Femtosecond laser dissection in *C. elegans* neural circuits

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ABSTRACT

The nematode *C. elegans*, a millimeter-long roundworm, is a well-established model organism for studies of neural development and behavior, however physiological methods to manipulate and monitor the activity of its neural network have lagged behind the development of powerful methods in genetics and molecular biology. The small size and transparency of *C. elegans* make the worm an ideal test-bed for the development of physiological methods derived from optics and microscopy. We present the development and application of a new physiological tool: femtosecond laser dissection, which allows us to selectively ablate segments of individual neural fibers within live *C. elegans*. Femtosecond laser dissection provides a scalpel with submicrometer resolution, and we discuss its application in studies of neural growth, regenerative growth, and the neural basis of behavior.

INTRODUCTION

An animal's behavior is encoded in the structure and function of its neural network. Understanding how neural networks form and how neural networks allow an animal to perform perceptual, computational, and behavioral tasks are the central questions in neurobiology. The nematode *C. elegans* is a powerful model organism for studies of neural development and behavior. Its entire neural circuit consists of 302 neurons that form the same wiring diagram in every adult worm.[1] Therefore, behavioral programs are hard-wired into this invariant circuit. The relative simplicity of the worm suggests the plausibility of a complete map between worm behavior and the structure and function of its neural circuit. However, we still know very little about how the worm builds its circuit in the course of development and about how the circuit carries out behaviorally relevant computation.

The main obstacle in physiological analysis of the *C. elegans* neural circuit has been a lack of tools. The worm as a model organism was "invented" by geneticists. Enormous efforts have been applied to making genetic and molecular biological tools: the worm genome is completely sequenced, it is straightforward to find important molecules through forward and reverse genetic techniques, it is straightforward to inactivate these molecules through genomic deletion or RNA inactivation. However, the satisfactory analysis of a neural network also requires tools to manipulate and monitor the structure and function of the neural network *in vivo*. The development of physiological methods has not kept pace with the development of genetic methods for technical reasons. It is hard to imagine minimally invasive electrophysiological techniques: electrical recording of neural activity in *C. elegans* requires either taking a neuron outside the animal[2], or taking the animal apart[3]. However, the small transparent nematode lends itself to emerging techniques in optical physiology, which are allowing researchers to track the activity of single neurons using light microscopy. Optics holds the key to minimally invasive ways to manipulate and monitor the *in vivo* activity of neural networks.

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[1] J.G. White, E. Southgate, J.N. Thomson, S. Brenner, "The structure of the nervous system of the nematode *C. elegans*" Philos. Trans. R. Soc. Lond. B Biol. Sci. **314**, 1-340 (1986).

[2] M.B. Goodman, D.H. Hall, L. Avery, S.R. Lockery, "Active currents regulate sensitivity and dynamic range in *C. elegans* neurons" Neuron **20**, 763-772 (1998).

[3] J.E. Richmond, E.M. Jorgensen, "One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction" *Nat. Neurosci.* **2**:791-797 (1999).

We have used *C. elegans* as a test-bed for the development of femtosecond laser dissection as a tool to manipulate the connectivity of neural networks inside the live animal.[4] Laser irradiation has long been used to kill individual neuronal cells in *C. elegans*. The standard technique employs nanosecond laser pulses to kill the cell bodies of targeted neurons in *C. elegans* larvae, which then develop into adults with behavioral deficits[5]. Nanosecond pulses have sufficient spatial resolution to selectively ablate individual cell bodies, which are several micrometers in size, but their resolution is too coarse to target slender neural structures like dendrites, which can have submicrometer diameters and spacing. Here, we show that femtosecond laser ablation delivers submicrometer resolution, and can be implemented in studies of the neural basis of behavior and studies of neural regeneration in adult *C. elegans*.

