

High-Throughput On-Chip *in vitro* and *in vivo* Genetic/Compound Screening Technologies

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Overview of group:

Our Lab is working on development and applications of technologies for studying and engineering neural processes. Both *in vivo* and *in vitro* neural regeneration and degeneration is being studied by femtosecond laser nano-surgery and on-chip multi-photon imaging as well as microfluidic *in vitro* and *in vivo* on-chip high-throughput screening technologies using the model organism *C. elegans*, primary mammalian neurons as well as human embryonic stem cell derived neurons.

1. High-throughput On-chip Small-animal Screening

Sponsors

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Project Staff

Christopher Rohde, Cody Gilleland, Fei Zeng, Chrysanthi Samara

Overview

The size and complexity of the instrumentation used to study large vertebrate animal models prohibits their use in high-throughput assays for rapid identification of new genes and drug targets. Because of this, researchers turn to simpler organisms, and the advantages of using small invertebrate animals as model systems for human disease have become increasingly apparent. The nematode *Caenorhabditis elegans* (*C. elegans*) is a powerful model organism due to a number of useful properties including its small size, optical transparency, rapid developmental cycle and the availability of a wide array of species-specific genetic techniques. However, since the first studies on *C. elegans* in the early 1960s, little has changed in how scientists manipulate this tiny organism by manually picking, sorting, and transferring individual animals. The reliance on manual techniques means that large-scale forward- and reverse-genetic screens can take several months to years to complete. The high-throughput techniques that exist in *C. elegans* require assays to be significantly simplified in order to be even partially automated.

Fortunately, many of the properties that make *C. elegans* a useful model organism also make it well suited to manipulation in microfluidic channels. This has enabled us to create components for an integrated, whole-animal, high-throughput sorting and large-scale screening platform to perform drug and genetic assays with sub-cellular resolution. Microfluidics have previously been used to perform novel assays on *C. elegans* for specific applications including generation of oxygen gradients [1], worm culturing/monitoring during spaceflight [2], optofluidic imaging [3], and maze exploration [4]. We have designed microfluidic devices [5][6] from multiple layers of the flexible elastomer poly(dimethyl siloxane) [7] that can be combined in various configurations to allow a multitude of complex high-throughput assays such as mutagenesis, drug and RNAi screens. These devices are: (i) a small-animal sorter that enables highly-stable of immobilization live, unanesthetized animals; (ii) an array of microfluidic chambers for simultaneous incubation, immobilization, sub-cellular-resolution imaging and independent screening of many animals on a single chip; and (iii) a microfluidic interface to large-scale multiwell-format libraries that also functions as a multiplexed animal dispenser.

Technology Background

On-chip high-throughput immobilization and sorting

The high mobility of *C. elegans* requires them to be immobilized in order to examine cellular and sub-cellular features. This is most commonly done using anesthesia such as sodium azide (NaN₃), levamisole, or tri-caine/tetrimisole. However, anesthetics can have negative or uncharacterized side-effects on biological processes. Cooling can also be used to reversibly immobilize animals, however this can also have unpredictable effects, especially since many *C. elegans* strains are especially sensitive to temperature variations. Additionally, these immobilization techniques are not suitable for assays requiring physiologically active animals, such as investigations into germ-line proliferation, development, or neurophysiology.

We have developed a microfluidic small-animal sorter that enables rapid and repeatable sub-cellular-precision immobilization of animals. Animals enter the chip through the inlet channel and can be continuously re-circulated. A single worm is captured via suction by a microchannel held at a low pressure, which eliminates the problem of simultaneously capturing multiple animals. The remaining animals are then flushed from the chamber, and microfluidic valves [8] are closed to isolate the single animal. The pressure in the single suction channel is then raised and the pressure in the multiple-suction channel on the opposite side of the channel is lowered. This partially immobilizes the animal against the multiple aspiration ports, but the animal is still capable of some movement. To completely immobilize the animals, we create a seal around them that restricts their motion completely [6]. This is done by using a 15–25 µm-thick flexible membrane that separates two elastomer layers. The top channel can be rapidly pressurized to expand the thin membrane downwards. The membrane wraps around the animals and forms a tight seal, completely immobilizing them in a linear orientation. Fig. 1 (a) shows a diagram of this process, and Fig. 1(b) shows an image of an immobilized adult animal in the device. Fig. 1(c) shows superimposed bright-field and fluorescence images taken at high magnification.

