Large-scale Transgenesis and Nerve Regeneration in *C. elegans*

by

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Abstract

Caenorhabditis elegans (C. elegans) is a widely studied model organism due to their fully mapped neural network of 302 neurons and amenable genetics. Their small size and short life cycle allows for rapid studies to be conducted; however, after decades of use the manual methods of manipulation have remained relatively unchanged. This thesis details the development of largely automated, high-throughput, optical, robotic, and microfluidic technologies for laser neurosurgery screening and transgenesis in C. elegans.

Discovery of molecular mechanisms and chemical compounds that enhance neuronal regeneration can lead to development of therapeutics to combat nervous system injuries and neurodegenerative diseases. By combining high-throughput microfluidics and femtosecond laser microsurgery, we demonstrate for the first time large-scale in vivo screens for identification of compounds that affect neurite regeneration. We performed over ten thousand microsurgeries at single-axon precision in the nematode C. elegans at a rate of 20 seconds per animal. Following surgeries, we exposed the animals to a hand-curated library of approximately one hundred small-molecules and identified chemicals that significantly alter neurite regeneration. In particular, we found that the PKC kinase inhibitor staurosporine strongly modulates regeneration in a concentration- and neuronal type-specific manner. Two structurally unrelated PKC inhibitors produce similar effects. We further show that regeneration is significantly enhanced by the PKC activator prostratin.

Microinjection is an essential and widely used method for C. elegans transgenesis. Traditional injections have remained low-throughput for decades due to the laborious nature of the manual procedure which prohibit large-scale transgenesis screening. This thesis details the development of the C. elegans Automated Micro-Injection (CAMI) system for high-throughput microinjection which could be broadly useful for analysis of gene function through insertion of CRISPR/Cas9, cDNA or RNAi vectors in future applications. Using the standard roller phenotype the transformation efficiency in creating transgenic lines is comparable to manual injections. We demonstrate the utility of the system by deep-phenotyping a subset of 6 mutants backgrounds selected from the million mutant project. We introduced 12 fluorescent reporters to label 38 neurons in each genetic background in order to search for defects in development.
Acknowledgements

To my family who are friends and friends who are family.

To the makers of Gleevec, thank you.
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Chapter 1

Introduction

This thesis provides context for the research into the use of high throughput screening technologies to create custom transgenic lines and using whole animal models to study \textit{in vivo} neural regeneration. The first section explains the motivation behind this research and introduces some of the relevant disease models.

1.1 Thesis Motivation

The adult mammalian central nervous system has very limited capability of regenerating its axons after traumatic injury, which has been attributed to both extrinsic signals of the inhibitory glial environment as well as intrinsic neuronal factors. The discovery of cell-permeable small molecules that enhance axon regrowth can potentiate the development of efficient therapeutic treatments of spinal cord injuries, brain trauma, stroke, and neurodegenerative diseases. Identification of such molecules will also provide valuable tools for fundamental investigations of the mechanisms involved in the regeneration process. Currently, small-molecule screens for factors affecting neuronal regeneration can only be performed in simple \textit{in vitro} cell culture systems. However, these systems do not replicate conditions \textit{in vivo}. In addition, off-target, toxic or lethal effects often manifest only \textit{in vivo}. Thus, a thorough investigation of neuronal regeneration mechanisms requires \textit{in vivo} neuronal injury models.

\textit{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 prevent large-scale studies in these animals. The nematode \textit{Caenorhabditis elegans} is a simple, well-studied, invertebrate model-organism with a fully mapped neuronal network comprising 302 neurons. Its short developmental cycle, simple and low-cost laboratory maintenance, and genetic amenability can allow quick
identification of the molecular targets of screened compounds, and can lead to the discovery of novel genetic and molecular pathways.

Figure 1.1 The nematode, *Caenorhabditis elegans* (A) Adult stage C. elegans with length of 1mm and diameter of 50μm (B) Cultured in bacteria on agar plate. Image credits: wormbook.org.

Until recently however, the small size of *C. elegans* (~50 in diameter) prevented its use for investigation of neuronal regeneration mechanisms. We previously demonstrated femtosecond laser microsurgery as a highly precise and reproducible injury method for studying axon regrowth in *C. elegans*. The non-linear multiphoton absorption of the incident femtosecond pulse allows sub-cellular-resolution surgery of nematode neuronal processes with minimal out-of-plane absorption and collateral damage. Furthermore, due to the *C. elegans* stereotypic anatomy and hermaphroditic reproduction, the same neurons can be repeatedly axotomized at the same distance from the soma in isogenic animal populations, significantly enhancing reproducibility of assays. Recent studies have used this technique to investigate how factors, such as animal age, neuronal type, synaptic branching, and axon guidance signaling influence regeneration. In combination with screens on a β-spectrin mutant strain that exhibit spontaneous neurite breaks, this technique has also revealed that axon regrowth depends on the activity of MAP kinase pathways.

However, the high motility of wild type nematodes causes a significant throughput challenge. Precise laser axotomy and imaging at the cellular level require orientation
and immobilization of animals. Traditional immobilization methods, such as the anesthetics sodium azide (NaN₃), levamisole and tricaine/tetramisole, have significant and/or uncharacterized effects on nematode physiology, which may affect the regeneration process. In addition, anesthetics need several minutes to take effect, and are thus incompatible with high-throughput screening. Other techniques that can be used to reversibly immobilize C. elegans include trapping of nematodes in wedge-shaped microchannels, cooling and exposure to CO₂. However the physiological consequence of such exposure to low temperatures and CO₂ are unknown.

Microinjection is an essential and widely used method for C. elegans transgenesis. Currently, injections are performed manually and methods have remained relatively unchanged for decades (Stinchcomb et al., 1985). Traditional injection methods are laborious and low-throughput which prohibit large-scale studies. We developed the C. elegans Automated Micro-Injection (CAMI) system for high-throughput microinjection which enables large-scale transgenic screens of nervous system morphology, as used here, and which will be broadly useful for analysis of gene function through insertion of CRISPR/Cas9, cDNA or RNAi vectors in future applications. The overall system development strategy is detailed in Figure 1.2. This core system can be iteratively improved for both precision and speed in addition to being adapted to solve other challenges.
Figure 1.2 CAMI system overview and strategy. By taking advances in engineering from many disciplines and combining them into high-throughput systems we are able to perform precision experiments for large-scale studies using whole animal models to address a variety of disease models. The engineering advances on the left side will be continuously improved and hardware/software components and algorithms can be upgraded over time.
Cody Gilleland led the technical development and design of all aspects of the platform for precision instrumentation technology and adapted the hydrogel immobilization technique for microinjection. Cody Gilleland, Adam Falls and James Noraky developed the algorithms for worm feature detection and target mapping through rapid scanning and precision stitching of images. Adam Falls and Cody Gilleland refined the needle shape and developed the procedures and troubleshooting efforts to prevent needle clogging. Cody Gilleland and Adam Falls performed the injections for the mutant screen. Cody Gilleland performed injections and refined all techniques during the rol-6 efficiency study. Max Heiman designed the biological assays, generated reagents, scored phenotypes, performed all downstream analysis, and provided feedback as we refined the injection technology. Mehmet Fatih Yanik supervised the project and provided feedback at all times.

