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On chip Cryo-Anesthesia of *Drosophila* Larvae for High Resolution *In vivo* Imaging Applications

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Abstract

We present a microfluidic chip for immobilizing *Drosophila melanogaster* larvae for high resolution, *in vivo* imaging. The chip creates a low-temperature micro-environment that anaesthetizes and immobilizes the larva in under 3 minutes. We characterized the temperature distribution within chip and analyzed the resulting larval body movement using high resolution fluorescence imaging. Our results indicate that the proposed method minimizes submicron movements of internal organs and tissue without affecting the larva physiology. It can be used to continuously immobilize larvae for short periods of time (minutes) or for longer periods (several hours) if used intermittently. The same chip can be used to accommodate and immobilize larvae across all developmental stages (1st instar to late 3rd instar), and loading larvae onto the chip does not require any specialized skills. To demonstrate the usability of the chip, we observed mitochondrial trafficking in neurons from the cell bodies to the axon terminals along with mitochondrial fusion and neuro-synaptic growth through time in intact larvae. Besides studying sub-cellular processes and cellular development, we envision the use of on chip cryo-anesthesia in a wide variety of biological *in vivo* imaging applications, including observing organ development of the salivary glands, fat bodies and body-wall muscles.

TOC image

We present a microfluidic chip for cryo-immobilizing *Drosophila melanogaster* larvae for high resolution, *in vivo* imaging of neurobiological processes and other applications.

Author Contributions

A.R.C., R.I., C.C. and N.C. designed the research; A.R.C. fabricated the microfluidic devices; A.R.C and R.I. performed the experiments; A.R.C. and R.I. analyzed the data; R.H. and Y.F. contributed UAS-mito-Dendra2 flies; A.R.C and R.I. wrote the paper.

Keywords

Cryo-anesthesia; membrane; PDMS; immobilization; *Drosophila* larvae; mitochondrial transport; microfluidic chip; *in vivo* imaging

1. Introduction

Drosophila melanogaster is a widely used organism for modeling human biology and diseases at the behavioral, cellular and molecular level [1] [2] [3]. The larval stage has a translucent cuticle and a simple neuroanatomy that is amenable to *in vivo* imaging [4] [5] [6] [7]. However, one of the biggest challenges associated with live imaging of *Drosophila* larvae is achieving an acceptable degree of immobilization which is required for observing cellular and sub-cellular events with high spatial, optical resolution without affecting the animal's physiology.

Current immobilization techniques include dissection [8] [9], the use of anesthetics [10] [11] or glue, and squeezing the larva's body [12]. Methods such as dissection are lethal, which therefore prohibits long term imaging. Glue is toxic, provides irreversible immobilization and is difficult to implement for very young animals. The use of anesthetics –such as isofluorane [11] [12] or desflurane [11] - has many advantages, but they are known to affect the animal's neurophysiology and neural activity [13] [14] [15]. Additionally, imaging must be restricted to short intervals in order to allow recovery between doses [14] [13].

Conventional anesthetics pose a threat to the user's safety and being regulated chemicals, require installing several safety components. The most popular mechanical immobilization techniques involves capturing the larva by flattening it between two microscope slides using light pressure, by placing water in between a coverslip and glass slide to hold the larva with capillary pressure [12], or by taping the larva onto a coverslip [16]. While these methods can be easily implemented, the degree of control is not ideal and the immobilization is not necessarily adequate in order to allow stable high resolution imaging of sub-cellular processes in all regions of the larvae. Furthermore, the imaging duration is limited and larval recovery can be difficult, if not impossible, if too much pressure is inadvertently used to flatten the animals [17]. In order to image reliably cellular/sub cellular events while maintaining proper body orientation over longer periods, an easy to use, reversible, low cost and chemical-free immobilization method is needed.

With the recent development of micromachining and soft lithography fabrication techniques [18] [19], novel microfluidic devices and systems have emerged for manipulating small organisms. Many research groups have developed microfluidic devices that immobilize un-anesthetized larvae. The microfluidic device developed by Yan *et al.* [20] immobilizes 3rd

instar larvae using a mechanical microcompressor. While proving to be a versatile platform, it requires the use of complicated and expensive laser and CNC fabrication processes in addition to PDMS soft lithography. Ghaemi *et al.* [17] constructed two pneumatically-driven microfluidic devices to immobilize and perform calcium imaging in 3rd instar larvae. Although the principle of operation is simple and standard soft lithography fabrication techniques were used, the architecture of the devices is complicated and capable of accommodating only 3rd instar larvae. Mondal *et al.* [15] developed a microfluidic device that can immobilize larvae at different developmental stages, but the loading process is tedious and the larva have to be squeezed against the microfluidic walls. Ghannad-Rezaie *et al.* [21] developed microfluidic devices for short term *in vivo* imaging. The device mechanically compresses the larva against a coverslip while imaging. Although good immobilization results were obtained without the use of a complicated setup, the chip could accommodate only 3rd instar larvae. Younger larvae were poorly immobilized whereas older larvae did not fit in the chip. While the device significantly reduced movement compared to freely moving larvae, the level of immobilization was not satisfactory for imaging internal structures. Frequent, small body movements did not allow high spatial resolution imaging of subcellular structures (such as neuro-muscular junctions and axons) inside the larval body.

In this work, we present a temperature based immobilization approach for high resolution *in vivo* imaging of sub-cellular structures and processes in *Drosophila* larvae. Specifically, we developed a microfluidic chip that creates a cold micro-environment around the larval body while effectively anesthetizing it. The microfluidic chip will henceforth be referred to as the cryo-larva chip. The experimental setup is made up of commercially available, off the shelf parts making its assembly and replication straight forward. While cryo-anesthesia has been previously implemented by Chung *et al.* [22] and Rhode *et al.* [23] to immobilize other model organisms such as *C.elegans*, a system dedicated towards performing low temperature immobilization of *Drosophila* larvae for high magnification *in vivo* imaging has not been demonstrated until now.

The cryo-larva chip can be used to study a wide variety of biological processes, including the transport of organelles and proteins at the cellular/sub-cellular level as well as neuro-synaptic growth demonstrated here. Using the chip, we observed *in vivo* mitochondrial trafficking in neurons and the developmental growth of synapses at the neuromuscular junctions (NMJs). Nearly all studies of mitochondrial transport in neurons have previously been conducted in isolated cells in culture, while observation of the developmental growth of synapses is often inferred from cultured neurons “aged” over the course of a few days. Our technology therefore provides a powerful alternative for studying those events within intact animals *in vivo*.

Apart from its demonstrated use for *in vivo* neurobiological studies, the cryo-larva chip based immobilization method can help many other fields of *Drosophila* biology, since it should also allow for imaging over time of the salivary glands, fat bodies, body-wall muscles, and other commonly studied larval organs. Additionally, with the popularity of the use of genetically-encoded fluorescent reporters, live tissue is often required to utilize many newly developed fluorescent probes that provide a readout of real-time cellular physiology which is abolished with dissection and fixation. Some examples include autophagy reporters

(mCherry-GFP fusions that measure pH in autophagosomes) [24] [25], intracellular pH indications (pHluorins) [26] [27], various calcium reporters (including GCaMPs and CaMPARI) [28] [29], and ATP biosensors (ATeam and Perceval) [30] [31]. Our chip will allow such reporters to be now used in intact animals, and changes in these physiological readouts can be tracked and compared within different genetic and environmental manipulations.

2. The Microfluidic chip and the cooling system

The Microfluidic Cryo-Larva Chip

The proposed immobilization method incorporates a PDMS/glass microfluidic chip with a two-layer architecture (Figure 1). The first layer ('Layer 1') contains a 5.0 mm long, 1.6 mm wide and 170 μm thick immobilization microchamber which accommodates the larva. The immobilization microchamber is surrounded by a network of microchannels that is held under vacuum applied through the vacuum port. The vacuum creates a reversible and strong seal between the PDMS, oil, and glass coverslip holding the larva in place after it is manually loaded onto the chip. After imaging is completed, the vacuum is released and the larva can be easily removed from the chip. The chip has also two ports (indicated as 'food inlet' and 'food outlet' in Figure 1(a)) for providing food to the larva if needed. The second layer ('Layer 2'), contains a 5.5 mm long $\times$ 2.8 mm wide $\times$ 100 μm thick microchamber (the 'cooling' microchamber) and sits on top of the immobilization microchamber. The two microchambers are separated by a 10 μm thick PDMS membrane. Chilled coolant fluid is passed under moderate pressure (~ 3 psi) through Layer 2 via the coolant inlet/outlet ports. This creates a low temperature microenvironment ($\sim 5^\circ\text{C}$), while at the same time the PDMS membrane deflects and compresses the larval body against the glass coverslip. Even though the membrane semi-restricts large body movements, cooling the larva down to $\sim 5^\circ\text{C}$ is the dominant factor that anesthetizes the larva body. This dual approach cryo-anesthesia and mechanical compression results in effective immobilization allowing stable, long term, high resolution imaging.

