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Trapping the nematode on a micro chip for the future of science

John X. J. Zhang [a]
[a] Ph.D., Department of Biomedical Engineering , The University of Texas , at Austin, Austin, Texas E-mail:
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Trapping the nematode on a micro chip for the future of science

John X. J. Zhang[1]

[1]Ph.D., Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas

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At the basis of neuroscience is the correlation between behavior and neural signals. This relationship is studied using a variety of techniques, including fluorescent intensity measurements of genetically encoded calcium indicators. The simple structure and conservation of some basic developmental pathways between vertebrates and *Caenorhabditis elegans* makes it an ideal model for studies employing this technique, but it has been difficult to apply neural stimuli in a controlled way to such a small organism. New microfabrication techniques allow the creation of "worm traps" via a combination of lithography and microfluidic regulation. These micro-scale devices have made possible the immobilization of nematodes in the absence of glue (which could affect the worms' neural activity), in addition to offering more control over the application and removal of stimuli than was possible in previous experimental setups. Chronis *et al.* link an increase in AVA (a type of motor neurons formed by bilaterally symmetric interneuron pairs) neural activity to the initiation of anterior-traveling body waves, and observed an increase in ASH (a type of sensory neurons responsible for a wide range of avoidance behaviors in response to chemical and physical stimulations) neural activity in response to stimulation by high osmotic strength solutions. The microfabrication developments described can be combined with recent advances in the biological sciences, in particular the developmental embryology, to yield solutions to important problems, such as the detection of low concentration substrates and the analysis of homeostasis and embryonic development. [DOI: 10.2976/1.2806028]

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CORRESPONDENCE
John X. J. Zhang:
John.Zhang@engr.utexas.edu

Elucidating the neuronal basis of behavior is a cornerstone of both cognitive science and neurobiology. Classically, this has been pursued through the biochemical approach of using agonistic and antagonistic drugs to evoke and explore the effects of different neurotransmitter receptors on emotion. This has helped illustrate the role played by different brain centers, such as the hippocampus or amygdala, in fear and apprehension (Engin and Dallas, 2007). Such studies have been used to develop pharmacological agents for neurologically based behavior disorders, such as naltrexone, which decreases alcohol craving by preventing the activation of the μ and κ opioid receptors responsible for pleasure. However, the primary drawback to this method is the widespread distribution of many neurotransmitter receptors, which complicates the establishment of links between neural pathways and behavior. There has additionally been research that has directly stimulated individual neurons with needle-based electrodes.

Another important method of associating behavior with areas of the brain is magnetic based imaging. Two methods are primarily used: magnetoencephalography (MEG) and functional magnetic resonance imaging (fMRI). In both modalities a human subject is made to perform a task, and the areas of the brain recruited to perform it are visualized. MEG detects the magnetic field associated with action potentials, which is generated by an electric current of flowing ions. The imaging employed by fMRI detects blood oxygenation levels by assessing the magnetic state of hemoglobin, which is different for deoxyhemoglobin and oxyhemoglobin. This method has its own

critical limitations: it does not explain mechanism, merely showing the region of the brain activated, and it is dependent on the time required for blood to reach the appropriate region of the brain, thus failing to represent neural processes in real time.

More direct, single-neuron imaging became available with the introduction of genetically encoded Ca^{+2} indicators (GECI), which were used in transgenic worms, flies, zebrafish, and mice to visualize transient calcium movement involved in action potentials. With this innovation, neural activity could be quantified by its relationship to GECI fluorescence. This method offered the highest "resolution," since measurements could be taken at the true neuronal level, instead of merely imaging macroscopic regions of the brain (Pologruto *et al.*, 2004).

However, the GECI method operates under several constraints involving the size and nature of the animal model used, and, consequently, the nature of the environment that provided stimulus. An ideal subject is nearly transparent, which minimizes the loss of signal from calcium-associated fluorescence. Additionally, it is compact, which would limit the complexity of its nervous system and facilitate the process of making correlations between behavior and measured signal. One model organism meeting these requirements is *Caenorhabditis elegans*, which is a small nematode (1 mm in length) that has a simple nervous system made up of 302 neurons. It has been extensively researched, and its genome is fully sequenced. It also has a large repertoire of behaviors, including responses to mechanical, thermal, and chemical stimuli.