Phenotyping genes in model organisms plays an important role in understanding human disease. The ability to deliver genetic vectors at large scale to create transgenic *C. elegans* could accelerate the assignment of *in vivo* gene function. Although large numbers of *C. elegans* mutants are available and genetic engineering techniques like CRISPR/Cas9 work efficiently, it still remains a great challenge to deliver such genetic vectors, to express large numbers of tissue-specific transgenic reporters to phenotype these mutants, and also to perform epistasis analysis with RNAi injection to such large numbers of genetically engineered mutants (with millions of possible mutant/reporter/RNAi combinations). Here we present a platform for high-throughput engineering of transgenic *C. elegans* lines. The animals are immobilized in a temperature sensitive hydrogel mixture using a standard multi-well platform. Computer vision algorithms are integrated with microscope automation and precision robotics for gonad microinjection at an average rate of less than 30 seconds while maintaining similar transformation efficiency as manual injections. We demonstrate transgenic screening of neuronal morphology through rapid generation of stable transgenic lines by introducing combinations of multiple fluorescent reporters (each combinatorial reporter mix includes 3 different neuron-specific promoters) into six different mutant lines. Deep-phenotyping of each mutant line was performed by quantifying neurite lengths across
36 neuron types. We are currently investigating phenotypes of short dendrites that manifest in anchoring defective mutants and awaiting confirmation.

**Keywords:** *C. elegans*, high-throughput transgenesis, phenotyping, automation, computer vision, robotics, neuronal defect

**Introduction**

Recent advances in sequencing technology have now made it feasible to genotype hundreds of thousands of patients to develop medically related associations with human gene variants. In order to take advantage of this new information and rapidly probe gene function and identify new targets we must also scale up the genetic modification and phenotyping of model organisms. The immense scale of the available genetic engineering resources such as CRISPR/Cas9 and potential permutations of experiments reaches into the millions yet current methods of delivering these vectors remain the rate-limiting step preventing powerful large-scale applications (Figure 1A). The downstream tools for analyzing the newly generated transgenic lines are also advancing in the areas of sequencing, imaging and behavioral assays. The gene mutations can have impact across multiple cell types and processes and thus should be fully characterized by deep-phenotyping with multiple fluorescent reporters for each background. Large *C. elegans* mutant banks have been generated (Million Mutant Project) yet many remain relatively uncharacterized beyond a single element. We present here a system architecture for rapid engineering of transgenic *C. elegans* lines at large scales.

Their small size, ease of culture, rapid development, wealth of genetic techniques and large mutant libraries make *C. elegans* a powerful model organism for understanding human disease. Microinjection is an essential and widely used tool for creation of transgenic *C. elegans* [1] and has enabled a number of significant discoveries; however, the laborious nature of the injection technique has remained relatively unchanged for decades. Although traditional systems are low in cost, manual injections are laborious and low-throughput which inhibit large-scale transgenic studies. Advances in genome sequencing technology are enabling large-scale gene mapping efforts yet revealing their function at large-scale remains a major challenge. With the introduction of gene synthesis technologies and the CRISPR/Cas9 technique (etc) for rapid genome editing the limiting factor now lies in the process of manual microinjection.
We present the *C. elegans* Automated Micro-Injection (CAMI) system which enables new applications for large-scale screens of gene function through powerful genetic techniques such as gene insertion by DNA plasmids, RNA interference, CRISPR/Cas9, and injection of small-molecules (Fig 1A). In their natural state, *C. elegans*’ neurons are transparent and cannot be visualized. Fluorescent labels must to be introduced to visualize their morphology. We have demonstrated the utility of the system by performing a transgenic screen of neuronal defects by introducing fluorescent reporters into a mutant library (Fig1B).

![Current *C. elegans* transgenesis pipeline productivity](image)

**Figure 2.1 Applications for large-scale *C. elegans* transgenesis.** (A) Many reagents and mutants are currently available yet the major bottleneck lies in the manual process to deliver these vectors. (B) By combining fluorescent tissue reporters with a wild-type strain the neurons in the amphid sense organ located in the head of the nematode are...
characterized for shape and dendrite length. Note these specific images were not taken of lines created by the injection system but the CAMI system was used to successfully re-create these lines. (Image credit: Max Heiman)

The CAMI hardware system (Fig 2.2B) is composed of a high-speed camera mounted on an automated inverted microscope with temperature controlled incubator, micromanipulator for needle positioning, piezo unit for needle vibration pulsing, and pneumatic valves for pressurized dispensing of reagents.

The conventional oil-based method requires significant manual intervention to place worms against a dry agar pad where only a few animals can be processed at a time before the animals die due to desiccation after roughly 5 to 10 min. CAMI is completely software controlled increasing speed and precision while reducing human error and user fatigue enabling large-scale studies. The hydrogel immobilization technique (Pluronic F-127, [1]) can integrate with existing industrial plate/liquid handling systems and enable hundreds of worms to be immobilized and recovered en masse following injection. This gel immobilization technique has been demonstrated using microfluidics [2,3] and warming channels but has not been adapted or demonstrated to enable microinjection.