The height and overall dimensions of the immobilization microchamber are large enough to accommodate larvae of different developmental stages (1st instar to late 3rd instar). In order to achieve membrane deflections large enough to mechanically restrict the larva, a 15:1 mixing ratio was used for fabricating the first PDMS layer. This reduces the elastic modulus by a factor of ~ 2 [32] when compared to the 10:1 mixing ratio used for the second PDMS layer.

The Cooling System

The microfluidic cryo-larva chip, that sits on the stage of an inverted microscope, is connected to a closed-loop, cooling system that consists of a peristaltic pump, a cooling module and a reservoir (Figure 2).

The peristaltic pump (GE P-1, GE Healthcare Life Sciences) circulates the coolant through the system.

The cooling module consists of two thermoelectric Peltier units (TEC1-12710, 50W), each mounted on an aluminum heatsink (41 mm × 41 mm × 13 mm), and a flow chamber that is sandwiched between the two Peltier units. A small fan is attached to each aluminum heatsink in order to remove the generated heat. The coolant leaves the flow chamber at a below-zero temperature and, after passing through the chip, returns to the closed reservoir.

When immobilization is not required or the larva needs to be unloaded, the coolant is diverted away from the chip into the reservoir through a three port stopcock valve and bypass tube. Polyvinyl tubing is used for all fluidic connections. Silicone rubber tubing is used in the peristaltic pump. The length of tubing used is optimized to accommodate the cooling system around the inverted microscope while minimizing any parasitic heat gain (Supplementary Figure 1).

3. Results and Discussion

3.1. Microfluidic chip and system characterization

We used an infrared camera (FLIR SC600) to obtain the temperature distribution within the chip. The average temperature of the immobilization microchamber was measured to be $5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ at a coolant flow rate of 3.2 ml/min. (Figure 3), indicating a uniform temperature profile within the microchamber. Upon stabilization of the immobilization microchamber temperature to $\sim 5^{\circ}\text{C}$, the low-temperature environment was maintained over a period of several hours.

A concentrated salt water solution (5M sodium chloride) was used as the coolant fluid. We used salt in order to eliminate freezing of the water (the coolant reaches below zero temperatures in the flow chamber). Additionally, it allows us to pre-cool the coolant before use (typically placed in a freezer at -10°C for 1–2 hours). It was observed that the pre-cooling step reduced the response time needed for the temperature to stabilize by $\sim 50\%$. (Supplementary Figure 2). We define the response time of the system as the time needed for the temperature of the immobilization microchamber to reach 90% of its steady state temperature after the flow of the coolant has started. Increasing the flow rate of the coolant also reduces the response time (Figure 4) from ~ 2 minutes (at flow rate of 1.7 ml/min) to ~ 1 minute (at flow rate of 3.2 ml/min).

All experiments were conducted with the average ambient relative humidity maintained below 15% for optimal cooling. Higher humidity levels, especially above 50%, resulted in condensation on the tubing as well as on the cold surfaces of the Peltier modules that compromised the cooling performance of the system leading to reduced immobilization.

3.2. Time required for larval immobilization

An experiment was performed to quantify the dual effect of mechanical compression and cryo-anesthesia upon larval movement. Individual larvae were loaded into the chip and GFP-expressing neuronal cell bodies along the ventral nerve cord (VNC) were imaged (we used a confocal microscope at 250× magnification). A video where each frame is a single optical plane image (Section 4.4) was acquired for a total duration of 9 min (the flow of the coolant was turned on at 0.5 min and turned off at 5.5 min).

For each acquired frame, we selected the centroid of a cell body along the VNC that was visible in all frames in the video. The absolute distance moved by the centroid between consecutive frames was tracked over time using Metamorph software (Figure 5). Results show that with no coolant flow through the chip, the larvae were active and crawling in the immobilization microchamber. Once the coolant started flowing through the chip, rapid immobilization was observed with movement decreasing and stabilizing in under 3 minutes (Supplementary Movie 1). As soon as flow was turned off, the larvae started to rapidly regain mobility inside the chip and recovered completely within 15 minutes after removal from the chip.

Furthermore, it takes ~ 1 min before any significant reduction in the body movement is observed. At that time point, the temperature has decreased to ~ 10 °C (see Figure 4 for a flow rate of 3.2 ml/min) and the corresponding average body movement is ~ 5 µm (Figure 5). That result demonstrates that there is a correlation between the temperature of the immobilization microchamber and the magnitude of the body movement.. We selected 5 °C as an optimum temperature at which body movements are negligible (< 0.5 µm) while chilling-induced injury is minimized.

3.3. Characterization of larval movement

To validate the proposed method we recorded the movement of various neuronal structures during immobilization under high magnification (400×).

Individual larvae were loaded onto the chip and pre-cooled coolant was placed into the reservoir. The flow rate was set to 3.2 ml/min and coolant flow through the chip was turned on. After 4 minutes, a video where each frame is a collapsed z-stack of ~7 images of GFP-labelled neuronal structures of interest was collected over a 2 minute duration (Section 4.4) before diverting flow away from the chip using the bypass tube. Acquiring a collapsed z-stack of a focal volume within each larva instead of a single image of a focal plane allowed continuous observation of sufficient features of interest throughout the 2 minute period despite photobleaching effects and any potential movements along the z axis by the peristaltically deflecting membrane. To identify whether the low-temperature environment in the immobilization microchamber (4–6 °C) results in larval immobilization, a control experiment was performed with the coolant flowing through the chip without activating the Peltier units.

For every acquired frame, three features that were visible across all frames were selected; mitochondria within NMJ terminals, mitochondria within segmental nerve axons, and neuronal cell bodies within VNCs (Figure 6(a), (c)). The distance moved by the centroids of these features in between consecutive frames was estimated by tracking them over time and then averaged (Figure 6). Mitochondrial labeling was used for this analysis because the separated, distinguishable puncta (Figure 6) allowed for tracking of individual small features through time. Upon the initiation of coolant flow, we noticed that the pressure of unchilled coolant (still at room temperature) caused some reduction in larval movement. This is because the flexible membrane mechanically compresses the larva against the coverslip if there is coolant flowing through the chip, irrespective of coolant temperature. Additionally, mechanical compression aids optical access by bringing internal body structures close to the

coverslip which are otherwise hard to observe. However, under the influence of purely mechanical compression, the larvae are not adequately immobilized to enable steady, high resolution imaging of sub-cellular structures necessary for performing neurobiological studies. The average movement of the neuronal structures was further minimized when chilled coolant was flowed through the chip (Figure(s) 6–8), thereby clearly indicating the immobilizing effect of cryo-anesthesia. This demonstrates that mechanical compression of the flexible membrane and the low-temperature environment act synergistically to promote larval immobilization. Imaging with the chilled coolant led to a significant improvement in imaging quality (Supplementary Movies 2–4). Figure 7 shows the total trajectory of distances moved over a 2 minute duration, comparing chilled verses unchilled (at room temperature) coolant flow. The trajectories obtained with chilled coolant flowing through the chip are far more localized, further supporting the results shown in Figure 6. Histograms showing percentage of total number of movements occurring over a 2 minute duration (Figure 8) illustrate that immobilization of NMJs, VNCs and axons with the proposed method is predominantly due to the cryo-anesthetic effect of flowing chilled coolant through the chip. The probability density estimates fitted onto the histogram data (Figure 8) indicates that bigger movements (greater than one pixel) are much less likely to occur while imaging with chilled coolant use compared to with the control. Apart from having a larger spread of data points, the maximum distance moved was also larger while imaging all three neuronal structures for the control compared to with using chilled coolant as demonstrated by the box and whisker plots in Supplementary Figure 3.