Since *C. elegans* is such a small, simple organism, there are challenges to providing stimulus in a controlled environment. Traditionally, the specimen is physically immobilized with glue, following which a chemical stimulus is delivered over the worm by flowing it over the substrate. The shortcoming of this design is that it generates more than one stimulus, which makes it difficult to determine if the resulting signal is evoked by the chemicals, or if it is a response to the mechanical stimulation associated with immobilization and the flow of media over the worm. Additionally, little control could be exerted over both the temporal and spatial distribution of the chemical stimulus. Newer methods involving environments created by microfabrication techniques, in particular microfluidic devices (Xia and Whitesides, 1998; Thorsen *et al.*, 2002), can allow a more precise control over both independent and inter-dependent variables.

MICROFLUIDIC CHIP FOR NEUROSCIENCE

The term "chip" is used as a nod to the manufacturing process of the device, which is commonly used to create silicon computer chips (Chronis *et al.*, 2007). "Microfluidics" refers to devices which can process microscopic volumes of solutions through a series of channels with diameters of only a few hundred microns. The new field of microfluidics has the potential to influence numerous subject areas in biology (Thorsen *et al.*, 2002). Recent research projects made possible by microfluidic advances include the exploration of the archaeal light-driven chloride pump (NpHR) from *Natronomonas pharaonis* for temporally precise optical inhibition of neural activity (Zhang, 2007), and the development of a method for assembling *Drosophila* embryos in a microfluidic device to study the development network (Dagani *et al.*, 2007).

Microfluidic devices are created using soft lithography, a nonphotolithography technique based on self-assembly and replica molding (Teng *et al.*, 2006; Thorsen *et al.*, 2002). The channel is designed using AutoCAD and made into a chrome mask, which is then spin casted to deposit a thin layer of photoresist. An optically clear elastimer, polydimethylsiloxane (PDMS), is poured over the photoresist, cured, and peeled off. The channel pattern is left imprinted in the PDMS. To create the final product, the PDMS strip is stuck to an activated glass cover slip. Using this fabrication technique, microchannels of arbitrary size, length, and complexity can be created easily.

The best way of obtaining more realistic data would be to study the model organism in a controlled environment where external factors, other than the ones specifically used in the tests, do not impact the behavior of the organism. Ideally, observing behavioral processes *in vivo* would result in a more accurate quantitative account of neuronal activity influencing behavior. To overcome these factors, Chronis *et al.* developed a new technique for combining the emerging technology of microfluidics with *C. elegans* imaging. Microfluidic devices control fluid flow on a micro scale and have two requirements: leak-proof microvalves and a method of controlling the valves. The work of Chronis *et al.* expands on previous studies of neuron correlation to movement and behavior in response to osmotic pressure. Using two microfluidic devices, the activity of AVA interneurons (a type of motor neurons formed by bilaterally symmetric interneuron pairs) and ASH sensory neurons (a type of sensory neurons responsible for a wide range of avoidance behaviors in response to chemical and physical stimulations) are monitored and correlated with systemic behavior. It has been suggested that AVA neurons are involved in backward movement, functioning in tandem with ASH neurons to carry out complex multimodal behaviors, including changes in osmotic pressure. By utilizing microfluidics to monitor and measure neural activity in live *C. elegans*, Chronis *et al.* have substantiated this previously unconfirmed hypothesis.

Chip design and fabrication

For the experiment described, two chips were designed. The first step in the process was the creation of master molds via photolithography. Silicon wafers were made by spin casting (spinning of a disk-shaped mold along its central axis, and then pouring molten silicon into the mold to create disk-

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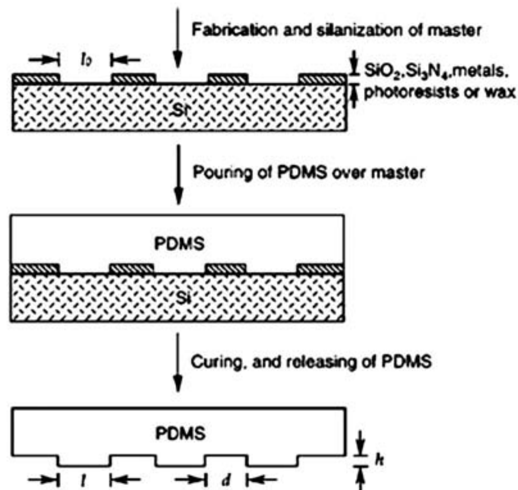