A temperature sensitive hydrogel is used to pipette transfer animals from standard culture on agar plates to a multiwell plate with a thin cover glass bottom. The multiwell plate is then placed on to a peltier unit inside an incubator at 15°C and the animals are settled against the cover glass by gravity. The peltier unit is then activated by applying 4 amps for 3 minutes which raises the temperature of the gel to 25°C causing it to harden into a gel state immobilizing the animals directly against the cover glass surface. The multi-well plate is then mounted onto the microscope stage with a temperature incubator below at 28°C holding the gel temperature to 25°C. This allows animals to be imaged while heated to an ambient temperature in a physiologically stable environment that is open to robotic manipulation. Animals have shown to recover after being immobilized in the gel for 60 min in comparison with the standard method using halocarbon oil which causes animals to desiccate in less than 10 min. Computer vision algorithms detect animal locations and targeted gonad locations (Fig 2.2C). The autofocus laser system brings the gonad into view and the user makes a small XY and focus alignment for precision targeting. The needle is then brought into the image plane enabling axial penetration of the cuticle and gonad sheath with assistance from piezo vibration. The reagent is dispensed followed by axial removal of the needle. The XY gonad target locations are stored
in memory enabling the user to transition between injection sites while remaining in 20X magnification eliminating the need to change magnification. This system is capable of performing injections at a rate of less than 30 seconds in comparison with manual techniques at ~2 min per animal where the user can only sustain operation for 2-3 hours per day. The gel is then diluted with M9 media and placed in a shaking incubator at 15°C for 10 min to dilute the gel followed by pipetting the animals onto a seeded agar pad for recovery. To maintain independent transgenic lines the injected P0 worms are then placed on single plates followed by separating F1s to individual plates to enable independent line generation.
Figure 2.2 CAMI System overview and operation. (A) C. elegans are immobilized in a custom hydrogel mixture for injection by first pipetting the hydrogel solution into a well plate and allowing the worms to settle flat against the glass bottom at 15°C. The hydrogel is then solidified by increasing the temperature to 25°C. Microinjections are then performed in less than 30 seconds each and then recovered by diluting the hydrogel with M9 media and pipetting onto agar plates for follow up screening. After 8 days the transgenic animals are screened and picked from the plate. (B) The system hardware is controlled by a custom unifying software interface that enables each of the hardware
components to work together in symphony. The hardware configuration is labeled in the diagram where a micromanipulator is added to an automated microscope with incubator. (C) Custom computer vision algorithms detect the targeted regions for injection by drawing a spline along the animal and then selecting a distance along the spline for targeting in the 20X magnification. (D) With the worm immobilized flat against the glass the targeted region is then focused and the needle is hovered about 100µm above the gel. The needle is then rapidly inserted through the hydrogel and into the targeted region with 2 microns of precision with piezoelectric vibration assistance. The plasmid is then dispensed into the gonad region and the needle is quickly removed to the hover position and the XY stage is translated to the next injection location.

Table 2.1 Microinjection timing

<table>
<thead>
<tr>
<th>Step</th>
<th>Mean Time ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. XY Stage Travel</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>2. Z-focus / Fine alignment</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>3. Needle Insertion</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>4. Plasmid Dispense</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>5. Needle Exit</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Total</td>
<td>25 ± 3</td>
</tr>
</tbody>
</table>

*Mean ± SD

Table 2.1 and Figure 2.3 Microinjection timing and steps in 20X magnification. The average time per injection over 60 animals. Each step is broken down and quantified in the table with the correlating demonstration in the diagram.

Once the injection targets have been mapped the stage is moved to each new XY location while remaining in 20X magnification. The autofocus laser system keeps the target near the focal region. A small XYZ adjustment (typically less than 10 µm) is made to target the specific gonad location and the needle is inserted directly into the target location while avoiding damage to the gut. A piezoelectric vibration assists with penetration of the cuticle and gonad sheath while a small constant (always on) pressure prevents clogging of the needle tip due to biological debris. To prevent clogging from debris in the injection mixture an aggressive spin procedure was implemented (see methods section). After injection the needle is pulsed and vibrated locally in the plasmid solution to clear
any debris and also determine if the needle is clogged and requires further attention to unclog the needle tip.

Table 2.2 Transformation Efficiency. We demonstrate that the system can be used to reliably generate transgenic lines. We injected 39 PO young adult animals with the standard rol-6 vector to induce the roller phenotype and demonstrate that our precision platform using hydrogel immobilization successfully produces transgenic lines with similar transformation efficiency as manual microinjection techniques using the oil-based method.

Procedure

The animals are grown on standard agar plates seeded with OP50 bacteria. The agar plates are then washed with a pluronic gel solution (25% pluronic, 10 mM Sodium Azide) and poured onto a dish with a cover glass bottom and placed into a 15°C incubator allowing the worms to settle to the bottom (Fig. 2.2A). The Sodium Azide causes the worms to relax and straighten out as the gel provides mechanical restraint to immobilize the worms in a physiologically viable and reversible manner through dilution and cooling.

The near perfect planarity enables image processing algorithms to quickly detect the worm location and features to map injection targets. In all images the young adult worms are roughly 1mm in length and 60-100 μm in diameter.
Figure 2.4 Worm planarity in gel preparation for immobilization (A) The glass bottom well plate is covered and placed on top of the peltier unit and copper plate with level to ensure even thickness of hydrogel. The peltier unit is sandwiched between two thermally conducting copper plates to evenly transfer the heat to the bottom of the well plate. Notice the electrical tape surrounded by the blue box used to level the shelf. A plastic cover is placed over the well plate to retain heat and moisture while a level en-
sures that the hydrogel is evenly coated to prevent uneven drying of the gel leading to desiccation of the animals (B) Worm out of Z-plane without peltier heating method. (C) Worm in Z-plane flat against the cover glass using the quick peltier heating method that hardens the gel before the worms are able to crawl to the top of the gel away from the heated glass surface. (D) Image of worms successfully immobilized in the hydrogel as the well plate is placed on the microscope stage above a 2X objective. This well plate is custom made to have long troughs for needle entry access over a large area and can be custom ordered at large scales. These custom plates were washed with ethanol and reused.

After waiting for 5 min the dish at 15°C to allow the worms to settle the hydrogel is heated to 25°C by applying a 4A current to the peltier unit for 3 min to allow the gel to solidify to keep the worms flat against the glass surface. This allows the hydrogel to quickly transition from a gel state to a hardened state. The plate can then be moved to the stage of the microscope while the worms remain immobilized securely against the glass (Fig 2.4). If the worms are placed in the hydrogel mixture at 15°C and allowed to settle to the bottom then moved to the microscope the movement will cause the worms to become out of plane. The heated incubator alone does not allow for the quick temperature changes necessary to harden the gel before they crawl out of plane.