3.4. Evaluating performance of alternative coolants

The main disadvantage of using 5M salt water solution as a coolant is that over time, salt precipitates out of the solution and can clog the coolant inlet/outlet ports if the device is not rinsed thoroughly after use. Furthermore, besides requiring a more rigorous cleaning/rinsing protocol, any accidental leakage of the coolant onto metallic microscope parts can lead to corrosion, which could cause significant and expensive long term damage. The metal flow chamber sandwiched in between the two Peltier modules can also get corroded over time, which can lead to release of debris that might enter the chip along with coolant flow and obstruct the fluidic channels. This may ultimately cause the membrane to burst under fluid pressure, which would cause permanent damage to the chip.

To overcome these disadvantages, we explored the use of two other commonly available, non-corrosive coolants with subzero freezing points: 40% ethanol and 50% glycerol solutions. Video frames of collapsed Z stacks (under 400× magnification) of mitochondria at NMJs, cell bodies in VNCs, and axonal mitochondria in segmental nerves were collected over 2 minute intervals and analyzed using the previously described experimental procedure (Section 4.4). 50% glycerol solution proved to be too viscous and affected peristaltic pump performance (flow rate was significantly reduced). Furthermore, as a result of its dense nature, movement artifacts were created along the z direction while imaging due to very large membrane deflections which in turn increased pressure on each larva leading to poor immobilization. 40% ethanol solution, being relatively less viscous, did not produce any significant reduction in flow rate (comparable to 3.2 ml/min with the flow rate potentiometer setting on the pump being constant) and did not produce any perceptible movement artifacts.

Additionally, the immobilization microchamber stabilized to the same final steady state temperature of $\sim 5^{\circ}\text{C}$ in ~ 1.8 minutes without requiring any precooling. This is comparable to the performance of pre-cooled 5M salt water solution maintained at -10°C in the external coolant reservoir (Supplementary Figure 4). Furthermore, the quality of immobilization within the VNCs and axons was comparable to the results obtained with 5M salt water solution. However, the structures at the NMJs were not as well immobilized by 40% ethanol solution (Figure 9). Given that PDMS is gas permeable and ethanol is highly volatile, it is likely that vapors of ethanol may reach the larva in the immobilization microchamber and affect the quality of immobilization. Ethanol is a well-known stimulant and has been proven to affect mobility in adult fruit flies [33] by inducing muscle contractions, but its effect on larvae are less well understood. To minimize any ethanol vapors from reaching the micro immobilization chamber, a $2\text{ }\mu\text{m}$ layer of parylene c was sandwiched in between two $10\text{ }\mu\text{m}$ PDMS layers creating a $22\text{ }\mu\text{m}$ thick PDMS-parylene c-PDMS membrane separating the immobilization microchamber from the cooling microchamber. Parylene c is sandwiched in between two thin PDMS layers instead of depositing it as a single layer on the bottom surface of the device in order to minimize any crack formation [34].

After the parylene c coating, the average movement of the neuronal structures at NMJs imaged with 40% ethanol coolant were significantly lower, and were comparable to results obtained using 5M salt water solution (Figure 9). Since the total membrane thickness is larger and parylene c is stiffer compared to PDMS (Young's modulus is three orders of magnitude higher), the resulting membrane deflection and therefore pressure being applied on the larvae is lower even though the final steady state temperature remains the same. Given that the quality of immobilization is similar despite membrane deflection being significantly lower in the parylene c coated chips, it may be concluded that the cooling effect is the dominant factor contributing towards causing immobilization compared to the effect of mechanical compression. In fact, making the membrane stiffer results in lower mechanical pressure being applied on each larva as it is being compressed against the coverslip implying the potential to have improved survival post immobilization. Therefore, apart from using 5M salt water solution, 40% ethanol solution can be used as an alternative coolant choice by using the modified chip post parylene c deposition.

It was seen that for the 5M salt solution, the flow rate increases with pump motor speed, which is adjusted with the flow rate potentiometer setting on the pump. However, for heavier more viscous fluids like 40% ethanol and 50% glycerol solutions, increasing load on the pump limits one's ability to control flow rate. Therefore, for these fluids, the steady state temperature and system response time will not reduce even if the flow rate potentiometer setting is increased beyond 4.

All the imaging experiments were performed at the same flow rate potentiometer setting for ease of comparison between different coolant performances. Additionally, higher flow rates would exert greater fluid pressure on the membrane causing it to deflect more and overcome the vacuum pressure generated by the syringe, leading to the chip detaching from the glass cover slip. Since it is possible for 5M salt water solution to reach lower temperatures with higher flow rates, in theory, it might be possible to achieve even better immobilization with increased pump motor speeds while using a stronger negative pressure source like a vacuum

pump to maintain the chip on the coverslip. However, due to larger membrane deflection caused by higher flow rates, the fluid pressure acting on the membrane (Supplementary Figure 5) would also lead to a larger mechanical compressive force on the larvae which could lead to reduced survival. A negative effect on the health of the larvae due to increased mechanical pressure could in turn potentially lead to a lower quality of immobilization. Therefore, the flow rate should be increased with caution, especially while using the cryo-larva chips without parylene c.

3.5. Larval survival

To determine the effect of cryo-anesthesia on larval survival, we subjected batches of 3rd instar larvae to repetitive fifteen minutes long cooling periods at 4 °C with a gap of 1 hour in between consecutive exposures. Even after six consecutive cycles, survival remained unaffected (Supplementary Figure 6). A trial was considered a success if the larva was alive after being subjected to each cooling cycle.

While 3rd instar larvae showed good survival even after multiple imaging sessions, most of the 1st instar larvae did not survive long enough to develop into 2nd instars even after being subjected to single 10 minute imaging sessions on the microfluidic chip containing a PDMS membrane. However, survival of young animals was increased significantly when the parylene coating was used: 90% of the 1st instar larvae survived to develop into 2nd instars after being subjected to single 10 minute imaging sessions on the microfluidic chip containing a PDMS-parylene c-PDMS membrane. Being both thicker as well as stiffer, the mechanical compressive force applied by the PDMS- parylene c-PDMS membrane on the larvae is significantly lower compared to the pressure applied by just the PDMS membrane. This seems to confer significantly improved survival of the fragile 1st instar larvae.

3.6. Use of the cryo-larva chip to track mitochondria within neurons *in vivo* reveals stationary nature of synaptic mitochondria and heterogeneous mitochondrial turnover

As an application of the new immobilization method for *in vivo* imaging, we observed the trafficking of mitochondria from the cell bodies of larval motoneurons to their synaptic axon terminals through time. To track the trafficking of mitochondrial contents in neurons, we used the photo-convertible Dendra2 protein targeted to the mitochondrial matrix (mitoDendra2), which was expressed in a subset of motoneuron using Gal4/UAS system. Intact 3rd instar larvae were immobilized using the chip (without parylene c), and were subjected to focal application of UV light to irreversibly convert mitoDendra2 from green to red fluorescence specifically in the cell bodies in the VNC (section 4.4). With the immobility of the animal, the photo-conversion is specific to the VNC only, as no converted (red) mitoDendra2 can be observed at t=0h in distal regions like the axons and NMJ (Figure 10). The trafficking of red (photoconverted) mitochondria from cell bodies to axons and the axon terminals was then observed through time via successive imaging in the larval chip (Figure 10(a), (b)). Because of the non-invasive nature of the proposed method, the same neuronal structures within a single animal could be analyzed through time. Between imaging sessions the larvae were removed from the chip and allowed to crawl and feed unrestricted on grape-agar plates. Neither the photo conversion nor multiple imaging sessions in the microfluidic chip were toxic to the animals (Data not shown). Moreover, the degree of photo

conversion (Supplementary Figure 7) and the distribution of converted mitoDendra2 at different time points was reproducible from animal to animal (Figure 10).