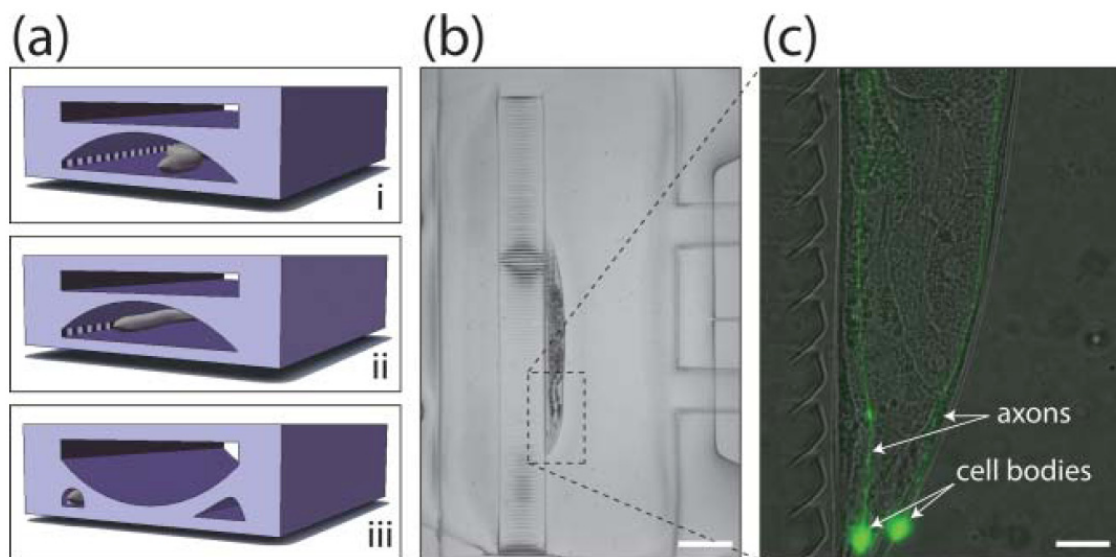


Figure 1: Microfluidic immobilization of *C. elegans*.

Following immobilization, the worm can be imaged at sub-cellular resolution using high-resolution optics. The stability achieved is sufficient to allow three-dimensional imaging and sub-cellular manipulation using femtosecond laser micro-surgery (Section 2.2). Following phenotype identification the captured animal is released and can be directed to one of two collection channels.

To quantify the effectiveness of our microfluidic immobilization versus regular anesthesia, we tracked the cell bodies of touch neurons labeled with green fluorescent protein (GFP) using a software algorithm. To track the movement of all cell bodies, we captured movies at 50x magnification of *pme4::gfp* animals immobilized either by the anesthetic NaN_3 at concentrations $10 \mu\text{M}$ (the highest concentration for which we could recover animals) or by our microfluidic device. To identify the cell bodies, the frames from the movies were first thresholded and the large connected regions were identified. The centroids of these regions were recorded for each frame, and their instantaneous velocities were calculated. The movement of the immobilized animals is comparable to their motion when deeply anesthetized.

We tracked the lifespan and brood size of 25 animals that were each immobilized for 1 min using 15 PSI of pressure in the immobilization channel to see if our devices affect the health of the animals. Note that the pressure applied to the animals by the membrane is not the full 15 PSI, due to the resistance of the membrane to flexing. The immobilized population was compared to a control population that was not run through the device. The mean lifespan of the immobilized population was 17.3 days (s.d. = .05 days) and 16.9 days for the control population (s.d. = 4.0 days). We used the Graphpad Prism software package to perform the log-rank (Mantel-Cox) test. The p-value is 0.8947, which suggests there is no statistically significant difference between the lifespans of the two populations. Both populations also produced normal brood sizes, and were free of axonal blebbing.

Femtosecond-laser micro/nanosurgery enables precision ablation of sub-cellular processes with minimal collateral damage [9] and we have previously employed this technique to perform the first axonal regeneration study in *C. elegans* [10,11]. However, manually preparing an animal for surgery, imaging and recovering it afterwards are laborious. Additionally, the effects of long-term anesthesia on these 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. Fig. 2(a) shows an image of a *pme4::gfp* animal whose touch neuron process has been cut. This provides a powerful tool for the discovery of potential drugs and genetic factors affecting neural degeneration and regeneration.

Another application requiring an even higher degree of stabilization is multi-photon microscopy [12]. 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, [13] which is especially significant in assays that require animals to be imaged at multiple time points. Fig. 2(b) and Fig. 2(c) show two volume reconstructions of *pmec4::gfp* animals acquired using two-photon microscopy of non-anesthetized live animals immobilized in our device.