The automated Nikon Ti eclipse microscope allows the changing of objectives, filters, shutters and cassettes to enable rapid changes to quickly respond to imaging demands.
Figure 2.5 Precision instrumentation system and controls (A) The unified software interface enables the user to interact and control the entire system largely by mouse control. The second screen allowed for simultaneous updating of the code to refine the system. The microscope is secured to an anti-vibration air table while the incubator is placed on the ground to avoid vibration from the oscillating fan. (B) An adjustable height table hosts the manual instrument controls to prevent vibration from user interaction.
and allows the user to sit or stand on demand. Controls from left to right: sutter XYZ precision manipulator, perfect focus Z height adjustment, XY stage joystick (C) The Nikon Ti eclipse microscope is equipped with DIC optics, perfect focus laser system, and 20X/2X objectives on a rotating turret. The manipulator hosts the needle and holder with piezo vibration device.

Figure 2.6 Systems Integration. This diagram demonstrates how the components are controlled with a custom software package. This custom software enables non-compatible hardware devices to work together in symphony by hacking the hardware interface of these devices. This also enables rapid software prototyping and is highly adaptable to other applications.
Figure 2.7 Rapid four well scanning. Custom well plate is scanned at maximum speed in the X direction while the camera captures a video. The well plate was cut from a standard plastic well plate mold using a ban saw. A cover glass bottom was then super-glued to the bottom. This allows for optimal scanning and open access for needle intervention. For future applications custom plastic molds can be designed and fabricated with glass attached by multiple companies. These well plates were rinsed with ethanol and re-used with minimal contamination.
Figure 2.8 Montage build and worm selection. Fiducial markings provide location references when stitching together each of the rows. Using an intensity threshold and object area function the worms are selected for analysis at full pixel resolution. This allows us to perform precision processing on a subset of this larger image in the next step.
Figure 2.9 Feature detection and gonad mapping. (L) The worm is selected using intensity thresholding and object area detection. A spline is then drawn through the center of the animal and distances are measured. As these images are stored they will be incorporated into neural network training data to improve speed and selectivity of our detection algorithms. (R) The robust algorithm automatically adapts to unique phenotypes like the dumpy shown here with a short wide body.
Figure 2.10 Precision image stitching. The stage is run at full speed scan while the camera records a video of the passing frames. Our algorithm then stitches these together within one pixel of accuracy. The algorithm uses MATLAB toolboxes for corner detection and mapping then estimates a best fit approximation. The animals are approximately 1 mm in length.
Figure 2.11 Traveling algorithm for nearest neighbour path optimization. The waypoints are formed and incorporated into a greedy nearest neighbour travelling algorithm[4] to find the most efficient path to include each waypoint. This algorithm was implemented by James Noraky. Since the target locations are mapped in XY stage coordinates this allows the user to remain in 20X as they move from worm to worm without need for changing of objectives and adjusting coarse Z focus. This helps to streamline the microinjection process by quickly presenting injection targets.
Figure 2.12 Screenshot of the CAMI software interface. The image shown here is in 20X magnification DIC of a young adult hermaphrodite worm. The needle enters the worm
from the right side at a 45 degree angle from the cover glass surface then pressurizes to dispense the reagent forcing the gonad arm to expand. The controls allow the user to iteratively adjust the needle position and apply short pulses (100 ms, 60 psi) to ensure the needle is in the correct position and adequate vector delivery is occurring.

The stage moves to the XY location and autofocuses on the region. The needle has been calibrated to be in alignment with the image plane so the needle is brought directly into the focal plane and inserted into the gonad. The needle tip has been characterized so that every needle is pulled with an open tip which prevents the need for breaking and reduces variability between needles. This also reduces the time needed to replace and re-fill a needle after a failed break attempt.

Discussion

Conventional microinjection techniques are unable to support large-scale phenotyping efforts. We presented a system architecture for rapid transgenesis in *C. elegans* with transformation efficiency similar to traditional methods using the standard roller phenotype assay. We further demonstrated its utility by deep-phenotyping a mutant library for neuronal defects. With advances in genome editing and synthesis technology making it feasible to produce millions of permutations of potential experiments we must also advance the technologies for introducing these vectors at large scale. The CAMI system lends itself to wide adoption because it is made from robust, commercially available components eliminating the need for expensive microfabrication equipment commonly used in microfluidic approaches while shifting the complexity to custom software that can be rapidly adapted and prototyped. This core system could be increasingly valuable with time due to the advances in machine learning, robotics, computer vision algorithms, and advancing camera technologies. The modular nature of the platform allows each hardware component to quickly exchanged if a component is damaged enabling the potential for high-throughput systems to run at large scales in an industrial setting.

Methods

*C. elegans* culture and million mutant library. Nematodes were grown and recovered post-injection at 20°C in NGM agar plates. Standard procedures were followed for *C. elegans* strain maintenance and genetic crosses. Nematode strains used in this study included: [CGC: rol-6], [Million Mutant Project: T23F1.5, cutl-14], [Knockout: cut-1, cut-5, cut-6]
Preparation of injection mixtures: In order to remove particulates that could contribute to needle clogging the reagents underwent an initial cleaning by centrifugation. The reagents were spun for 10 min (25000 rpm, 4°C) and the top 90 percent of the solution was transferred to a clean tube. This step was repeated once. When not in use the reagents were stored at -20°C. Prior to loading into needles for microinjection the reagents were thawed and centrifuged as described above. Fluorescence imaging was performed by the DeltaVision imaging system in the lab of Max Heiman.

![Image with needle design and refinement](image)

**Figure 2.13 Custom needle design and refinement.** The needles are adapted from the bee-stinger shape in the Sutter P-97 Pipette Cookbook. The broad shaft allows for rigidity in passing through the viscous hydrogel while the thin tape enables delicate penetration of the worm cuticle and gonad sheath without cause excessive damage. Our needle design is highly sensitive to room humidity and we also include a pre-programmed air purge for 60 sec before beginning the pulling procedure. We find that our needle pulling is more successful when the weather outside has low humidity which influences our lab conditions.
**Equipment List**

Box filament, Sutter P-97 needle puller, *in vivo* scientific incubator, Allied Vision Technologies Camera, Optical Vibration Damping Table, Prior ProScan II XY robotic stage, micromanipulator, pneumatic valves, MATLAB software and toolboxes

Optics: Nikon Ti eclipse microscope, filter wheel, condenser turret, Eppendorf needle holder, Objectives: 20X, 2X, DIC components

**Acknowledgements**

We thank Christoph Engert of the Horvitz Lab and Victor Ambros for helpful discussions and advice. We also thank Joseph Steinmeyer and Mark Scott for helpful advice in troubleshooting hardware issues.
A number of technologies were developed in the initial and intermediate stages that could serve as useful to other applications. This chapter details these technologies that may not be featured in future manuscripts.