The data suggest that mitochondrial contents distribute rapidly from cell bodies to axons where they merge with unconverted (green) mitochondria resident in axons, presumably via mitochondrial fusion (Figure 10(c)) [35]. Within 5 hours after photoconversion of the mitochondria in cells bodies, most of the mitochondria within axons contained both red and green mitoDendra2 protein, while only a few (green only, white arrows) remained devoid of converted mitoDendra2. This represents a population of axonal mitochondria that has not been subjected to fusion with cell-body derived mitochondria within this short time frame. Within 12 hours, the vast majority of the mitochondria in axons are both red and green, which suggests a continued mixing of mitochondrial contents throughout the axon with mitochondria derived from the cell body at the time of photoconversion (Figure 10(c)). Previously, mitochondrial fusion in axons was only assessed by observing individual events captured via time-lapse imaging [35], limiting the chance of observing a fusion event to only a few minutes at a time. With this new method, mitochondrial dynamics in axons can be assessed globally over the course of days, through time, and in the same animal.

At a further distance from the cell body, at the ends of the axon are the axon terminals at the NMJ synapse. We were able to observe the trafficking of cell-body derived mitochondria to the terminal through time by capturing the mixing of red mitochondria with the green/unconverted mitochondria that were resident in the NMJ (Figure 10(c)). Surprisingly, the time lapse imaging revealed that, in contrast to their high degree of motility in axons [36] for which we could not track the same mitochondria across individual time points (Figure 10(c)), mitochondria at NMJ synapses are stationary, maintaining the same relative position and shape between imaging sessions over time courses as long as 12 hrs. By observing the same pool of synaptic mitochondria over time, it can be inferred that the contents of the synaptic mitochondria are dynamic: the increase in red fluorescence, accompanied by a decrease in the green fluorescence (Figure 10(d)) suggests that the contents of synaptic mitochondria are slowly replaced over time with new material from the cell body. With this new method, we were able to quantitatively measure this delivery of cell-body derived mitochondria to the NMJ by plotting the frequency distributions of the average red to green fluorescent intensity ratio per mitochondria of individual mitochondria at the different time points (Figure 11(a)). On the population level, there is a clear progression of the increased delivery of cell body derived (red) mitochondria to the NMJ (higher red:green ratio) as time from conversion increases as shown by the distinct peaks in the distributions at 5 and 12h post conversion.

The immobility of individual mitochondria at the NMJ makes it straight-forward to recognize and quantify the changes that occur to individual mitochondria through time (Figure 11(b),(c)). As shown by the broad distributions in the 5 and 12h post conversion frequency distribution, incorporation of cell-body derived mitochondria is not homogenous to all NMJ mitochondria (Figure 11(a)), especially at the 5h time point. At 5h after photoconversion of cell body mitochondria, converted mitoDendra2 appears within some (Figure 11(b), pink arrowhead) but not all of the synaptic mitochondria (Figure 11(c), blue arrowhead), with some variation in intensity changes of green and red fluorescent intensities

within individual mitochondria, even in the same position in the NMJ (Figure 11(b)). Within 12 hours, nearly all of the synaptic mitochondria contain photo-converted mitoDendra2 (Figure 10(d)), but with varying degrees of green and red intensities on the individual mitochondria level as shown by the red:green ratios of 19 individual mitochondria that were tracked across the 3 time points (Figure 11(c)). This time course implies that although on the global level, there is consistent delivery of mitochondria to the NMJ (Figure 11(a)), there seems to be non-uniform turnover of contents from individual mitochondria at the NMJ synapse that can be tracked with this method. We would ultimately be interested in understanding more about this heterogeneous turnover of mitochondria, and how it correlates with other subcellular events occurring in the NMJ synapse in the future.

In trafficking experiments, co-localization of multiple fluorescent markers acquired sequentially (on the order of milliseconds of separation) is essential for proper interpretation of results. Therefore, mitochondrial identification and tracking is heavily dependent upon quality of immobilization, which directly influences how well the fluorescent features are spatially co-localized across image frames. To test how reliably our chip can immobilize larvae on short term time-scales that would be necessary for co-localization of dual fluorescent markers, we quantified the co-localization of fluorescent sub-cellular features in between image frames acquired 8 seconds apart (average time taken by image acquisition system to acquire image frames from both red and green channels). Co-localization has been evaluated both in terms of estimating correlation between (Section 4.7) the frames (Figure 12(a)) as well as quantifying percentage areal overlap of objects (Section 4.8) identified in and common to both frames (Figure 12(b)). The greater the value of correlation and percentage of areal overlap, the higher the degree of co-localization. Furthermore, it was observed that while co-localization of features at the NMJs was comparable to the results obtained from the chip developed by Ghannad-Rezaie *et al.* [21] which mechanically immobilizes the larvae, the co-localization of features at the VNCs and axons is significantly improved with the proposed cryo-anesthetic method compared to the purely mechanical immobilization approach [18] by 30% and 50% respectively.

3.7. Use of the cryo-larva chip to observe neuro synaptic growth *in vivo*

Because of the powerful genetic tools available, the *Drosophila* larval NMJ has been a widely used model for studying and understanding the cellular machinery involved in synaptic growth. Various screens have been used to identify genetic mutations that result in the altered growth of the NMJ, typically assessed by observing the morphology of the NMJ at a later stage (ie, 3rd instar) [37]. This post-mortem analysis can be used to infer much information about synaptic growth, but few studies have been able to assess developmental growth, since this requires successively imaging the same larvae through multiple developmental stages [38] [39]. The method proposed in this paper can be used to observe the growth of synapses at the NMJs as the larvae grow dramatically in size, starting from the 1st instar and all the way up to the 3rd instar developmental stage. This is a significant improvement over the method previously developed by Ghannad-Rezaie *et al.* [21] in which the same device could not be used to image different larval sizes. Individual 1st instar larvae were immobilized and their NMJs imaged with the microfluidic chip containing a PDMS-parylene c-PDMS membrane. The same animals were then placed on the chip after they

developed into 2nd and then 3rd instar larvae after 24 and 48 hours respectively and their NMJs were re-imaged. The growth of synapses at the NMJs, both in terms of size as well as number of branches, can be easily observed across all larval developmental stages as seen in Figure 13. Although shown here is observing the developmental growth of a normal NMJ, with the wealth of genetic tools and mutations available from decades of research in the *Drosophila* NMJ field, the details of many of the synaptic growth defects can be looked at in a more in-depth manner using this method to track growth dynamics from 1st through 3rd instars larvae. Further, although we are observing the simple morphological growth in this particular example, with the abundance of endogenously tagged protein traps [40] and fluorescent reporters available in the *Drosophila* field, subcellular changes can be observed with this same method at different developmental stages of NMJ development.

3.8. Comparison of cryo-larva chip to other approaches of immobilization

Even though low temperature larval immobilization has not been demonstrated for *Drosophila* before, cryo-anesthesia has previously been implemented to immobilize other model organisms such as *C.elegans*. Chung *et al.* [22] developed a fully automated microfluidic platform for performing high throughput microscopy and sorting of *C.elegans* with flow based pressure driven loading and robust temperature control on chip. While this platform has been very successfully implemented for worm populations studies, it would not be possible use a pressure driven flow based loading mechanism as the larvae cannot be maintained in a fluid suspension like *C.elegans*. Larvae would not be able to survive inside a fluid without having access to air while being manipulated by pressure driven flow inside tubing. Additionally, the *Drosophila* larval cuticle is stiffer than the *C. elegans* cuticle, making them more susceptible to mechanical stresses during flow based manipulation. A flow based pressure driven loading approach cannot guarantee loading larvae with their ventral surface facing downwards which is the required orientation to perform most imaging applications. Furthermore, a significant change in device dimensions as well as architecture would be required to accommodate *Drosophila* larvae due to their significantly larger sizes. Rhode *et al.* [23] developed a high throughput technology for screening *C.elegans* inside multiwall plates by cryo-immobilizing and imaging worms using a custom made Peltier element array. While the method can be successfully used to perform imaging at the cellular level for *C.elegans*, *Drosophila* larvae being much larger and having a more turbid cuticle compared to *C.elegans* require slight mechanical compression for good optical accessibility, especially for imaging more internal structures and sub-cellular processes which is not possible with the platform developed by Rhode *et al.* [23].