RESULTS

2.1 Submicrometer resolution of femtosecond laser dissection

In a recent study, Shen et al. established that tightly-focused femtosecond laser pulses closer to the ionization threshold (3 nJ) can generate a laser-induced plasma that is only 300 nm in size.[6] The combination of ultrashort duration of the laser pulse, low energy, and high thermal conductivity of an aqueous medium also minimize the long-range effects of laser irradiation such as mechanical stress, bond-breaking, or heating. For example, calculations show that although the plasma reaches a temperature of 10,000 K, the temperature rise 1 μm from the focal point is at most 10 °C and lasts only 2 μs. We sought validation of the submicrometer precision of femtosecond laser ablation by visualization and with functional tests.
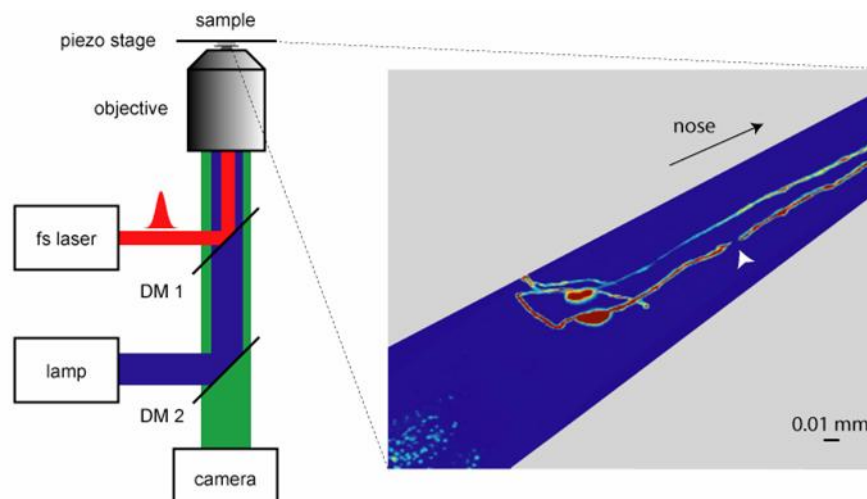

Fig. 1. Schematic of the laser apparatus and sample fluorescence image of the head of *C. elegans* showing a GFP-labeled neuron with a severed fiber in pseudocolor.

A schematic diagram of the apparatus permitting simultaneous fluorescence imaging and femtosecond laser ablation is shown in Fig. 1. In the original version of the apparatus, near infrared pulses were generated by an amplified titanium:sapphire laser system (λ_{center} = 800 nm), with pulse durations of about 100 fs. By serial insertion of neutral density filters and Kepler telescopic lenses, we adjusted the pulse energy to about 3 nJ and overfilled the back-aperture

[4] S.H. Chung, D.A. Clark, C.V. Gabel, E. Mazur, A.D.T. Samuel, "The role of the AFD neuron in C. elegans thermotaxis analyzed using femtosecond laser ablation" BMC Neuroscience, submitted.

[5] C.I. Bargmann, L. Avery, "Laser killing of cells in *Caenorhabditis elegans*" Methods Cell. Biol. **48**, 225-250 (1995).

[6] N. Shen, D. Datta, C.B. Schaffer, P. LeDuc, D.E. Ingber, E. Mazur, "Ablation of cytoskeletal filaments and mitochondria in cells using a femtosecond laser nanoscissor" Mech Chem Biosys in press.

of a Zeiss Plan Apochromat 63x, 1.40-NA oil-immersion objective. The same objective was used to image the sample by epifluorescence microscopy using a Coolsnap CCD camera (Roper Scientific Photometrics, Tucson, AZ). In a more recent version of the apparatus, we generate near infrared pulses with a cavity-dumped laser, which generates pulses with durations of about 100 fs and energy >30 nJ (KMLabs, Boulder, CO). For this application, we favor the cavity-dumped laser system for its relative simplicity and stability in comparison to the amplified titanium-sapphire laser system.

We verified the spatial resolution of femtosecond laser ablation in C. elegans by ablating a submicrometer segment from a nerve fiber. Using the lipophilic fluorescent dye DiO, we stained the subset of amphid neurons that have ciliary projections through openings in the worm's cuticle skin[7]. The fasciculated amphid dendrites are enclosed and separated by sheath cells, so it is straightforward to visualize separate fibers. A single dendrite within the bundle was severed by placing it at the focal point of femtosecond laser pulses. Pulses with energies below the ionization threshold lead to photobleaching at the focal point, which can be distinguished from ablation by the rapid recovery of fluorescence due to diffusion of fluorophore into the intact fiber. Figure 1B shows the dendritic bundle both before and two minutes after ablation with 3 nJ pulses at the ionization threshold; after ablation the central dendrite is cut while neighboring dendrites as close as 500 nm are not visibly damaged.
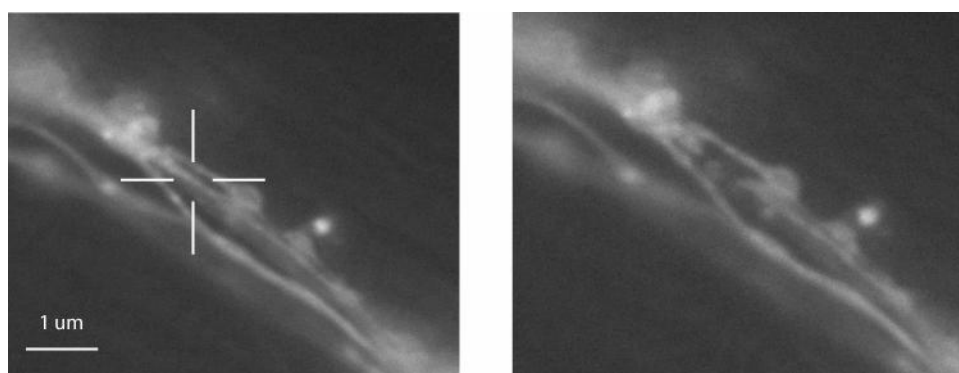