The entire well is scanned with a 2X objective and stitched together to form a montage of the entire well plate. The montage is then processed to find the worm location. Currently, this algorithm detects 90 percent of worm target locations which has been sufficient to complete screens in a timely manner. In all images the worms are roughly 1mm in length and 60-100μm in diameter. These techniques were developed but were not necessarily used in the final design of the system but should be investigated for use in further development of the system.
Figure 3.1 Stitched montage of immobilized worms in 2X. The images are then scaled down and resized to bring only a single worm into the field of view. The image is pre-processed by using dilation, erosion, and skeleton functions to remove the outer perimeter and map the head and tail regions. The head and tail regions are then removed and the largest region of white are then processed to fit a statistical measure to indicate gonad regions.

Figure 3.2 Pre-processed worm image to remove head and tail and isolate worm features. A spline is drawn along the midline of the worm and the isolated regions are mapped with distances along the spline to determine gonad regions. The centroids of the detected gonads are localized and the XY locations are stored. The current algorithm detects about 60 percent of the visible gonads. This algorithm was not used for screening.
Figure 3.3 Spline and Gonad Detection. Once the gonads regions have been detected we must then process to determine if it is accessible from the right side. We propose an algorithm that draws a line from the gonad centroid to a distance to the right of the worm (~200 μm). If this line does not cross the spline of the animal then the gonad is listed as accessible for injection. The green arrows indicate that the gonad is accessible. Any test lines that cross the spline or any other debris will be rejected and not included in the injection path. In order to safely penetrate the worm without damaging the gut this algorithm could be developed. The needle should only enter and access gonad locations from the right side to prevent excess damage by puncturing through the animal.
Figure 3.4 Microinjection system including rotation stage. The previous CAMI system design integrated with existing microinjection systems by the addition of a custom rotating stage, pneumatic valves for pressurized dispensing of reagents, and micromanipulator for needle positioning on an inverted microscope.

Computer vision algorithms will detect positions of worms and their gonads and calculate the optimal angle for needle approach. A custom rotation stage will automatically orient the animals for microinjection and the system will autofocus on the gonad and bring the needle into the image plane.
Figure 3.5 Rotation software interface. Once a worm is located a special algorithm calculates the safe approach angle that would allow the needle to access the worm without breaking on the surrounding components. A series of red and green lines would allow the user to select the optimal approach angle. The XY stage would then translate as the theta stage rotates to the correct location. This technology worked great but it required that the user switch back and forth between 2X and 20X since the mechanical rotation aspect was not perfect due to the slack in the belt. This was improved by using gears and belts with teeth while only rotating in the same direction each time to keep tension on the belt. We decided to move to the well plate method for easier adoption until more precise rotation components are commercially available.

In order to speed the autofocus aspect of the system we developed a custom algorithm. The Nikon microscope piezo Z-drive has a handshake delay of 0.5 sec per movement which causes significant delays as autofocus requires multiple steps as images are acquired. We move in a single sweep and take video frames ~100 frames/sec and correlate each frame location with a Z height as we score each frame with a focus score. We scan the entire worm in Z-plane (150µm) and then move to the correct Z height immediately after processing with a total time of 2-3 sec with accuracy of 3µm. The handshakes of the communication with the Nikon Z controller prevented fast updating of the Z position so we switched to the perfect focus unit as funds were made available to ease adoption by others by using simple commercially available components.
Figure 3.6 Autofocus algorithm. This algorithm determines Z-movement in the video to correlate frames with Z-height. The Y axis correlates to Z movement while the X axis corresponds to the frames taken. Data1-4 represent the different algorithms we tested. These algorithms processed the total variance of an image (Y-axis) with the frame sequence number (X-axis). The spikes indicate the top and bottom edges of the worm.
This section is developed and written in collaboration with Max Heiman at Children's Hospital Department of Genetics as a potential use of the microinjection technology at large scale. Max Heiman designed all aspects of the biological screen related to his research direction. He selected a subset of the mutants from the million mutant project and generated or acquired each of the fluorescent labels that were later injected and screened using the CAMI system by Cody Gilleland.

The biological screen necessitated the development of methods to process multiple unique experiments (Fig. 1A) with a single preparation step. The ability to immobilize the worms for over one hour enables multiple experiments to be processed but the samples must be separated into different wells.
Multiwell: enables 6 unique experiments in parallel

Figure 4.1 Six well plate design for parallel combinatoric experiments. We implemented the six well design that allows the worms to be pipetted into the designated region where the needle can access the bottom of the well for injection without coming into contact with the edges of the plate. This enables a small area that can be stitched together using 12 images (3X4, 2X magnification) with precision open loop XY motion control. The marker dots serve a dual purpose as a fiducial for image registration and location for the worms to be safely immobilized for injection.

Motivation for transgenesis screening

The wiring of the nervous system is partly genetically programmed, but the search for “matchmaking” molecules that instruct each neuron’s choice of targets has remained frustratingly incomplete. *C. elegans* offers the advantages of a small nervous system with highly stereotyped anatomy, and is thus a promising model in which to comprehensively define all factors required to control assembly of a complete portion of a nervous system. The principles identified in this simple system will provide a framework for thinking about nervous system assembly in more complex systems. This is an ambitious goal, and requires the development of new technology for high-throughput mu-
tant screening to genetic saturation; for rapidly introducing cell-specific markers into a large number of mutant backgrounds; and for careful quantification of the effect of each mutant on each neuron subtype. The methods presented here are designed for ready adoption by other labs, and will be broadly applicable for high-throughput labeling and screening of single neurons. These methods will thus greatly expand the toolkit for dissecting nervous system assembly and function in this model system.