Among the several approaches that have been developed for *Drosophila* larval immobilization, the method presented by Heemskerk *et al.* [11] enables *in vivo* imaging of larvae using desflurane with an immobilization chamber that is easy to implement. However, desflurane, a regulated chemical substance, requires additional safety components such as a gas collection assembly, transfer tubing, gas disposal assembly and gas disposal tubing making the use of desflurane complicated. The device developed by Heemskerk *et al.* seems to be amenable for use with other gas anesthetic agents like CO₂, which is relatively easier to implement than desflurane. Given that the cryo-larva chip has a two layer architecture with a liquid impermeable, but gas permeable PDMS membrane separating the two layers, chilled

coolant flow through the chip could be easily replaced with a gas anesthetic like CO₂. This feature makes the cryo-larva chip an “off the shelf” versatile alternative to previous devices used to immobilize larvae since it can utilize both liquid coolants, and potentially anesthetic gases (as previous shown by Ghannad-Rezaie *et al.* [21]).

Even though anesthetics like CO₂ are attractive choices for immobilization, chemical vapors have relatively more significant negative effects on larval physiology compared to chemical free cold immobilization. A study conducted by Badre *et al.* [41] concludes that apart from effects such as blocked synaptic transmission at the neuromuscular junction by decreased sensitivity to glutamate, an exposure to 100% CO₂ for 30 minutes will kill *Drosophila* larvae. On the other hand, a study conducted by Kostal *et al.* [42] concludes that viable larvae may be stored in quiescence at low temperatures (3–9°C) for up to two months, with larval survival showing further improvement with a fluctuating thermal regime (stored at 5°C for 20 hours followed by 4 hours at 11°C for 60 days) indicating that exposure to warmer temperatures allows repair of potential chill injury. These results reflect positively on the utility of the cryo-larva chip that also subjects larvae to less than 10 minutes of cold temperatures (4–6°C) followed by recovery over extended periods at room temperature. Even though the studies have been conducted over long durations under the fluctuating temperature regime, the results presented by Kostal *et al.* [42] provide evidence of larvae re-establishing ion balance, repair by heat shock protein assisted processing, replenishment of depleted ATP, management of oxidative damage and synthesis of cryo-protectants, all of which would be expected to take place using the cryo-larva chip. Additionally, it was observed that approximately 80% of the larvae imaged with the cryo-larva chip subsequently pupariated which demonstrates minimal long term physiological effects due to the cryo-immobilization method. Even though cooling the animal will effect biological processes on the order of tens of minutes, the intended use of the cryo-larva chip is to cool the animal for short periods of time (2–4 minutes) only to perform imaging, and then allow the animal to recover and develop back at normal temperature to provide a series of snapshots through time of long term processes like mitochondrial transport and synaptic growth that takes places over several hours if not days. It is not meant to perform shorter term (on the level of tens of minutes) time-lapse imaging of a physiological process while the animal is being cooled inside the chip, for which the method developed by Heemskerk *et al.* [11] would be a better alternative.

The method developed by Heemskerk *et al.* [11] avoids mechanical compression to minimize tissue distortion which is very effective for applications where imaging of soft internal structures such as the wing disc is required. In applications in which the maintenance of the tissue architecture without distortion is absolutely necessary, the mechanical compression would be a downside, and the method utilized by Heemskerk *et al.* could be a better alternative. However, the mechanical pressure and subsequent tissue distortion would be generally consistent in different imaging sessions of the same animal, and therefore would be accounted for in comparisons through time. In our application, we notice no significant distortion of the structures we imaged (VNCs, axons, NMJs) in comparison to fixed larval tissue from dissected animals that are not compressed in any way. Further, *Drosophila* larvae have a turbid cuticle and slight mechanical compression is required for good optical accessibility, especially for higher resolution imaging of sub-

cellular structures along the dorsal and ventral surfaces that are relatively much less distorted when squeezed compared to lateral organs like the wing disc. The PDMS membrane in the cryo-larva chip improves optical focus by pushing the larva against the glass coverslip. Additionally, since it is critical to have the animal lie straight with its ventral surface facing downwards on the coverslip to be able to locate anatomical landmarks while imaging, the mechanical compression applied by the membrane maintains proper body orientation and minimizes tissue distortion by preventing the larva from crawling around/coiling/twisting/flipping inside the chamber before the cooling effect immobilizes the animal. Furthermore, any potential effects of mechanical compression on cellular dynamics within the animal, can be minimized by making minor modifications to device dimensions such as making the membrane thicker and/or stiffer as already demonstrated by improved survival of 1st instars with the parylene membrane.

Simpler, physical constraint type techniques using capillary pressure developed by Nienhaus *et al.* [12] or using tape to mechanically immobilize larvae developed by Restrepo *et al.* [16] are convenient choices for imaging events at the tissue or cellular level. However, there are excessive small body movements that continue to take place while the animal is physically constrained with these mechanical approaches that will have a more substantial negative impact on higher resolution image quality while studying sub-cellular processes like mitochondrial transport. These movements also increase recording time as more image frames need to be acquired to correct for movement. Therefore, by supplementing the partial immobilizing effect provided by a mechanical constraint with cryo-anesthesia, the cryo-larva chip minimizes these small body movements, both in terms of magnitude and frequency. This greatly improves imaging quality and reduces the time to image sub-cellular structures/processes at high magnifications.

Additionally, the cryo-larva chip and associated cooling system is a universal approach as it can be easily implemented with both inverted as well as upright microscopes. Given that some high resolution imaging studies are performed on an inverted microscope, placing a Peltier unit with heat dissipation modules directly on top of the animal or coverslip while imaging will require the construction of specialized mechanisms to prevent excess mechanical loading of the animal, as well as the microscope's piezo-stage. Implementing this approach will therefore significantly increase system complexity that would have to be specific to the user's microscope configuration. Furthermore, if transmitted light is needed for a specific application, the cryo-larva chip will allow light to pass through which would not be possible if the Peltier is directly placed on the animal. Therefore, even though biologists have been known to immobilize adult flies easily by simply placing them directly on the surface of a Peltier element or ice in order to image cellular features under an upright microscope, implementing this approach would have to be able to be accommodated by the microscope configuration and not be universally usable, especially for imaging sub-cellular features, with a high degree of control like the cryo-larva chip set up.

4. Experimental Methods

4.1. Device fabrication

Soft lithography was used to fabricate the microfluidic device. Master molds for Layer 1 and Layer 2 were micro fabricated on two different silicon wafers. A 170 μm mold was created for Layer 1 by spinning and patterning SU-8-2100 photoresist on a silicon wafer. Similarly, a 100 μm mold was created for Layer 2 by spinning and patterning SU-8-2050 photoresist on another silicon wafer. To fabricate Layer 1, a 15:1 PDMS mixture ratio was spun cast at 400 rpm for 50 secs over the 170 μm mold and cured at 65 °C for 5 hours. To fabricate Layer 2, a 10:1 PDMS elastomer to curing agent mixture was poured over the 100 μm mold and allowed to cure for 5 hours at 65 °C. Layer 2 (~ 3.5 mm thick) was peeled off from the 100 μm SU-8 master mold and the coolant inlet/outlet ports were punched using a sharpened, 19-gauge needle (0.031 inch I.D., O.D: 0.042 inch O.D.; Kathetics). Layer 2 was then aligned and air plasma bonded (50 W, 250 mTorr, 60 s) to Layer 1. The two layer PDMS structure was then peeled off from the 170 μm SU-8 master mold followed by the creation of vacuum and food ports using the sharpened, 19-gauge needle that was previously used to create fluidic ports in Layer 2. Leak proof access to Layers 1 and 2 was provided using polyvinyl tubing (0.023 inch I.D., 0.038 inch O.D.; BD Intramedic) connected via a steel pin (0.016 inch I.D., 0.025 inch O.D.) to the coolant inlet/outlet and vacuum ports on the device.

For fabricating devices with PDMS-parylene c-PDMS membranes, the same master molds for Layers 1 and 2 were used. Layer 1 was fabricated by spinning 15:1 PDMS elastomer to curing agent mixture at 400 rpm for 50 secs and curing it at 65 °C for 5 hours. A 2 μm parylene-c polymer using the physical vapor deposition (PVD) process [43] was deposited in a conformal manner onto the surface of PDMS on Layer 1. A 15:1 PDMS elastomer to curing agent mixture was spun cast at 400 rpm for 50 secs over the parylene c layer and cured at 65 °C for 5 hours to create a 22 μm thick PDMS-parylene c-PDMS membrane. Layer 2 was fabricated with coolant inlet/outlet ports punched into it and plasma bonded to Layer 1, ports for vacuum and food were punched and fluidic access was provided as previously described.

