A MICROFLUIDIC CHIP FOR IMMOBILIZING AND *IN VIVO* IMAGING OF *DROSOPHILA* LARVAE

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ABSTRACT

We present a 2-layer microfluidic PDMS chip for the long-term immobilization and *in vivo* imaging of *Drosophila* larvae. Using a combined CO_2/mechanical immobilization mechanism, we achieved high on-chip survival rates (up to 85% during a 10-hour time course) while we eliminated body movement which is required for high resolution imaging. We demonstrated the use of this technique for performing time-lapse imaging of axonal sprouting over a 12-hour period after laser axotomy. The noninvasive nature of the proposed method enabled for the first time to study *in vivo* axonal regeneration after laser injury. Our results suggest that the proximal stump of the injured axon remains dormant for 7 hours before regeneration is initiated.

KEYWORDS: Microfluidics, Axonal Regeneration, *In Vivo* Imaging, *Drosophila* larva

INTRODUCTION

Despite the great potential of using *Drosophila* larva as a model organism for studying various biological processes at the molecular and cellular level [1, 2], several technical issues need to be addressed in order to perform *in vivo* imaging and quantify the relevant phenomena. For high resolution imaging, the larva body needs to be exceptionally stationary during immobilization. On the other hand, time-lapse imaging of long term events such as new axonal growth after injury requires a gentle immobilization technique which will not affect the larva physiology. Conventional immobilization approaches involve dissection or the use of chemicals (e.g. chloroform or isofluorane) to anesthetize the larva body. While the use of anesthetics has many advantages, anesthetics are known to inhibit neuronal activity and alter neuronal physiology, while safety concerns can become an important issue [3]. Because larvae can survive only short doses of these chemicals, time lapse imaging must be restricted to time intervals of 2 hours in order to allow recovery between doses of the anesthetic [3]. In order to *in vivo* image cellular events on a broader time scale (e.g. up to several hours), a chemical-free immobilization method is needed.

Here, we report the development of novel microfluidic PDMS chip for the long-term immobilization and *in vivo* imaging of *Drosophila* larvae (Figure 1). The chip, termed the 'larva chip', employs a dual-immobilization mechanism: it mechanically restricts the larva body while simultaneously delivers carbon dioxide gas (CO_2). It minimizes the stress on the larva (recovery takes place in less than 30 seconds) and allows time-lapse *in vivo* imaging to be performed over extended periods of time. The larva chip can be efficiently utilized for imaging various cellular events which occur on different time scales, ranging from milliseconds up to several hours. While in this work we focus upon cellular responses to targeted neuronal injury, the proposed approach can be broadly used for *in vivo* imaging of various biological processes in *Drosophila* larvae.

EXPERIMENTAL

The larva chip is a 2-layer PDMS microfluidic chip and enables the long-term (up to 10 hours) immobilization of single larvae. The first PDMS layer of the chip contains a 3.5 mm long, 1.5 mm wide and 170 μm thick PDMS microchamber (the 'immobilization microchamber') and it is reversibly attached to a glass coverslip. The immobilization microchamber is designed to snugly fit the body of an early-stage 3rd instar larva (the 3rd instar larva body is ~ 200 μm thick). To avoid larva starvation, the immobilization chamber is connected to two microfluidics channels that supply grape juice to the larva head. The microchamber is surrounded by a microfluidic network that is held under vacuum in order to maintain a strong seal between the PDMS and the coverslip. A second PDMS layer is vertically integrated into the first PDMS layer to deliver a 95:5 % mixture of CO_2 : air to the immobilization microchamber through a 10 μm thick PDMS membrane. CO_2 is supplied under moderate pressure (5 psi) to the chip resulting in the deflection of the PDMS membrane that collapses into the larva body. The combination of pressure that is applied to the larva body through the PDMS/glass walls and the delivery of CO_2 is sufficient to completely immobilize it. Additionally, internal body structures, such as segmental nerves which contain motoneuron and sensory neuron axons, are brought closer to the coverslip, allowing for high resolution imaging through the use of high numerical aperture microscope objectives (Figure 1C). Using this dual (CO_2/mechanical) immobilization approach, larvae can be kept alive for more than 10 hours (Figure 2).