Background

A long-standing question in neuronal development is whether there exists a generalized “code” by which neurons recognize their targets. The Heiman Lab previously used the sensory anatomy in the nose of *C. elegans* to define two secreted extracellular matrix (ECM) factors, DEX-1 and DYF-7, that are required for target recognition by one type of sensory neuron, called the amphids (Heiman and Shaham, 2009) [5]. These neurons are born at the nose tip, anchor to a target there, and then stretch their dendrites out behind them as the cell bodies crawl away. When target recognition fails, neurons do not anchor properly, and dendrites fail to extend. DEX-1 and DYF-7 are strongly required for amphid neurons to recognize their target at the nose but, according to preliminary experiments, make a weaker contribution to the ability of other nearby sensory neurons to recognize their own distinct targets. This suggests DEX-1 and DYF-7 may be two components of a larger set of local ECM that act combinatorially to define the target of each neuronal class. The goal is to determine whether such a generalized code exists, or whether each neuron type uses its own bespoke mechanisms to identify its target.

To identify mutants affecting every component required for each sensory neuron type to recognize its target, we generated a collection of transgenic strains bearing cell-specific CFP, YFP and RFP labels in 12 sensory neuron types in the amphid of the *C. elegans* nose and separated by strain containing the labels: [ADF, AWC, AFD], [CEP, OLL, OLQ], [URX, URY, FLP], [BAG, IL1, IL2]; no cell-specific markers are yet identified for the URB type). These strains are shown in Figure 4.2 and the cassette labels a total of 38 neurons divided over 4 strains. Once these markers were developed and shown to be stable we could then used them to phenotype mutant backgrounds. Fluorescent labels can also introduce variability when using them in high-copy extrachromosomal arrays and have previously shown defects that were artifacts of the marker itself.
Figure 4.2 Sensory neurons fluorescently labeled in wildtype background. The amphid sense organ is composed of the neurons labeled above and corresponds to the color code of the text for YFP, CFP and RFP labels. We selected these channels so they could be easily distinguished from one another. These labels are integrated at high-copy in extrachromosomal arrays for brighter expression enabling easier detection and imaging. The strains were generated using the CAMI system and imaged on the DeltaVision system in the Heiman Lab. (Image credit: Max Heiman)
Figure 4.3 OLQ sensory neuron showing a potential developmental defect (needs confirmation). The wildtype background on the left shows proper dendrite extension. The cut-5 background is demonstrating an anchoring defective phenotype. The arrow points to the short dendrite that did not properly anchor at the tip of the nose as the cell body crawled away during development. This phenotype is currently being confirmed and is exhibited in roughly 10 percent of the animals.

The coding of neural specificity identified here will establish a framework of rules to help explain how a nervous system of any size may be assembled. Moreover, the meth-
odologies we establish will set the stage for a new era of high-throughput analysis of this simple nervous system, allowing manipulations of dozens of cell types and hundreds of genes simultaneously.

The CAMI system demonstrated successful transformation of problematic strains with delicate cuticles and odd shapes due to cuticle and other mutations. The precision, delicacy and needle refinement enabled injections with low collateral damage and prevented the guts from spilling out since the animals are under pressure in the gel.
The ability to rapidly load animals from a well plate enabled us to perform precision laser neurosurgery with single axon precision on more than 10,000 worms. During the screening process we realized that roughly one third of the time the animal needed to be rotated in order to visualize and cut a specific axon. Cody developed a system and method to rapidly rotate the animals on chip using a system of off-chip valves that increased the throughput by a third bringing it down to 20 seconds per laser surgery. This technology demonstrated the first large-scale in vivo nerve regeneration screen and with single axon precision. This screen resulted in the discovery of compounds that significantly enhance in vivo nerve regeneration. The compound libraries were kindly provided by Prof. Steve Haggarty of Massachusetts General Hospital that were chosen based upon their ability to induce neurite outgrowth in mammalian cell culture. Cody Gilleland, Chrysanthi Samara, Christopher Rohde, and Mehmet Fatih Yanik conceived and designed the research. Christopher Rohde developed the majority of the software control with user interface and design also contributed by Cody Gilleland. Cody Gilleland engineered the system for large-scale operation by developing off-chip valves and techniques for loading animals reliably and rotating them on-chip. Cody also developed troubleshooting techniques for fabrication reliability, cleaning debris/bleaching which are featured in a Nature Protocol. Chrysanthi Samara and Christopher Rohde interpreted the drug screening results. Mehmet Fatih Yanik supervised the research during the entire process.

This co-first author work was first published in Proceedings of the National Academy of Sciences:

Discovery of molecular mechanisms and chemical compounds that enhance neuronal regeneration can lead to development of therapeutics to combat nervous system injuries and neurodegenerative diseases. By combining high-throughput microfluidics and femtosecond laser microsurgery, we demonstrate for the first time large-scale in vivo screens for identification of compounds that affect neurite regeneration. We performed thousands of microsurgeries at single-axon precision in the nematode *Caenorhabditis elegans* at a rate of 20 seconds per animal. Following surgeries, we exposed the animals to a hand-curated library of approximately one hundred small-molecules and identified chemicals that significantly alter neurite regeneration. In particular, we found that the PKC kinase inhibitor staurosporine strongly modulates regeneration in a concentration- and neuronal type-specific manner. Two structurally unrelated PKC inhibitors produce similar effects. We further show that regeneration is significantly enhanced by the PKC activator prostratin.

**Introduction**

The ability of neurons in the adult mammalian central nervous system to regenerate their axons after injury is extremely limited, which has been attributed to both extrinsic signals of the inhibitory glial environment [6] as well as intrinsic neuronal factors [7-9]. The discovery of cell-permeable small molecules that modulate axon regrowth can potentiate the development of efficient therapeutic treatments for spinal cord injuries, brain trauma, stroke, and neurodegenerative diseases. Identification of such molecules can also provide valuable tools for fundamental investigations of the mechanisms involved in the regeneration process. Currently, small-molecule screens for neuronal regeneration are performed in simple in vitro cell culture systems. Such screens have already revealed large numbers of chemicals that enhance regeneration and/or affect cellular morphogenesis, yet many of these hits still remain untested in vivo. In addition, most in vitro studies do not translate to animal models and also fail to reveal off-target, toxic, or lethal effects. Thus, a thorough investigation of neuronal regeneration mechanisms requires in vivo neuronal injury models.
In vivo investigation of neuronal regeneration has been performed mainly in mice and rats. However, their long developmental periods, complicated genetics and biology, and expensive maintenance prevent large-scale studies on these animals. The nematode Caenorhabditis elegans is a simple, well-studied, invertebrate model-organism with a fully mapped neuronal network comprising 302 neurons. Its short developmental cycle, simple and low-cost laboratory maintenance, and genetic amenability make it an ideal model for large-scale screens, rapid identification of the molecular targets of screened compounds, and discovery of novel signaling pathways implicated in regeneration.

