

Microfluidic *in vivo* Screen Identifies Compounds Enhancing Neuronal Regeneration

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Abstract—Compound screening is a powerful tool to identify new therapeutic targets, drug leads, and elucidate the fundamental mechanisms of biological processes. We report here the results of the first *in vivo* small-molecule screens for compounds enhancing neuronal regeneration. These screens are enabled by the microfluidic devices we have developed for *C. elegans*. The devices enable rapid and repeatable animal immobilization which allows high-throughput and precise surgery. Following surgery, animals are exposed to the contents of a small-molecule library and assayed for neuronal regeneration. Using this screening method we have identified several compounds that enhance neural regeneration *in vivo*.

I. INTRODUCTION

THERAPEUTIC treatment of central nervous system pathologies, such as spinal cord injuries, brain trauma, stroke, and neurodegenerative disorders, will greatly benefit from the discovery of small molecules that enhance neuronal growth after injury. Identification of a diverse repertoire of such molecules and of their cellular targets can also provide important tools for fundamental investigations of the mechanisms involved in the regeneration process. Currently, small-molecule screens for factors affecting neuronal regrowth can only be performed using simple *in vitro* cell culture systems. However, these systems do not truly represent *in vivo* environment. Importantly, off-target, toxic or lethal effects of chemical compounds may only manifest *in vivo*. Thus, the thorough investigation of neuronal regeneration mechanisms requires *in vivo* neuronal injury models.

In vivo neuronal regeneration studies have been performed mainly in mice and rats. However, their long developmental periods, complicated genetics and biology, and expensive maintenance limit large-scale studies in these animals. We previously demonstrated femtosecond laser microsurgery as a highly precise and reproducible injury method for studying axonal regeneration mechanisms in the nematode

Caenorhabditis elegans (*C. elegans*) [1], [2]. Wild type nematodes move constantly, and to perform precise laser axotomy or imaging at the cellular level, animals must be immobilized. We have developed microfluidic on-chip technologies that allow automated and rapid manipulation, orientation, and non-invasive immobilization of *C. elegans* for sub-cellular resolution imaging and femtosecond-laser microsurgery [3], [4]. These technologies enable a variety of high-throughput genetic and compound assays.

We report here the first *in vivo* small-molecule screens for compounds enhancing axonal regeneration. The compound library we have screened yielded molecules targeting a wide variety of cellular processes, including cytoskeletal components, vesicle trafficking components, and protein kinases that enhance neuronal regeneration.

II. MICROFLUIDIC SMALL-ANIMAL SORTER

A. Device Layout and Operation

We developed a microfluidic small-animal sorter that can rapidly isolate and immobilize individual animals. The sorter consists of control channels and valves (gray) that direct the flow of worms in the flow channels in different directions (Fig. 1) [3]. A worm is captured in the chamber by suction via the top channel while the lower suction channels are inactive. The chamber is then washed to flush any other worms in the chamber (blue line) toward the waste or back to the circulating input. The chamber is isolated from all of the channels and the worm is released from the top suction channel to be restrained by the lower suction channels (red line). The aspiration immobilizes animals only partially, and it is not sufficient to completely restrict their motion. In order to fully immobilize the animals, we create a seal around them that restricts their motion completely. This is done by using a 15–25 μm -thick flexible sealing membrane that separates a press-down channel from the flow channel below (Fig. 1e) [4]. The press-down channel can be rapidly pressurized to expand the thin membrane downwards, wrapping around the animals and forming a tight seal which completely constrains their motion in a linear orientation. The image acquisition and processing are then performed, and the worm is either collected or directed to the waste, depending on its phenotype. Quantitative analysis of the immobilization stability shows that it is comparable to chemical anesthetics, and there was no change in the lifespan or brood size of the immobilized animals [4]. Additionally, visual observation of the animals and their