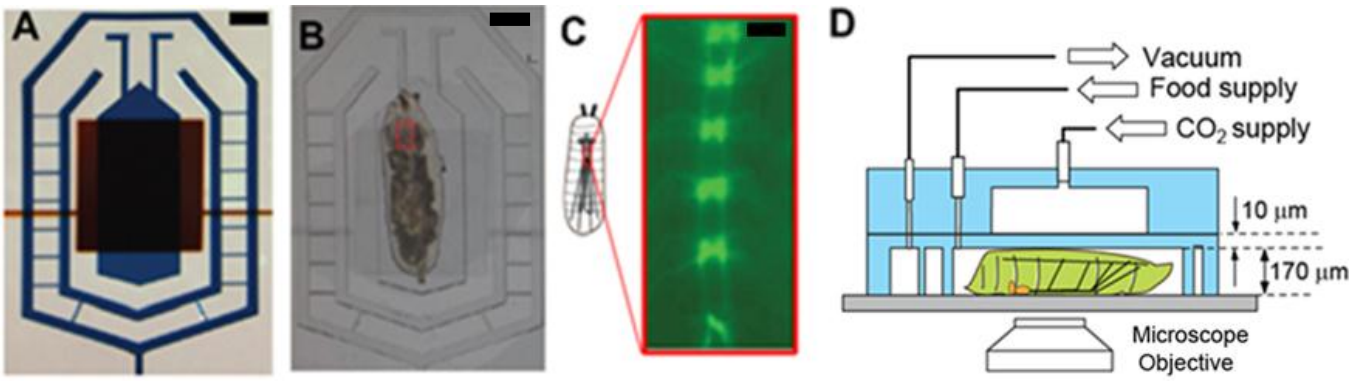

Figure 1. (A) The 2-layer PDMS chip for larva immobilization. The first PDMS layer (labeled with blue color) has an immobilization microchamber and is connected to two microfluidic channels to supply food to the larva head. A microfluidic network surrounding the chamber is used to create a tight seal between the PDMS and the glass coverslip. A second PDMS layer (labeled with red color) is vertically integrated into the first PDMS layer to deliver CO_2 through a 10 µm thick PDMS membrane. Scale bar, 1 mm, (B) Brightfield image of an immobilized, 3rd instar larva. Scale bar, 1 mm, (C) Fluorescent image of GPF-labeled axonal processes after larva immobilization Scale bar, 20 µm. (D) Schematic of the 2-layer architecture of the PDMS chip.

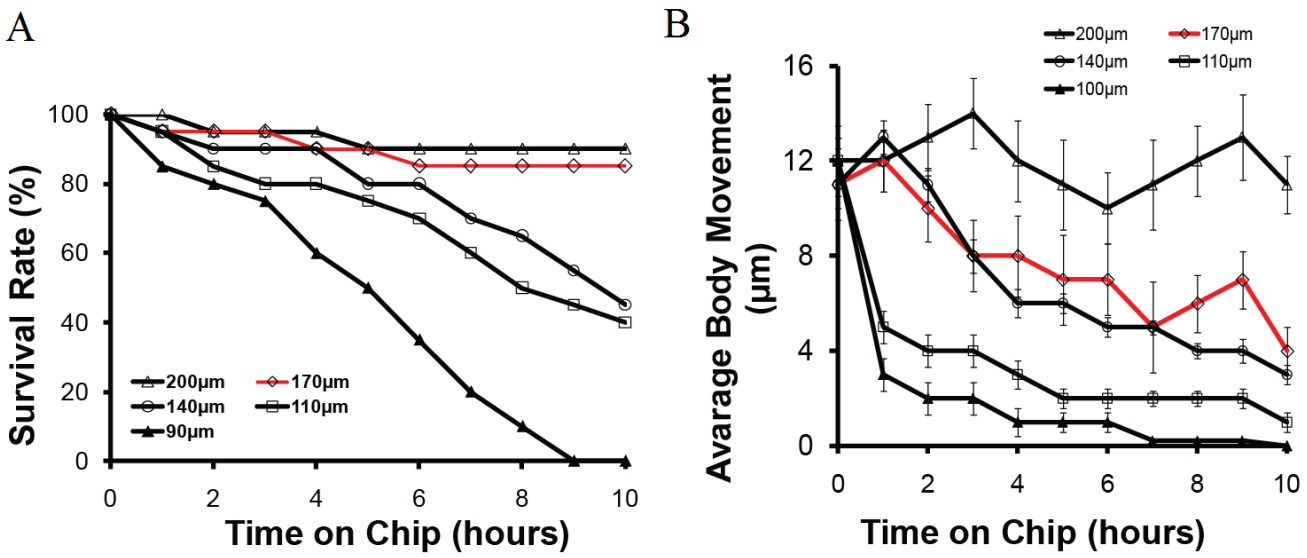

Figure 2: Larva survival rate (A) and average body movement (B) versus time on-chip. Larvae were periodically immobilized throughout the 10 hour time course (30s for every 5 min).The optimum immobilization microchamber thickness of 170 µm resulted in less than 10 µm of average body movement and survival rates up to 85%.

