating the importance of immobilization.

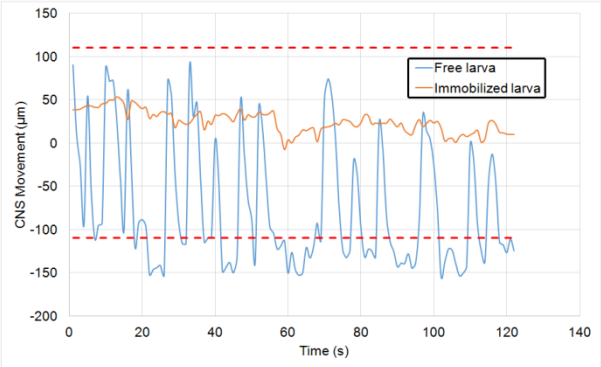


Fig. S.3: The movement of the CNS (based on the movement of the CoA of the CNS) for a free mobile (blue line) and an immobolized larva (brown line). The CNS of the non-immobilized larva could not be kept inside the field of the view (the red lines) due to the larva digging movement.

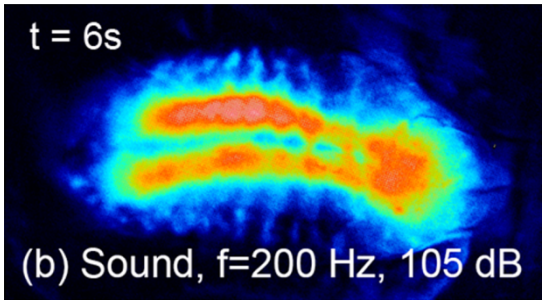
S.3 Statistical analysis, one-way ANOVA

Both chips demonstrated a statistically increase in CNS activities when the frequency of the sound signal was approaching to 200 Hz pure tone (see Fig. 8). Since depending on the intensity, the difference between 500 Hz and 200 Hz does not appear to be statistically significant in FlexiChip, a statistical analysis, one-way ANOVA was performed to statistically proof the results. To do this, the p-value of the one-way ANOVA (from summary data including the counts, means, standard deviations for each frequency) of two conditions was compared. In the first condition, the means and standard deviations of all frequencies was included in one-way ANOVA. While in the second condition, the mean and standard deviation of 200 Hz sound was excluded from the data. This analysis was performed on three different sound levels and the results were listed in Table S.1. The p-value of the first condition (when all frequencies were involved) in 95, 105 and 115 db sound level are respectively 4, 3 and 2 order of magnitude smaller than the second condition (when 200 Hz sound was excluded). Therefore, the results support that disregard to the sound intensity; the auditory response of the larvae to 200 Hz sound is statistically dominate compared to other frequencies.

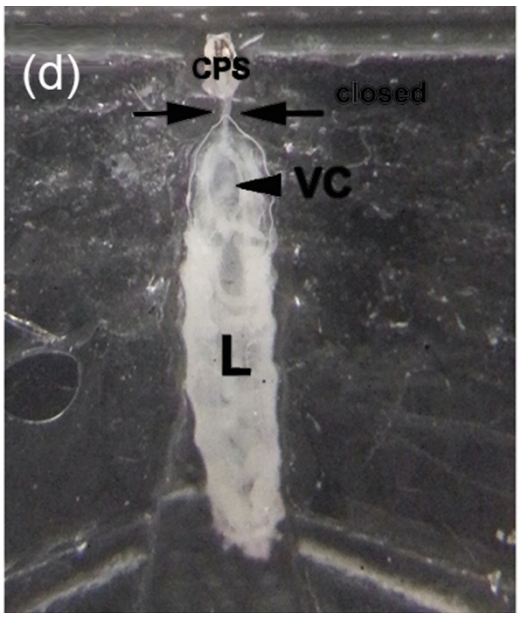
Table S.1: The p-value of one-way ANOVA (from summary data including the counts, means, standard deviations for each frequency) of two conditions

Sound Level	**p-Value**	
	All frequencies	All frequencies except 200 Hz
95 dB	7.08 E-6	6.71 E-2
105 dB	8.03 E-4	2.27 E-1
115 dB	6.15 E-5	7.89 E-3

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. In order to statistically reject the null hypothesis, one-way ANOVA (from summary data including the counts, means, standard deviations for each frequency) was performed 1000-5000 Hz data obtained from the pneumatic chip. The results indicate that it cannot be statistically proven (p-value higher than 0.001) any firm correlation between the results in the range of 500-5000 Hz for FlexiChip and the pneumatic chip at sound level of 95 dB), however, the peck in CNS response at 2000Hz inside the pneumatic chip at sound level of 105 and 115 dB can be statistically proven (p-value of 1.1 E-7 and 2.4 E-7, respectively).



Movie S1: Time-lapse movie of the fluorescent activities in the CNS of a larva while it was exposed to a 5s duration sound wave (200Hz and 105dB) in the Pneumatic Chip



Movie S2: Real-time movie of a loaded 3rd instar larva into FlexiChip ready for imaging