4.2. Animal preparation

For experiments tracking immobilization upon the onset of coolant flow (Figure 5), membrane targeted GFP was expressed in all motoneurons using the Gal4/UAS system (OK6-Gal4; UAS-mCD8GFP). For experiments measuring immobility at specific neuronal structures (VNC, Axons, or NMJ) (Figures 6, 7, 8, 9 and 10), mitochondria were labeled by driving expression of the fluorescent protein Dendra2 fused to the mitochondrial targeting sequence from cytochrome C oxidase subunit VIII under the control of a UAS promoter (UAS-mitoDendra2) [44]. Expression of UAS-mitoDendra2 [44] was driven using the m12-Gal4 driver line [21] [45] [46] which expresses in 2 motoneurons per hemi-segment allowing for the visualization of individual mitochondria through time. For photo-conversion experiments (Figure 10) UV light was applied using epi-fluorescence (UV dichroic, 405nm) which was focused onto the VNC using the microscope's condenser at full power for 8 seconds. The approximate region converted was ~25 μm circular region over individual pairs of motoneurons in the VNC, performed multiple times to convert all visible motoneurons in

the lateral VNC expressing mitoDendra2. Images of the VNC were taken before and after conversion, and initial t=0h images of the axons and NMJ were taken after photo-conversion. In between imaging sessions (at 5h and 12h), animals were removed from the chip and placed on grape-agar plates to freely crawl around and feed. All animals were 3rd instar larvae and were cultured and reared using standard *Drosophila* protocols.

For tracking synaptic growth (Figure 13), adult crosses were set 3 days prior to collection of late 1st instar/early 2nd instar animals. Larvae chosen for study varied slightly in size from 1–2mm in overall length, no animals >2mm were chosen for initial imaging on day 0. On day 0, collected larvae (genotype:M12-Gal4, UAS-mCD8RFP/+) were washed in water and placed on the chip containing a PDMS-parylene c-PDMS membrane. After imaging, animals were removed from the chip and placed individually on grape-agar dishes supplemented with yeast paste (dry baker's yeast mixed with water) in a 25°C incubator to freely crawl and forage for 24 hours before the next imaging session (the same procedure was followed in subsequent imaging sessions).

4.3. Larval loading and manipulation

Individual larvae were placed in halocarbon oil 700 (cat. # H8898 Sigma Aldrich Inc.), put on a glass coverslip (#12-544E, Fisherbrand), and then covered by placing the transparent chip on top using manual alignment under a stereo dissection microscope. A tight seal between the PDMS chip, oil and coverslip was created by applying vacuum pressure (600 mTorr) using a 20 cc syringe to the vacuum port of the microfluidic network in 'Layer 1' [47].

4.4. Fluorescence imaging

All *in vivo* imaging acquisitions were conducted using a spinning disk confocal system (Perkin Elmer), consisting of a Yokagawa Nipkow CSU10 scanner, and a Hamamatsu C9100-50 EMCCD camera, mounted on a Zeiss Axio Observer with an oil objective. Volocity software (Perkin Elmer) was used for image acquisition.

For experiments tracking immobilization upon the onset of coolant flow to determine the time required to immobilize the larvae (Figure 5), a single optical plane was captured with a 25× (0.8 NA) oil objective lens every 2 seconds for 9 minutes in total. In each typical imaging session, animals were kept in the chip for approximately 9 minutes.

For experiments measuring immobility at specific neuronal structures (VNC, Axons or NMJ) (Figure(s) 6, 7, 8, 9), a collapsed z-stack of the whole structure was acquired every 1 second over 2 minutes with a 40× (1.3 NA) oil objective lens. Each collapsed z stack, containing 6–7 z planes with step size 0.5–1.5 µm and exposure time of 100–115 milliseconds, took ~1 second to acquire. In each typical imaging session, animals were kept in the chip for approximately 10 minutes.

For photo-conversion experiments (Figure(s) 10, 11), each image was captured with a 40× (1.3 NA) oil objective lens. All images were confocal collapsed z-stacks taken of sequential red and green channels of neuronal structures at all time points. If slight movements occurred during the z-stack and red/green channels were slightly mis-aligned, another z-

stack was acquired until no movement was observed during the z-stack. Each collapsed z stack, containing 6–7 z planes with step size 0.5–1.5 μm and exposure time of 100–115 milli-seconds, took ~ 1 second to acquire. In each typical imaging session, animals were kept in the chip for approximately 5 minutes.

For tracking synaptic growth (Figure 13), imaging of NMJs was initiated two minutes after onset of flow of coolant. Typically, 3 NMJs per animal were imaged with a confocal z-stack using a 40 $\times$ (1.3 NA) oil objective lens and single channel acquisition. Each z-stack containing 6–7 z-planes, spaced 0.5 μm apart, was acquired with an exposure time of 100–115 milliseconds and took ~ 1 second to acquire. In each typical imaging session, animals were kept in the chip for approximately 5 minutes.

4.5. Quantification of on-chip larval movement

In order to quantify on chip larval movement and comment on the quality of immobilization, for every video acquired with the animal immobilized, three random features were selected after ensuring that they are visible across all collapsed z stack frames in the video (Figure(s) 6–9). For videos that were acquired of the larval VNCs, these features corresponded to fluorescently labeled neuronal cell bodies located at the VNC. Whereas for videos that were acquired of the larval NMJs and axons, the features corresponded to fluorescently labeled mitochondria. For a given video, the three chosen features were tracked across every frame and the absolute distance moved by their centroids in between consecutive frames was estimated using Metamorph 7.7.0.0 Imaging software. The video was accessed by the software in the form of 8-bit grayscale multi-tiff image frames. The features to be tracked were manually selected on the first frame by drawing a box around their periphery which formed their template images. The centroid of each box was defined as the corresponding feature centroid. After defining the template images, the search regions (the area in which the software should search for the feature to have moved to) around the features were also defined. The template matching algorithm [48] was then employed by the software to track the location of the features within the search regions in every frame. While running the template matching algorithm, the correlation coefficient threshold was set to 0.5. If the software failed to locate a feature in any given frame, tracking was stopped and the correct location of the feature was manually redefined by visual inspection before proceeding with automated tracking. The average distance moved in between frames was then calculated for every feature centroid followed by the estimation of the average distance moved across all frames for all three feature centroids. The optical resolution of the microscope setup that was used for distance estimation with 400 $\times$ magnification is 0.18 μm (1 image pixel corresponds to 0.18 μm) respectively.

In order to quantify the larval response time to immobilizing effects, for every video acquired before, during and after immobilization, a point (centroid of a cell body along the VNC) was selected that was visible across all frames (Figure 5). The point was then manually tracked across every frame and the distance moved in between consecutive frames was estimated using Metamorph 7.7.0.0 Imaging software. The optical resolution of the microscope setup that was used for distance estimation with 250 $\times$ magnification is 0.29 μm

(1 image pixel corresponds to 0.29 μm). Automated tracking with template matching was not possible as there was a lot of movement before and after immobilization.

4.6 Quantification of mitochondria fluorescent changes

Individual mitochondria were extracted from merged Z-stacks of confocal images of the NMJ using the Volocity software (Perkin Elmer), with an algorithm that selected individual puncta via a determined fluorescent threshold above background. From each mitochondria, an average fluorescent intensity of the whole mitochondria in the red and green channel were used to determine the red:green ratio. For population changes (Figure 11(a)), the average red:green ratio of pooled mitochondria at $t=0\text{h}$ (where there is no red fluorescence) was used to normalize all data. For measuring changes in individual mitochondria (Figure 11(c)), mitochondrial ratios at time points after $t=0\text{h}$ were compared to their original red:green ratio at $t=0\text{h}$ to determine a fold change. Only mitochondria that we were certain to be present and accounted for (based on visual landmarks) all time points were used for this analysis.

For measuring the conversion efficiency (Supplementary Figure 7), all converted cell bodies from a merged Z-stack image pre conversion and post conversion were compared in their overall average red:green fluorescent intensity ratios (similar to what is shown in Figure 10(b)). The ratios were compared to themselves to determine the fold change in the ratio displayed in the graph.