Figure 1. Microfluidic chip fabrication using soft lithography techniques. (Reprinted, with permission, from the Annual Review of Materials Science, Volume 28 ©1998 by Annual Reviews www.annualreviews.org)

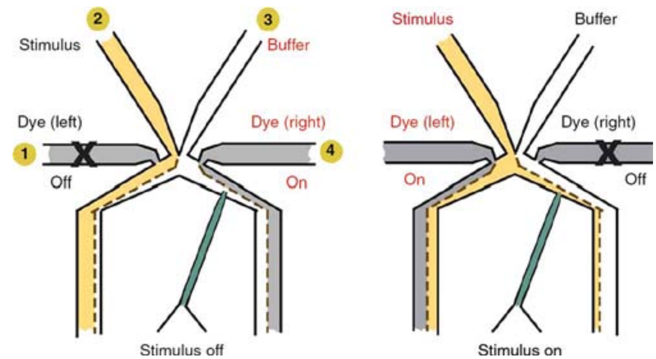

Figure 2. "Olfactory" chip. (Reprinted by permission from Macmillan Publishers Ltd: Nat. Meth. 4(9): 727–731, ©2007)

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shaped wafers), and then thin layers (28 microns) of photoresist were deposited over the wafers. Using AutoCAD software, patterns for the photolithography masks were designed, and the designs were then sent to a mask-making service to be converted into chrome masks. The silicon wafers were masked and exposed to UV radiation; afterwards, the photoresist was developed, the films etched, and the photoresist removed to create patterned surfaces on the wafers. Once the master molds had been constructed, soft lithography was used to make the actual chips. A silicone polymer (PDMS) was poured over the silicon molds and hot plates were used to harden the polymer (Fig. 1, adapted from Xia and Whitesides, 1998). Once the PDMS was cured, it was peeled from the master molds, treated with air plasma to activate the surfaces, and attached to glass coverslips. The resultant channels on the chips were determined by the patterns on the master molds. Inlets and outlets were created in the silicone polymer using a stainless steel needle, and hollow steel pins were attached to the inlets and outlets.

The "behavior" chip

The "behavior" chip consists of a single tunnel through which the worm crawls until it gets stuck. The setup is designed to correlate AVA interneuron activity with bodily crawling movements. The AVA interneurons are located in the thick, immobilized part of the worm, so they are easy to image. The image contrast comes from a genetically encoded fluorescent calcium indicator, G-CaMP. Calcium ions are an important indicator of neuron function; when a neuron fires, its intracellular calcium concentration increases dramatically for a short period of time. The microscope camera can detect this transient calcium increase because G-CaMP fluoresces much brighter in the presence of calcium. Thus, a neuron will signal activity by lighting up. The field of view of the microscope includes about 20% of the length of *C. elegans*, which is small enough to allow AVA neurons to be seen clearly, yet large enough to allow bodily movements to be seen, too. In the behavior chip experiment, researchers observed a stuck worm to see whether it was trying to crawl forwards or backwards, while simultaneously monitoring the activity of the AVA neurons. They found that AVA neurons were inactive when the worm was trying to crawl forward or was still, but became active whenever the worm tried to crawl backwards. This strong correlation infers that AVA neurons probably act directly in controlling backward movement. In theory, the AVA interneurons should activate just before the backward movement is initiated. Chronis *et al.* did not observe this time lag, but speculates that the optical signal coming from the neuron body may lag the signal from the distal processes, which act first. The behavior chip revealed a positive correlate between AVA interneuron activity and backward crawling motion in *C. elegans*.