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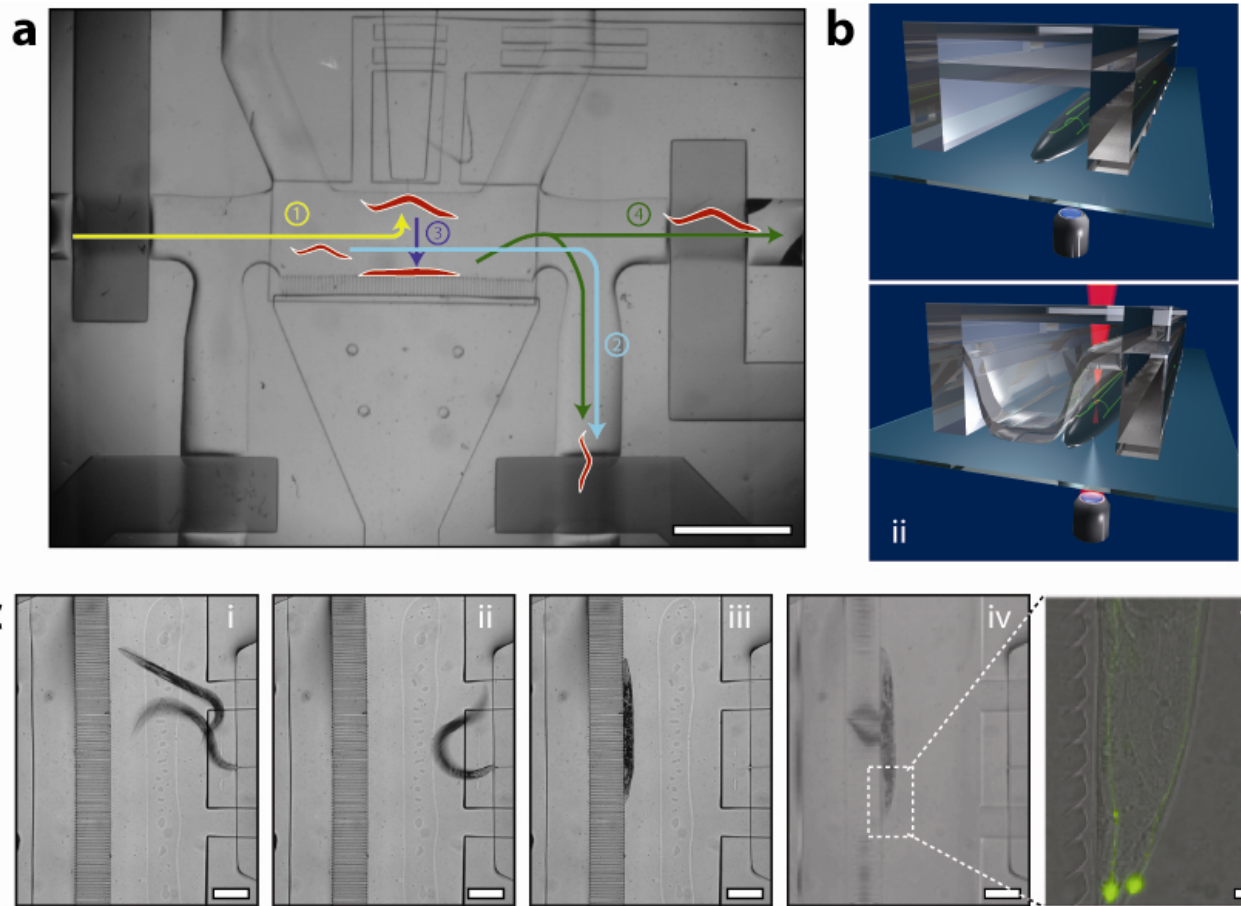


Fig. 1. Microfluidic immobilization for femtosecond laser surgery of *C. elegans*. (a) Micrograph of chip with numbered arrows showing microfluidic *C. elegans* manipulation steps. 1: Loading of nematodes and capture of a single animal by one aspiration channel. 2: Washing of the channels to remove and recycle the rest of the nematodes. 3: Release of the captured animal from single aspiration port, and recapture and orientation of it by a linear array of aspiration ports. 4: Collection of the animal after surgery. Scale bar: 1 mm. (b) Illustration of the final immobilization and laser axotomy: Once a single animal is captured and linearly oriented (i), a channel above it is pressurized pushing a thin membrane downwards (ii). This membrane wraps around the animal significantly increasing immobilization stability for imaging and surgery. (c) Single animal capture (i), isolation (ii) and immobilization (iii & iv). (v) shows combination bright-field of animal and fluorescent image of gfp-labeled posterior lateral mechanosensory neurons. Scale bars: (i)-(iv) 250 μ m, (v) 20 μ m.

neurons showed no signs of hypoxia or other distress.