Until recently however, the small size of C. elegans (~50 μm in diameter) prevented its use for investigation of neuronal regeneration mechanisms. We previously demonstrated femtosecond laser microsurgery as a highly precise and reproducible injury method for studying axon regrowth in C. elegans [10-12]. The nonlinear multiphoton absorption of the incident femtosecond pulse allows subcellular-resolution surgery of nematode neuronal processes with minimal out-of-plane absorption and collateral damage. Furthermore, due to the stereotypic anatomy and hermaphroditic reproduction of C. elegans, the same neurons can be repeatedly axotomized at the same distance from the soma in isogenic animal populations, which significantly enhances reproducibility of assays. Recent studies have used this technique to investigate how factors such as animal age, neuronal type, synaptic branching, and axon guidance signaling, influence regeneration [13, 14]. In combination with screens on nematodes exhibiting spontaneous neurite breaks due to dysfunction of β-spectrin, this technique has also revealed that axon regrowth depends on the activity of MAP kinase pathways [15, 16].

However, neuronal regeneration is a highly stochastic process requiring large numbers of animals to be screened. The high motility of wild-type nematodes causes a significant throughput challenge. Precise laser axotomy and imaging at the cellular level require orientation and immobilization of animals. Traditional immobilization methods using anesthetics, such as sodium azide, levamisole, and tricaine/tetramisole, have significant and/or uncharacterized effects on nematode physiology, which may affect the regeneration process [17]. In addition, anesthetics need several minutes to take effect, and recovery of nematodes from anesthesia requires exchange of media without losing animals, all of which hinder high-throughput screening. Other techniques that can be used to reversibly immobilize C. elegans include trapping of nematodes in wedge-shaped microchannels [18], cooling [19, 20], and exposure to CO2 [21,22]. However, the physio-
logical effects of exposure to low temperatures and CO₂ remain uncharacterized for many biological processes. In addition, none of these techniques has been adapted to perform large-scale chemical or RNAi screens using multiwell plates compatible with standard incubation and liquid-handling platforms.

We previously developed noninvasive mechanical means to immobilize *C. elegans* for high-throughput *in vivo* imaging and femtosecond laser microsurgery [23, 24]. Here, in order to facilitate large-scale screening of chemical libraries, we also developed a simple and robust mechanism to transfer nematodes from multiwell plates to microfluidic chips for neurosurgery and imaging. In combination with software we designed, we can load, image, and perform femtosecond laser microsurgery within 20 s per animal. We performed chemical screens using thousands of animals to test a hand-curated library of approximately 100 chemicals. We demonstrate that structurally distinct PKC inhibitors impair regeneration of *C. elegans* mechanosensory neurons. We also show that prostratin, a PKC activator, significantly increases neuronal regeneration.

**Results**

To enable chemical screens, we made several modifications to the microfluidic *C. elegans* screening technology we previously developed (Fig. 5.1A) [23, 24]. To incubate large numbers of animals in chemical libraries, we used multiwell plates, which are compatible with industrial liquid-handling platforms. We developed a method to rapidly, reliably, and repeatedly transport animals between standard multiwell plates containing chemicals and our screening chips (Fig. 5.1B). The multiwell plates are held at an angle, and a metal tube is inserted into each well until it reaches just above the well bottom. Because the animals settle near the well bottom, this allows rapid aspiration of animals without fluid being completely drained out of the wells. The channel array of the microfluidic screening chip is used to rapidly load multiple animals simultaneously into the main screening chamber (Fig. 5.1C, step 1). To capture an individual animal, the single aspiration port of the chamber is activated (Fig. 5.1C, step 2). The rest of the animals in the chamber are flown back toward the input (Fig. 5.1C, step 3) by brief application of a small pressure difference from the channel array. During this period, debris and air bubbles (which occasionally enter the chip during animal loading and adhere strongly to the chip surfaces) remain in the chamber. Next, by switching an off-chip valve, a stronger pressure pulse is applied to move debris or bubbles to the waste output (Fig. 1C, step 4), while the single aspiration port tightly holds the captured animal in the chamber. Sub-
sequently, all on-chip valves surrounding the main chamber are closed, isolating the single animal. The animal is then released from the single aspiration port and aspirated toward the channel array (Fig. 5.1C, step 5). This orients the animal linearly, making it easy to image and perform laser microsurgery. To increase the stability of immobilization, the channel above the chamber is then pressurized, pushing the thin immobilization membrane downward (Fig. 5.1 A and D). This fully constrains the animal motion for imaging and surgery [17, 24]. Once the animal is immobilized, the microscope and camera configuration automatically switches to high-resolution acquisition. We developed a simple software interface to quickly target the laser to the surgery position at a preset surgery distance from the soma of the neuron. The software requires the user to make only two mouse clicks to perform the entire surgery operation. The user first clicks on the soma of the neuron to be axotomized. The software then draws a circle centered on the soma where the radius of the circle is equal to the set surgery distance. The user next clicks on the intersection of the circle and the axon, which is the desired surgery location (Fig. 5.1E). The software automatically moves the laser target to this surgery position and performs the surgery. These enhancements significantly increase the throughput of our system: Our system can process animals within approximately 20 s on average, including off-chip loading and unloading of animals (Fig. 5.1F). Per animal, this is faster than the time previously reported for automated ablation of entire cell bodies alone (20), which requires less surgical precision.
Figure 5.1 Microfluidic \textit{C. elegans} manipulation for subcellular laser microsurgery and chemical library screening.