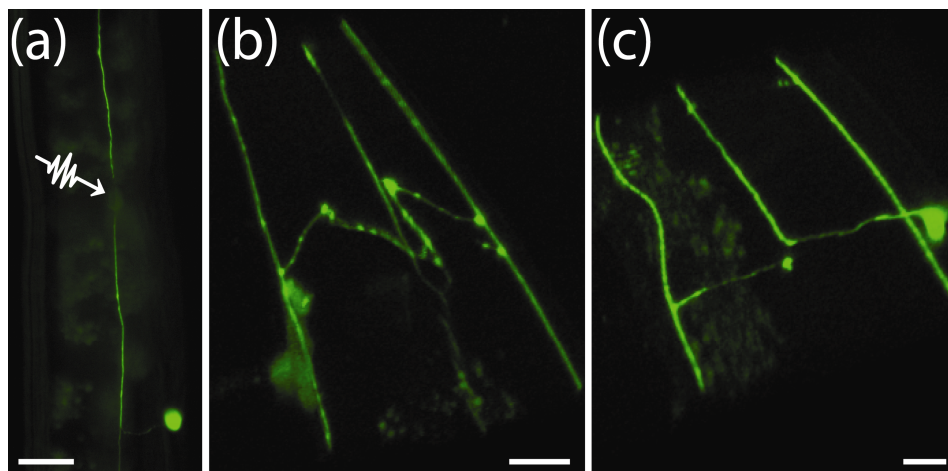


Figure 2: Microfluidic immobilization enables sub-cellular manipulation and three-dimensional imaging of live, awake animals.

Large-Scale Time-Lapse Studies Using Multiplexed Incubation chambers

High-throughput time-lapse studies on small animals are currently performed in multiwell plates by automated fluorescence microplate readers [14]. Because the animals swim inside the wells, only average fluorescence is obtained from each well, and cellular and sub-cellular details cannot be imaged. Although anesthesia can be used to immobilize the animals, the effects of long-term anesthesia prohibit many time-lapse screens, and anesthesia has detrimental effects on many biological processes. Additionally, animal loss can occur during media exchange. To address these problems, we designed the microfluidic-chamber device shown in Fig. 3(a) for worm incubation and for continuous imaging at sub-cellular resolution. Sorted worms can be delivered to the chambers by opening valves via multiplexed control lines [15].

Microchamber chips based on this design can be readily scaled for large-scale screening applications because the number of control lines required to independently address n incubation chambers scales only with $\log(n)$ [15]. The millimeter scale of the microchambers can allow hundreds of microchambers to be integrated on a single chip. To image animals, a flow is used to push the animals toward the posts arranged in an arc inside the chambers (Fig. 3(b) and 3(c)). This flow restrains the animals for sub-cellular imaging. The arrangement of the posts positions animals in a similar configuration, which simplifies the analysis of images. The medium in the chambers can be exchanged through the microfluidic channels for complex screening strategies, and precisely timed exposures to biochemicals (e.g., drugs/RNAi) can be performed. The use of microfluidic technology also reduces the cost of whole-animal assays by reducing the required volumes of compounds.

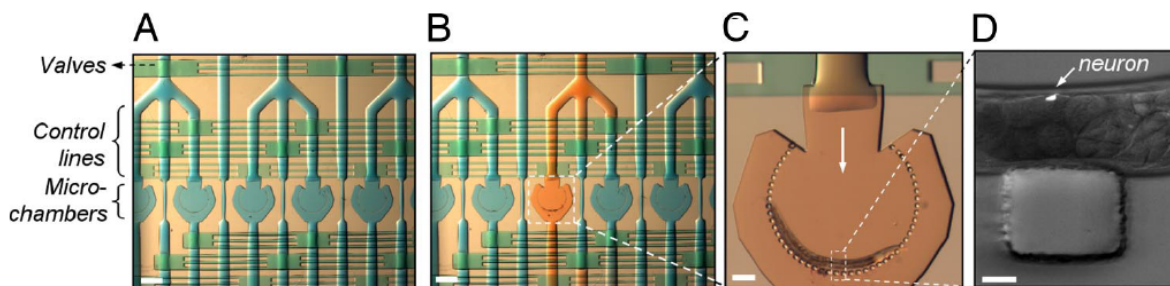


Figure 3: Individually addressable microfluidic screening chambers.

Well-plate interfacing

Interfacing microfluidics to existing large-scale RNAi and drug libraries in standard multiwell plates represents a significant challenge as it is impractical to deliver compounds to hundreds of microchambers on a single chip through hundreds of external fluidic connectors. The interface device in Fig. 4 addresses this problem. This microfluidic interface chip consists of an array of aspiration tips that can be lowered into the wells of microwell plates, which allows minute amounts of library compounds to be collected from the wells by suction, routed through multiplexed flow lines one at a time, and delivered to the single output of the device. Following delivery the lines of the chip can be easily and automatically washed to prevent cross-contamination. The output of the interface chip can then be connected to our microfluidic-chamber device for sequential delivery of compounds to each microchamber. Combining this multiwell-plate interface chip with existing robotic multiwell-plate handlers will allow large libraries to be delivered to microfluidic chips. The same device can also be used to dispense worms into multiwell plates, simply by running it in reverse.