Fig. 2. Before and after images of a dendrite targeted for femtosecond laser dissection, showing fluorescently labeled amphid neurons in *C. elegans*. The neuron in the center of the bundle is severed, without visibly affecting the collateral neurons that are less than 1 um away.

2.2 Inactivating sensory input by snipping sensory dendrites: behavioral analysis

C. elegans behavior is the product of computations carried out by neural pathways that can be traced through its neural wiring diagram. The advantage of studying animals with limited behavioral, sensory, and memory capacities is that the computational ingredients of behavior are transparent in the way that the animal executes specific behavioral decisions. In other words, metrics of overall behavior in *C. elegans* can be easily interpreted as metrics of neuronal contribution.

The AFDL and AFDR sensory neuronal pair plays a critical role in thermotactic behavior. In particular, these neurons contribute to the ability of the worm to bias its movements towards colder temperatures. When a normal worm experiences warming at temperatures above its preferred temperature (which tells the worm that it is headed in the wrong direction), the worm vigorously reorients itself. When the worm experiences cooling (which tells the worm that it is headed in the right direction), the worm tends to suppress reorientation. This behavioral phenomenon is straightforward to quantify by subjecting the worm to sinusoidal temperature cycles, and monitoring the occurrence of reorientation. Reorientation is more frequent during the warming phase than the cooling phase of a sinusoidal temperature cycle, and the amplitude of the modulation of reorientation rate is a measure of neuronal activity.

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[7] E.M. Hedgecock, J.G. Culotti, J.N. Thomson, L.A. Perkins, "Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes" Dev. Biol. **111**, 158-170 (1985).
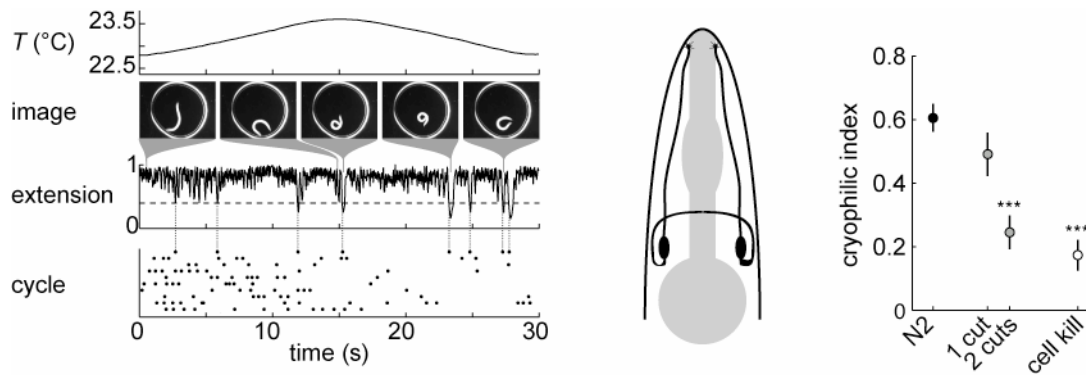
Fig. 3. Worms exhibit cryophilic bias by reorienting more frequently during the warming than the cooling phase of a thermal cycle. Reorientation can be flagged by monitoring worm extension: during forward movement extension oscillates around large values as bending waves propagate from head to tail whereas sudden dips in extension correspond to each reorientation event. A schematic of the AFDL and AFDR neurons in the head of the animal is shown, and the results with respect to cryophilic bias of snipping one dendrite near the nose of the animal, snipping both dendrites, or killing both neurons is presented.

We found that killing the AFD neurons tends to weaken the ability of the worm to generate cryophilic bias. An earlier report in which AFD was killed by laser irradiation with nanosecond pulses found that worms without AFD neurons tend to have atactic phenotypes (which is consistent with our observation) or cryophilic phenotypes (which is opposite of our findings).[8] Although it is difficult to reconcile the earlier report with our study, one reason for the discrepancy might be our use of a quantitative behavioral assay, which quantifies the strength of cryophilic bias, and not just whether the worm might exhibit bias or not. We also found that snipping both AFD dendrites weakens cryophilic bias to the same extent as killing both AFD neurons. This result allows us to conclude that AFD thermosensation is carried out at the dendritic tips. It is not obvious that this should be the case. Unlike chemosensory measurements, which must be localized to pores in the worm's cuticle skin, thermal conduction allows thermosensory measurements to be carried out anywhere inside the worm's body. Our results also allow us to conclude that AFD thermal sensitivity originates within AFD: snipping AFD dendrites leaves the synaptic architecture of the neuron intact, so AFD thermal sensitivity is not just transmitted to AFD by synaptic communication.