B. Applications

Femtosecond-laser micro/nanosurgery enables precise ablation of sub-cellular processes with minimal collateral damage [5] and we have previously employed this technique to perform the first axonal regeneration study in *C. elegans* [1], [2]. However, manually preparing an animal for surgery, imaging and recovering it afterwards are laborious. Additionally, the effects of long-term anesthesia on biological processes are not known. We can use our immobilization technique to repeatedly and rapidly immobilize animals and perform femtosecond-laser microsurgery with sub-cellular precision.

Another application requiring an even higher degree of stabilization is multi-photon microscopy [6]. This technique has the ability to perform optical sectioning with negligible out-of-plane absorption and emission due to its non-linearity. This dramatically reduces photobleaching and phototoxicity, [7] which is especially significant in assays

that require animals to be imaged at multiple time points. Both of these applications are illustrated in Fig 2.

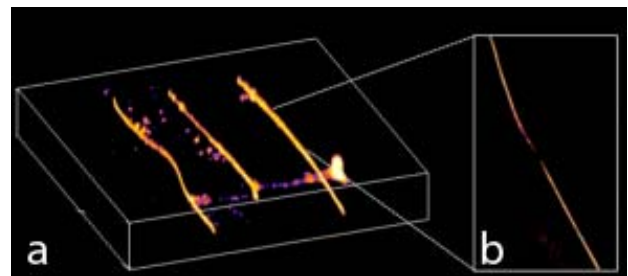


Fig. 2. On-chip small-animal immobilization allows for the use of many powerful optical techniques. Both (a) three-dimensional two-photon imaging and (b) femtosecond laser microsurgery can be rapidly and repeatedly performed on chip.

III. *IN-VIVO* SMALL-MOLECULE SCREENS FOR FACTORS AFFECTING NEURAL REGENERATION

The speed and accuracy of immobilization achieved by our microfluidic devices enables large-scale screening of chemical and genetic libraries. We have used these devices to screen for compounds affecting neural regeneration

following axotomy. Animals were subsequently exposed to elements of a small-molecule library containing a wide variety of compounds. By analyzing the length of regenerating axons following compound exposure, we identified several compounds that enhance neuronal regeneration *in vivo* (Fig. 3). By performing various RNAi screens using these laser and microfluidic technologies, we also identified genetic targets of these compounds.

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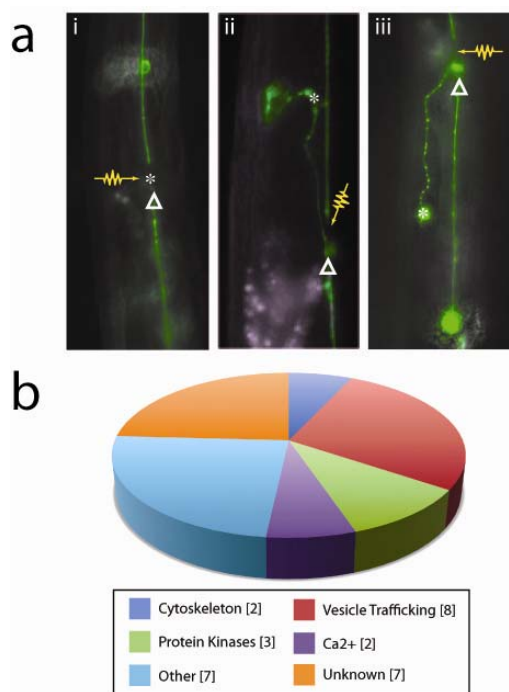


Fig. 3. (a) Illustration of some regeneration types observed following laser surgery and compound exposure. i) Short regeneration in control animal. ii,iii) Long regeneration in the presence of compound. Arrow indicates original surgery location, triangles (Δ) and asterisks (*) indicate start and end points of regenerated regions, respectively. (b) Results of *in vivo* small-molecule screen for factors increasing neural regeneration. Bracketed number indicates number of compounds discovered enhancing the regeneration process.

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