The "olfactory" chip

The "olfactory" chip was constructed similarly to the behavior chip, containing a narrow channel for trapping a young adult *C. elegans*. However, this channel design ensured that the stuck worm's nose would protrude into a flow stream delivering either a control solution or a highly molar solution. The setup was designed to correlate the ASH interneuron behavior with the detection of high osmotic pressure fluids. A diagram of the olfactory chip is shown in Fig. 2 (adapted from Nikos *et al.*, 2007). The liquid stimulus is switched by selectively blocking channels 1 or 4. One strange property of microfluidic devices is that two fluids running through the same channel will not mix unless there is a mixing mechanism. The olfactory chip takes advantage of this property and send fluorescent dyes down the output channels to monitor fluid flow and make sure there is no mechanical disturbance while switching stimuli. In this way, mechanical stimulus can

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be minimized and chemical stimulation isolated.

The olfactory chip experiments showed that ASH interneurons—a polymodal neuron that responds to mechanical, osmotic, and chemical stimuli—increase activity due to osmotic repellents *and* stimulus withdrawal. The first effect had been suggested in the literature, while Chronis *et al.* reported the second for the first time. The study reports a sustained, increased response (100–200% increase in G-CaMP fluorescence) to stimulus withdrawal. These observations indicate that ASH interneurons have a more complex multiphasic response than was previously thought.

THE BROADER IMPACT

Microfluidic chips have enhanced the ability to record and stimulate intact animals, opening up new research projects in the field of microfluidics and of neurobiology of microorganisms. With the lab-on-a-chip technology, components such as actuators, fluid handling devices and optical devices can be incorporated into microfluidic chips. This opens up a whole new range of experimental possibilities for delivering environmental stimuli of various kinds: pokes and bends, the controlled addition of complex and dynamic chemical and pharmacological elements, and heat and light (Zhang, 2007). Adding components such as waveguides and lasers could allow for the use of genetically targeted photoprobes and may potentially lead to the development of new optical recording techniques.

A topic of recent interest among engineers in the field of sensor design is the development of a device capable of measuring very low concentrations of chemical and/or biological agents. Recent research has shown how mammalian G-protein-coupled receptors (GPCRs) can be expressed in *C. elegans* to generate specific behavior in the presence of human ligands (Teng *et al.*, 2006). The olfactory circuit of *C. elegans* is extremely sensitive and selective, and with these recent genetic developments, one can easily see the possibility of using an "array" of the previously described "olfactory" chips coupled with organisms expressing different GPCRs to form a biosensor with a whole-animal sensing component. Were these to be developed, any compound that activates a known mammalian GPCR could feasibly be detected by the sensor array even at very low concentrations. Such a device could further research into novel methods for chemical sensors and/or biosensors. Whole-organism sensor arrays could be used in security applications for sensing harmful chemical agents like sarin gas.

CONCLUSION

One of the prime benefits of using microfabrication techniques to produce "worm chips" is the high degree of control over the dimensions, shape, and physical properties available to the researcher. This allows for the isolation of the stimulus, which is needed to accurately understand the behavioral response at the neural level. This is illustrated by the ability of Chronis *et al.* to ensure that chemical stimulation on the olfaction chip was limited to laminar flow at the head of the worm, which is a significant improvement over the use of agar plates and nonregional flow conditions. Furthermore, it is possible to create other forms of stimulation (such as thermal and electrical) using lithographic techniques with an equal degree of precision.

The behavior and olfactory chips were relatively simple first steps into the microfluidic environment of whole animals. In a more general sense, this research exhibits methods for the application of microfluidics to biological research. The primary reasons for the use of microfluidics in this experiment were that it allowed maintenance of complete environmental control and, at the same time, the assurance of the integrity of the living organism, with a greater degree of freedom than was possible using the previous "glue" method. This approach has previously been used in the analysis of other complex networks, such as the dynamics of homeostasis and the dynamics of embryonic patterning of *Drosophila melanogaster* (Lucchetta, *et al.*, 2005; Kastrup *et al.*, 2006) with results that showed its value in maintaining a highly controllable environment. Other complex biological networks such as protein aggregation could be studied with microfluidics.

Microactuators and microheating elements could be incorporated into future designs to provide isolated mechanical and thermal stimuli. Differently designed chambers could also be designed to study behavior that is currently difficult to observe, such as C bends (a sweeping arc of the worm body) and swimming. Combinations of the behavior and olfactory chips could be designed to correlate more complex behavior, or study neuron-neuron interactions in real time. Combinations of these tiny devices could be used to gain better understanding of complete neural circuits.

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