2.3 Regeneration of the peripheral nervous system in *C. elegans*

So far, we have discussed femtosecond laser dissection of sensory neurons in the head of the worm. We have never seen sensory neurons regrow, regenerate, or restore their own function, which might be analogous to the neurons in the central nervous system of other animals, that is neurons in the brain and spinal cord, which typically do not repair themselves after damage. In a recent study, Yanik et al. discovered that snipping motor neurons in *C. elegans* only leads to a transient disruption of motility.[9] The motor neurons in *C. elegans* regrow themselves within several hours after being severed, and the animal regains its ability to crawl! The ability of *C. elegans* motor neurons to regenerate might be analogous to the neurons in the peripheral nervous system of other animals, that is neurons innervating the body, which often repair themselves after damage.[10]

How do neurons regenerate? One possibility is that evolution has equipped certain neurons with specialized regenerative machinery. Another possibility is that certain neurons are able to reuse their machinery for initial growth during larval stages to also regenerate in the adult stage. Determining the signal that triggers regeneration as well as the molecular machinery that drives regeneration is of fundamental medical importance. Determining the trigger for regeneration will

[8] I. Mori, Y. Ohshima, "Neural regulation of thermotaxis in Caenorhabditis elegans" Nature **376**, 344-348 (1995).

[9] M.F. Yanik, H. Cinar, H.N. Cinar, A.D. Chisholm, Y. Jin, A. Ben-Yakar, "Neurosurgery: functional regeneration after laser axotomy" Nature **432**, 822 (2004).

[10] L.C. Case, M. Tessier-Lavigne, "Regeneration of the adult central nervous system" Curr. Biol. **15**, R749-753 (2005).

tell us why regeneration is arrested in the central nervous system, and might tell us how to circumvent that arrest. The possibility of genetic discovery of the regeneration trigger is enhanced by using *C. elegans*, which is the most genetically tractable animal model.

We have conducted a preliminary analysis of regenerative ability in other neurons in the "peripheral nerve system" of *C. elegans*. The PVM neuron is a mechanosensory neuron that enables the worm to reflexively avoid mechanical touch. This neuron has an axon that runs along the inside surface of the worm's skin and serves as a touch sensor. Touching the skin stretches the axon, which activates the neuron, which then activates motor neurons for crawling. Therefore, the properly directed growth of the PVM axon is the means by which worm skin is sensitized to touch. The soma of the PVM axon is located on the dorsal side of the animal near the tail. In the first larval stage of the worm (the L1 stage), an axon sprouts from the soma, grows directly to the ventral side, makes a sharp 90° turn, then grows straight in the anterior direction. Genetic analysis has shown that the properly directed growth of the PVM axon requires guidance cues established by chemical gradients of netrin and slit[11, 12]. We snipped the PVM axon near the soma in the last larval stage of the worm (the L4 stage), and discovered an interesting pattern of regeneration. The distal end of the original axon was allowed to deteriorate. The proximal end regrows, but can become lost in the first leg of the journey. The growing tip of the neuron wanders about until it finally reaches the ventral side, at which point it grows normally in the anterior direction.
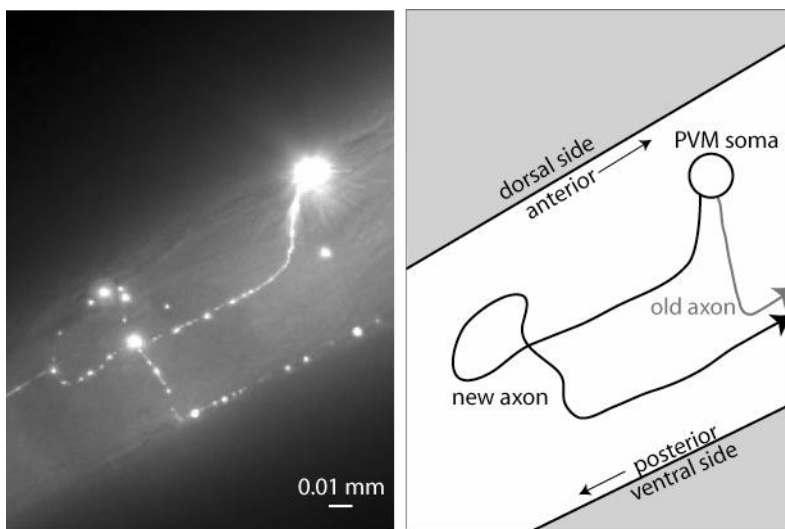
By systematically analyzing genetic mutants in *C. elegans* with defects in L1 stage growth or L4 stage regenerative growth, we will be able to assess whether the molecular determinants of initial growth and regenerative growth are shared or distinct. One explanation of the wandering regenerative growth of the PVM neuron is that the ventral/dorsal gradient of guidance cue has all but dissipated by the L4 stage.