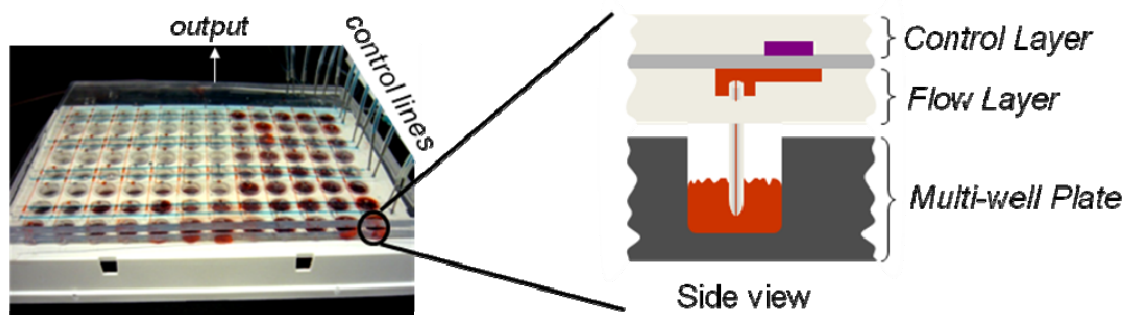


Figure 4: Microfluidic interface device for large-scale compound delivery.

Screening Strategies and Current Research

A variety of assays can be performed by combining the above devices in different configurations. Fig. 5 shows a setup to perform large-scale RNAi and drug screens with time-lapse imaging by combining our sorter, integrated microchambers, and multiwell plate interface chips. Although *C. elegans* is self-fertilizing and has perhaps the lowest phenotypic variability among multicellular model organisms [14], variations among assayed animals are still present, reducing the robustness of current large-scale screens. Sorting technology can be used to select animals with similar phenotypes (such as fluorescent marker expression levels) before large-scale assays to significantly reduce initial phenotypic variations [14, 16]. Feature extraction algorithms can be run on animals immobilized in the sorter or the incubation to screen thousands of animals on a single chip. The microfluidic immobilization method present in our small animal sorter is a rapid and highly repeatable technique for immobilizing small animals for imaging

and manipulation of sub-cellular processes without anesthesia. The stability is comparable to that of deep anesthesia and does not affect the lifespan or brood size of the animals. This enables high-throughput screening of cellular and sub-cellular phenotypes in whole animals, as well as the use of high precision techniques such as femtosecond-laser microsurgery and multiphoton microscopy on physiologically active animals. We are currently using these technologies to perform the first high-throughput investigation into the mechanisms of neural degeneration and regeneration following injury in *C. elegans*.

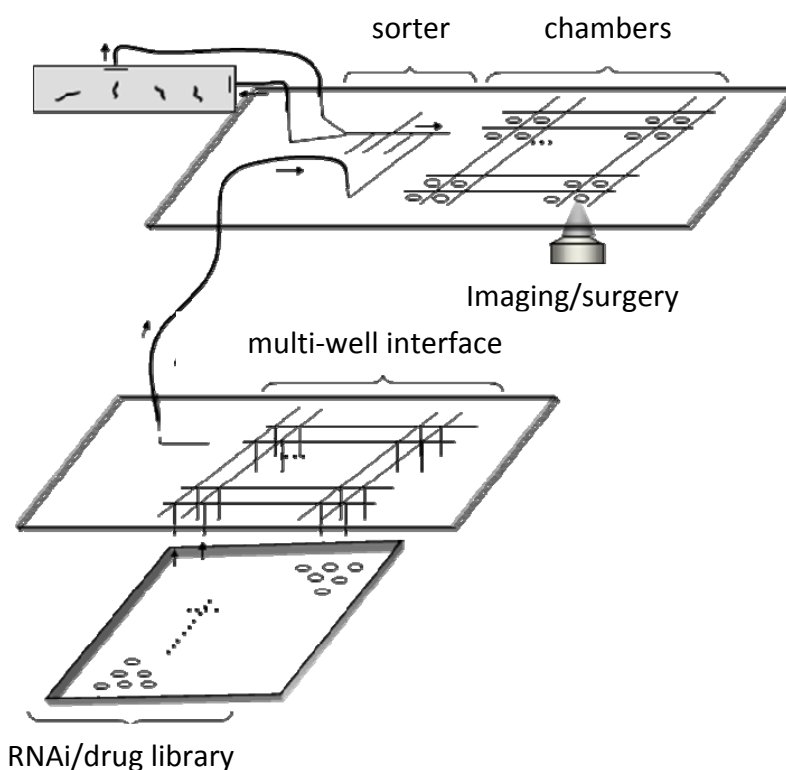


Figure 5: Experimental arrangement for RNAi/drug screening.

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“High-Throughput *in vivo* Genetic and Drug Screening Using Femtosecond Laser Microsurgery and Microfluidics”, C. Rohde, F. Zeng, M. F. Yanik, Invited Talk, CLEO, San Jose, 2008.

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