RESULTS

We used the larva chip to study and quantify regenerative axonal growth. Specifically, we conducted longitudinal time-lapse *in vivo* imaging of laser-injured single axons. Laser injuries were performed using a nanosecond 435 nm pulsed UV dye laser on single motoneurons [4]. We tracked the proximal stump of injured axons for a 12-hour period after injury (Figure 3) by collecting high resolution confocal images every minute throughout this time course. We observed that the proximal stump is relatively dormant for the first 7 hours after injury. However, between 7 and 12 hours after injury, new axonal sprouting is readily observed. By quantifying the change in the shape of proximal stump of the injured axon (data not shown), we noticed that F-actin, a well-known protein involved in vesicle and organelle movement as well as in cell signaling, is particularly dynamic between 10 and 11 hours after injury.

Those minute-to-minute changes in F-actin structure suggest the existence of a dynamic network of F-actin, which would be a fundamental component of a functional growth cone. It is intriguing that the dynamics are not observed immediately after injury, but require at least 7 hours after injury to initiate. Previously characterized transcriptional responses to injury require a similar time course in *Drosophila* neurons, so this time of dormancy may reflect the need for new gene expression or

transport of new material in order to form a new growth cone. In future studies it will be interesting to determine the cellular requirements for initiation of those dynamics.

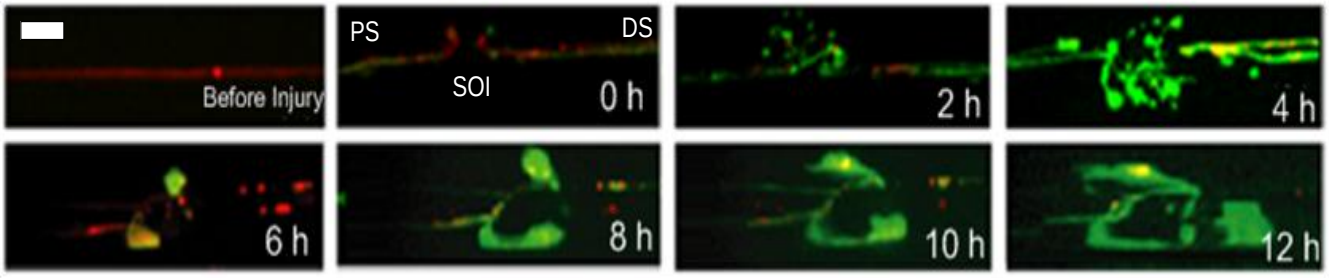

Figure 3: In vivo time-lapse images of the regeneration process 0-12 hours after laser axotomy. The proximal site (PS) of injury, the site of injury (SOI), and the distal site (DS) of injury are highlighted right after injury (0 h). The red color represents red fluorescent protein (RFP) expression that is localized at the membrane of the axon. The green color represents expression of F-actin. Scale bar, 10 µm.

CONCLUSIONS

We developed a novel 2-layer PDMS microfluidic chip for immobilizing single *Drosophila* larva. The proposed approach has several advantages over conventional methodologies: (i) it replaces the use of chemicals, allowing for *in vivo* imaging of unanesthesized animals, (ii) larvae do not need a recovery period after immobilization, imaging over a broad range of time scales is therefore possible, (iii) the immobilization conditions are reproducible and well-controllable, and (iv) the larva chip is simple to fabricate and its design can be easily adapted to immobilize larvae of different developmental stages or several larvae at once. Although we only described the use of the proposed immobilization technique to study responses to neuronal injury, we envision that the proposed larva chip can be broadly used to study many different cellular events *in vivo*. Examples include the formation of new synaptic contacts at neuromuscular junctions, the motility of cytosolic components in neurons, muscles, or glia and intracellular calcium signaling .

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