If initial growth and regenerative growth use the same mechanisms, then there must be a trigger for these mechanisms, which is activated early in development and can be reactivated by neuron severing. This trigger might be inactivated when the neuron grows to its full length. Perhaps the mature synapses at the distal end of the neuron send a retrigrade signal telling the neuronal cell body to stop growing. Investigating these possibilities is centrally important in the neurobiology of nerve cell regeneration, and will have important biomedical implications.

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[11] J.C. Hao et al., "*C. elegans* slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor" Neuron **32**, 25-38 (2001).

[12] C. Chang, T.W. Yu, C.I. Bargmann, M. Tessier-Lavigne, "Inhibition of netrin-mediated axon attraction by a receptor protein tyrosine phosphatase" *Science* **305**, 103-106 (2004).

2.4 Femtosecond laser dissection in other optically accessible preparations

Femtosecond laser dissection can be applied to any biological system that can be visualized with high numerical aperture optics. We have conducted preliminary studies using cultured neurons from the hippocampus of mouse. These neurons grow on the surface of a glass coverslip, and are easily visualized with our microscope. Mammalian neurons in *in vitro* culture exhibit many essential properties, including growth, synaptic communication, and even synaptic plasticity. For example, when the excitability of an individual neuron is forcibly reduced for several days, the strength of the input synapses are found to be elevated, restoring the average rate of action potential firing of the neuron to normal levels [13,14]. This response, referred to as homeostatic synaptic plasticity, occurs over several days and has become a topic of great interest in the field.
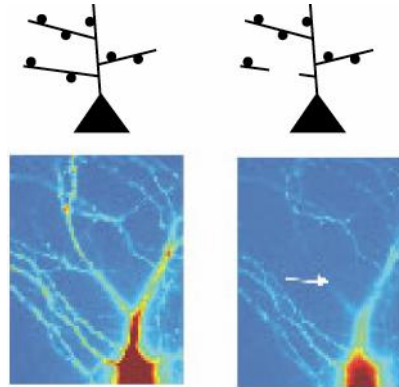

Fig. 5. Pruning Dendrites. Top figures schematically represent the experiment. In the bottom panels a cultured hippocampal neuron (14 days *in vitro*) is shown before and after a specific dendrite was severed using femtosecond laser dissection.

Several fundamental questions of great importance arise from experiments on homeostatic synaptic plasticity. How is the total input to a neuron maintained at a reasonable level? Are there sensors of activity distributed all over the neuron, or are they located in the cell body? Where is the information integrated? We can begin to perform empirical manipulations to address these questions using the instrument we propose to develop. To address the question of whether integration occurs in the soma, we can perform the following manipulation. Using laser microsurgery, we can sever some of the dendrites on just one side of the soma (Figure 5). One can then ask what happens to synapses on different dendrites of the neuron. Do the other synapses undergo compensatory increase in synaptic strength, so that the overall activity of the neuron can be restored? If there is a compensatory increase in synaptic inputs, where are these altered synapses – on all remaining dendrites, or only dendrites on the same side of soma as the severed dendrite? Needless to say, similar experiments can also be done for the output synapses by severing specific axonal branches.

CONCLUDING REMARKS

Femtosecond laser dissection provides a scalpel with submicrometer resolution, with which we can sever individual nerve fibers without causing collateral damage in optically accessible tissue. As we have shown, femtosecond laser dissection will be the driving force in many new investigations of neurobiology, applicable to studies in the neural encoding of behavior, neural plasticity, and neural growth and regrowth.

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[13] J. Burrone, M. O'Byrne, V.N. Murthy, "Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons" Nature **420**, 414-418 (2002).

[14] G.G. Turrigiano, K.R. Leslie, N.S. Desai, L.C. Rutherford, S.B. Nelson, "Activity-dependent scaling of quantal amplitude in neocortical neurons" Nature **391**, 892-6 (1998).