4.7. Quantification of correlation coefficient as a measure of co-localization

Image frames that are temporally 8 seconds apart at the start of each 2 minute video were chosen to be the green and red channels. Pearson's correlation coefficient [48] was calculated between these two frames (Figure 12(a)) using the CellProfiler software [49].

4.8. Quantification of % co-localization area

Image frames that are temporally 8 seconds apart (determined to be the average time to complete 2 color sequential z stack in our experiments) at the start of each 2 minute video were chosen to be the green and red channels. Objects (mitochondria and cell bodies) were identified in these two frames using the CellProfiler software. The objects were identified using the three-class Otsu thresholding method [50]. The % area of pixels that spatially overlapped between the two segmented images was then calculated and defined as % co-localization area (Figure 12(b)).

4.9. On chip temperature measurement

We estimated the temperature distribution created by the chilled coolant flowing through the microfluidic device as well as the temperature in the micro-immobilization chamber using non-invasive infrared thermal imaging [51]. Given the dimensions of the microfluidic device, the region of interest is too small to enable accurate contact measurement based conventional thermometer systems. A FLIR SC600 (measurement range: -20°C – 150°C , accuracy: $\pm 2^{\circ}\text{C}$ or 2%), 7.5 – 13 μm pre-calibrated long-range infrared thermal camera fitted with a FLIR T198059 close up lens (spatial resolution: $\pm 50 \mu\text{m}$) was used to measure the temperature of the immobilization microchamber in the chip. The chip was connected to

the cooling setup and mounted upside down with the coverslip facing the camera mounted on top. The coverslip was placed at the focal distance of the infrared lens with its plane lying perpendicular to the vertical axis of the camera. As it is difficult to measure the temperature of transparent surfaces, a piece of thin (100 μm) black tape with known surface emissivity (0.95) was cut to $\sim 5.5 \text{ mm} \times 2.8 \text{ mm}$ (size of the immobilization microchamber) and pasted on the coverslip over the immobilization microchamber. Images of the chip were then collected at 1 frame per second after starting coolant flow through the chip. Data acquisition and post processing of the infrared images was handled by the FLIR ExaminIR software. The software was also used to specify emissivity (0.95), ambient temperature (23°C) and ambient humidity corrections (15%). During data acquisition and/or during post processing, the minimum, maximum and average temperatures over the region of interest (region covered by the black tape) was estimated and plotted over time. Given that the coverslip ($\sim 150 \mu\text{m}$) and the tape ($\sim 100 \mu\text{m}$) are extremely thin, the time taken by these surfaces to equilibrate to the steady state temperature of the immobilization microchamber inside the chip maybe considered to be negligible.

4.10. On chip fluid pressure measurement

In order to estimate the pressure exerted by the PDMS membrane on the larva, the deflection of the membrane was first calibrated with the application of known static air pressure. The cooling microchamber was pressurized with air at known pressure using an air flow regulator (Elveflow microfluidic AF1 pressure pump) while plugging off the cooling microchamber outlet thereby causing the PDMS membrane to deflect. Membrane deflection was measured by optically focusing on the center of the membrane before and during pressurization using an upright microscope (Olympus BX-51), 10 $\times$ objective lens (MPLFLN10x, Olympus) and bright field illumination mode. Food color was added to PDMS during the fabrication of Layer 1 to enhance optical contrast and improve membrane deflection measurement accuracy. The calibration data was curve fitted using a 3rd order polynomial function (Supplementary Figure 5). After calibration was completed, the chip was connected to the cooling setup without adding the coverslip and coolant (5M salt water solution) was pumped through the cooling microchamber in the chip. The resulting membrane deflection under pressurized coolant flow was optically estimated and measurements were obtained at different flow rates of the coolant. The higher the flow rate, the greater the membrane deflection. Using the calibration curve and measured membrane deflection for a given flow rate, the fluid pressure being applied on the membrane was estimated.

4.11. Coolant preparation

5M salt water solution was prepared by mixing 73.05 g of sodium chloride (cat. # S9888 Sigma Aldrich Inc.) with 200 ml of ddH₂O while stirring. Additional ddH₂O was added until the final volume was 250 ml. 250 ml of 40% (v/v) ethanol solution was prepared by mixing 100 ml of 100% ethanol (cat. # 2701 Decon Laboratories Inc.) with 150 ml of ddH₂O by stirring. 250 ml of 50% (v/v) glycerol solution was prepared by mixing 125 ml of 100% glycerol (cat. # 536407 Sigma Aldrich Inc.) with 125 ml of ddH₂O by stirring.

4.12. Statistical significance test

The two tailed unpaired non parametric Man-Whitney test was performed to test the significance of the difference in mean of average movement measurements (Figure 6) from features at NMJs, VNC and axons between using the proposed method with chilled coolant flow and coolant flow at room temperature (control).

A one way ANOVA test followed by Tukey's multiple comparison test was performed to test the significance of the difference in mean of average movement measurements (Figure 9) from features at NMJs, VNC and axons between using the proposed method with chilled 5M salt water solution flow, chilled 40% ethanol solution flow above the PDMS membrane and chilled 40% ethanol solution flow above the PDMS-parylene c-PDMS membrane.

5. Conclusion

We developed and characterized a microfluidic method for immobilizing *Drosophila melanogaster* larvae which enables *in vivo* imaging in intact animals. The method uses a microfluidic cryo-larva chip to create a low-temperature micro-environment to deliver a combination of mechanical compression and cryo-anesthesia to immobilize individual larvae. A deformable membrane is used to mechanically compress each larva while simultaneously flowing chilled coolant over the PDMS/PDMS-parylene c-PDMS membrane and subjecting the larva to cold temperatures. The method is easy to implement, allows complete larval recovery and can be used to immobilize larvae of different developmental stages. Additionally, since the device is not permanently bonded to the glass coverslip, it can be subjected to multiple uses while making the larval loading process extremely quick and easy. Our results indicate that the proposed method is appropriate for repeated short-term (less than 10 minutes) immobilization sessions, with immobilization sufficient to perform high resolution imaging of sub-cellular structures.

Besides demonstrating high resolution imaging of larval NMJs, VNCs and axons, the proposed immobilization approach can have an impact on a variety of other imaging applications in *Drosophila* neuroscience, including neuronal laser microsurgery for studying axon regeneration, neuronal circuits and time lapse imaging for studying synapse development. Furthermore, technical challenges (mainly survival) have limited the successive imaging of the same *Drosophila* larvae across developmental stages without the use of anesthetics [38] [39]. The combined pressure/cooling immobilization of young larvae presented here allowed for an acceptable level of survival of imaging young larvae across their developmental ages. This alone will make this technique of high value for developmental biologists. Although the focus of this particular data was on the mitochondrial trafficking in neurons, the technique of *in vivo* local photo-conversion and trafficking that we were able to perform using this chip can be utilized for a variety of different long-term time lapse applications. These include imaging of subcellular trafficking, tracking of long range cell migration, and imaging proliferation dynamics. Having such a powerful tracking ability within an intact animal over multiple imaging sessions can be a great resource to many biological fields.

The inclusion of food ports as part of device architecture provides the possibility to maintain animals on chip and in between imaging sessions, similar to Ghannad-Rezae *et al.*'s [21] studies, in which food is delivered every 30 minutes to keep larvae alive on chip for 10 hours. Additionally, this provision will facilitate any attempt at transitioning to a more automated version of the system in the future to increase throughput.

Besides immobilizing *Drosophila* larvae, the cryo-larva chip has the potential to be used to study cellular biological events across developmental stages in other model organisms like *C. elegans*. Immobilization of *Drosophila* larvae is more difficult than that of *C. elegans* as they are much larger in size and therefore exert stronger body forces. Additionally, even if the outer larval body is completely immobilized by physical encapsulation, imaging internal organs of interest such as the CNS (Central Nervous System) capsule is extremely challenging as they can freely move inside the hemolymph-filled body cavity. Given that the proposed method successfully immobilizes *Drosophila* larvae for high resolution imaging, it is extremely likely that with minor modifications to the device architecture, the method will prove to effectively immobilize *C. elegans* as well, thereby expanding its potential applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG.1.

(a) Bright-field image of a 3rd instar larva immobilized in the microfluidic chip: The first and second PDMS layers are highlighted with blue color and yellow colors respectively. Passing the coolant fluid through the second layer decreases the temperature of the immobilization microchamber. (b) Stereoscopic image of the microfluidic chip (scale bar, 5 mm). (c) Cross sectional schematic of the chip that highlights its working principle. When there is no flow of the coolant fluid (left schematic), the larva freely moves in the immobilization microchamber (the PDMS membrane is not deflected). (d) When the coolant fluid is introduced, the larva is cryo-anesthetized and slightly compressed inside the immobilization microchamber.

FIG.2.
Schematic diagram of the experimental setup highlighting the components of the cooling system. The solid blue lines indicate coolant flow.

FIG.3.

(a) Thermograph of the microfluidic chip at a coolant flow rate of 3.2 ml/min (viewed from the glass side). The dotted black lines indicate the outline of the chip (scale bar, 5 mm). The white rectangle represents the location of the immobilization microchamber. (b) Thermograph showing the temperature variation in the immobilization microchamber (~ 2.5 °C difference between the center and the sides). (c) Maximum, average and minimum temperature values obtained over the region bounded by the white rectangle shown in (a) versus time. The cooling modules are turned on and coolant flow (3.2 ml/min) through the chip starts at 0 sec with the average temperature stabilizing to ~5 °C in under 5 min.

FIG.4. Average temperature of the immobilization microchamber versus time for different flow rates. Coolant flow through the chip started at 0 sec

FIG.5.

(a) Absolute distance moved by the centroid (point of intersection of the diagonals of the white box bordering the cell body and indicated as a red dot in (b)) of a cell body located at the VNC over time (acquired before (I), during (II) and after (III) immobilization). The dashed blue line represents average distance moved between frames for ten 3rd instar larvae. The solid green line represents best fit by non-parametric regression using locally weighted scatterplot smoothing. Error bars indicate standard error of mean of measurements taken from ten animals imaged on the same device. (b) A single frame showing the VNC and the centroid of a cell body (scale bar, 30 μm).

FIG.6.

Schematic representation of larval neuronal structures of interest. Confocal micrographs showing fluorescently labeled: (a) Mitochondria at an NMJ with red dots representing centroids of individual mitochondria. (b) Cell bodies at the VNC with red dots representing centroids of individual cell bodies. (c) Mitochondria along axons with red dots representing centroids of individual mitochondria. Plots showing the average of distances moved by centroids of : (a) mitochondria at NMJs, (b) cell bodies along VNCs and (c) mitochondria along axons in between video frames collected over 2 minutes. The videos were collected

with larvae loaded on the microfluidic chip with chilled as well as room temperature coolant (5M salt water) flowing (3.2 ml/min) through the chip respectively. Error bars indicate standard error of mean of measurements taken from ten larvae imaged on the same device. (scale bar, 11 μ m).

FIG.7.

Confocal micrographs showing the trajectories (shown as green, blue and yellow paths and marked by red numbers) moved by three randomly selected centroids of (a) mitochondria at NMJs, (b) cell bodies along VNCs and (c) mitochondria along axons in between video frames collected over 2 minutes. The videos were collected with larvae loaded on the microfluidic chip with chilled as well as room temperature coolant (5M salt water solution) flowing (3.2 ml/min) through the chip respectively (scale bar, 11 μ m).

FIG.8.

Histograms showing percentage of total number of movements in between frames of (a) mitochondria at NMJs, (b) cell bodies along VNCs and (c) mitochondria along axons in between video frames collected over 2 minutes. The solid lines (red and blue) represent probability density estimates fitted onto histogram data. The videos were collected with larvae loaded on the microfluidic chip with chilled (represented in blue) as well as room temperature (represented in red) coolant (5M salt water solution) flowing (3.2 mL/min) through the chip respectively. Pixel resolution is 0.18 μm .

FIG.9.

Plots showing average of distances moved by (a) mitochondria at NMJs, (b) cell bodies along VNCs and (c) mitochondria along axons in between video frames collected over 2 minutes. The videos were acquired with larvae loaded on the microfluidic chip (without and with parylene c) with chilled coolant (5M salt water and 40% ethanol) flowing through the chip. Error bars indicate standard error of mean of measurements taken from ten animals imaged on the same device.

FIG.10.

(a) Schematic representation of newly developed assay to measure mitochondrial trafficking from the cell-bodies of motor neurons in *Drosophila* larvae to axons and the NMJ synapse in intact larvae. While immobilized in the cryo-larva chip, whole larvae are subjected to focal application of UV light to their VNC to photo-convert labeled mitochondria (mitoDendra2). Converted cell-body derived mitochondria (red) can then be tracked as it is trafficked to distal neuronal processes. (b) Representative example of motor neuron cell-bodies on one side of the VNC expressing mitoDendra2 before (Pre-conversion) and immediately after (Post-conversion) photo-conversion. For all subsequent images in this figure: Green fluorescence is unconverted Dendra2, while red fluorescence is photo-converted Dendra2 protein (scale bar, 20 μ m) (c) Representative example of the mitochondria in axons of motor neurons from the same animal through time following photo-conversion (0 hours (h) represents immediately following conversion). Notice at 5h post-conversion (middle), most resident axonal mitochondria have fused with cell-body derived mitochondria (green + red). Some axonal mitochondria have not fused with mitochondria trafficked from the cell body

(green alone, arrowheads). By 12h, all axonal mitochondria contain content from trafficked cell-body derived mitochondria (green + red) (scale bar, 10 μ m). (d) Representative example of an NMJ synapse from the same animal tracked through time for the delivery of cell-body derived mitochondria. Top panel shows whole synapse, while bottom panels represent a high magnification view a single branch of the NMJ (boxed region). Notice at 5h post conversion (middle panel), there is some trafficked mitochondrial material in the NMJ shown by the weak red-fluorescence in certain mitochondria (arrowheads). By 12h post-conversion, all mitochondria in the NMJ contain mitochondrial material that was present at the cell-body at the time of conversion (scale bars, 20 μ m and 10 μ m).

FIG.11.

(a) Frequency distribution plot of the average red:green ratio of individual mitochondria at the NMJ from the three different time points observed in mitochondrial trafficking experiments (t=0h (blue), 5h (green) and 12h (red) post conversion). Each time point include >500 mitochondria from at least 6 NMJs originating from 3 different animals. Bars represent frequency histogram and dotted lines represent Gaussian fit curves for each individual time point. (b) Representative region of NMJ containing 4 recognizable stationary mitochondria across all time points (t=0h, 5h and 12h post conversion). Merged pictures are shown in top row, while middle row is un-converted (green) mitoDendra2 alone and bottom row shows

cell-body derived converted (red) mitoDendra2 alone. Colored arrowheads depict 2 different mitochondria with differing rates of incorporation of red material, and correspond to the colored lines in (c). (scale bar, 2.5 μ m) (c) Example plot of the individual red:green fluorescent intensity changes of 19 individual mitochondria across the three different time points shown in (b). Colored lines related to the corresponding colored arrowheads in (b). Note the differential changes in red:green ratios of neighboring mitochondria indicating a heterogeneous delivery and turnover of mitochondria in the NMJ.

FIG.12.

Box and whisker plots showing (a) correlation and (b) % co-localization area between red and green channels of mitochondria at NMJs, cell bodies along VNCs and mitochondria along axons. The image frames corresponding to the red and green channels are 8 secs apart. The videos were collected with larvae loaded on the microfluidic chip with chilled coolant (5M salt water solution) flowing through the chip. Each box extends from the 25th to 75th percentiles, the line in the middle is plotted at the median and the whiskers span between the smallest and largest data values (measurements taken from ten animals imaged on the same device).

FIG.13.

Representative images of growing NMJ terminals imaged successively during larval development in a single animal. To visualize the morphology of the NMJ synaptic terminal, a membrane tagged red fluorescent protein (UAS-mCD8-RFP) was driven in three motoneurons (onto muscles 26, 27, and 29) via the M12-Gal4 driver. Late 1st instar larvae were immobilized and imaged with the cooling chip on day 0 (left), and the same animal was imaged 24h (middle) and 48h (right) later. The different developmental stages of the animal are indicated on top. Arrowhead denotes the region of high magnification below and in inset, highlighting the growth and elaboration of one region of the NMJ. All pictures are at the same scale with scale bars of 20 μ m (whole NMJ pictures) and 2 μ m (high magnification images).