(A) Micrograph of dye-filled microfluidic chip. Red, control (valve) layer; yellow, flow layer; blue, immobilization layer. Scale bar: 1 mm. (B) Animal loading from multiwell plates. The multiwell plate is held at a 40° angle and a stainless steel tube is inserted to the well bottom. (C) Microfluidic \textit{C. elegans} manipulation steps. 1. Loading of nematodes. Dust, debris, air bubbles, and bacteria occasionally also enter the chip. 2. Capture of a single animal by the single aspiration channel. 3. Isolation of a single animal within the chamber by low-pressure washing of the channels to remove and recycle the rest of the nematodes. 4. Cleaning of channels by high pressure washing to remove debris and bubbles. 5. Orientation of the single animal by releasing it from the single aspiration port and recapturing it by the channel array. 6. Immobilization by pressurizing a thin membrane (see D). 7. Laser microsurgery (see E). 8. Unloading of the animal from the chip after surgery. (D) Illustration of the final immobilization process. Once a single animal is captured and linearly oriented (Left), a channel above the main chamber is pressurized pushing a thin membrane downward (Right). The membrane wraps around the animal, significantly increasing immobilization stability for imaging and surgery. Precise laser targeting of subcellular features is achieved using a femtosecond laser tightly focused inside the \textit{C. elegans} body by a high numerical aperture objective lens (see Materials and Methods). (E) Software interface to accelerate axon targeting for laser axotomy. A right mouse click on the cell body is used to identify the portion of the axon
at a set distance from the soma, and a left mouse click automatically moves this location to the laser focal point. (F) Average time per animal for screening steps. Total time per animal is from three independent experiments with 100 worms each.
Figure 5.2 *In vivo* chemical screen for small molecules affecting axonal regeneration.
(A) Primary target categories of the screened compound library. The dashed parts of the pie chart represent the percentage of compounds in each category affecting regeneration. The number of screened compounds and the percentage of the effective compounds in each category are denoted. (B) Common regeneration phenotypes observed 72 h following axotomy and compound exposure: (i) no axon regrowth, (ii) forward regrowth, (iii) backward regrowth, and (iv) regrowth with branching. Arrows and asterisks indicate start and end points of regenerated axons, respectively. For regrowth with branching, indicated start and end points are for the longest regrown branch. Scale bars: 20 μm. (C) Effects of protein kinase modulators in the regeneration of PLM neurites after laser axotomy. PLM neurons of L4 nematodes were axotomized 50 μm away from the cell body. Animals were incubated in the presence of kinase modulators for 48 h, and the lengths of the longest regrowing neurites were measured (**, P ≤ 0.01). Error bars denote the SEM, n indicates the total number of animals used in each case, and each bar shows one representative screen with its controls.

Using this technology, we screened *C. elegans* for regenerative effects upon exposure to a chemical library enriched for compounds that may affect neurite outgrowth in mammalian cell cultures in vitro [26, 27]. The potential targets of the small-molecule library that we screened included various kinases, cytoskeletal proteins, endocytic vesicle trafficking components, and nuclear processes (Fig. 5.2A). Such use of chemicals with a priori known targets facilitates delineation of molecular mechanisms involved in regeneration. To test the effects of these compounds on regeneration we axotomized mechanosensory neurons of *C. elegans*. These neurons have been used extensively for investigation of neurodegeneration in connection with human diseases [28, 29]. They grow long axonal processes devoid of large numbers of lateral branches, enabling highly precise microsurgery and subsequent imaging and characterization of outgrowing processes. Axotomies were performed on ALM mechanosensory neurons at larval stage 4 (L4) nematodes, approximately 200 μm away from the cell body. Following microsurgery, animals were incubated in the presence of small molecules at concentrations ranging from 10–20 μM. Neurite regeneration was assessed 72 h post axotomy by measuring the length of the regrowing processes (Fig. 5.2B). Fig. 5.2A shows a classification of the library compounds and the percentage of chemicals in each group that led to significant regeneration effects (i.e., P ≤ 0.05 in Student’s t test). The compounds screened, the number of animals treated with each compound, the effects on regeneration, and the statistical significances are provided in SI Text (Fig. 5.S1 and Table 5.S1). From our pre-
liminary screen, we identified a number of chemical compounds that significantly altered axon regeneration \((P \leq 0.05)\), among which were modulators of protein kinase activity (Fig. 2A). This observation, in conjunction with the recent studies implicating specific MAP kinases in the regeneration of nematode GABAergic motor neurons [15, 16], prompted us to further investigate the effects of kinase modulators in *C. elegans* neurite regeneration. We investigated the effects of this compound class on regeneration of PLM neurons, since regrowing ALM neurons pass near or through the dense and complex neuronal circuitry of the nerve ring and occasionally interact with its components, complicating analysis and interpretation of the results.

By performing laser axotomy on PLM mechanosensory neurons, we analyzed the effects of all the commercially available kinase modulators from our initial chemical library, which included staurosporine, wortmannin, LY294,002, H89, W7, PD 98,059, 50-E12, Y-27632, and dibutyryl-cAMP (Fig. 5.2C and Table 5.1). Known targets of these compounds are shown in Table 5.1. Compounds were tested at late larval stage and young adult nematodes, at concentrations ranging from 10 to 100 \(\mu M\) (Fig. 5.2C and Table 5.1). Staurosporine, a nonselective kinase inhibitor with high affinity for protein kinase C (PKC) [30], exhibited the strongest effects. Staurosporine administered at a concentration of 10 \(\mu M\) caused approximately a threefold decrease in the regrowth of PLM neurons 48 h after axotomy, whereas concentrations lower than 5 \(\mu M\) did not exhibit any significant effect (Fig. 5.3A). The effect was similar in L4 and young adult animals \((44.25 \pm 7.47 \text{ vs. } 112.86 \pm 9.23 \mu m, P = 2.89 \, E \cdot 07 \text{ in L4 animals}, 44.15 \pm 6.23 \text{ vs. } 89.32 \pm 7.11, P = 3.20 \, E \cdot 05 \text{ in young adults})\) (Fig. 5.52). In early larval stages, 0.5% DMSO (used as a solvent for staurosporine) was toxic; however, the few surviving L3 nematodes also exhibited decreased regrowth and strong morphological abnormalities. Although toxicity of DMSO is a limitation for using chemical libraries on young larvae, 0.5% DMSO is not toxic to either young adults or older animals, which is of significant value for pharmaceutical screens targeting degenerative diseases and regeneration.

Interestingly, the effect of staurosporine administration was specific to the neuronal type; although staurosporine affected PLM touch neurons, it did not alter regeneration of the ALM touch neurons, D-type GABAergic motor neurons, CEP dopaminergic neurons, or AWB olfactory neurons (Fig. 5.3B). Given that only the posteriorly located PLM neurons exhibited sensitivity to staurosporine, this differential effect could be attributed to physical barriers preventing staurosporine from reaching more anterior parts of the
C. elegans body. To investigate this possibility, we took advantage of GABAergic neurons, which can be found all along the nematode body. In contrast with its effect on PLM neurons, staurosporine did not alter significantly the regrowth of axotomized posterior GABAergic motor neurons, nor did it cause any difference in the response among GABAergic motor neurons in the posterior-, anterior- or mid-body of animals (Fig. 5.3D). These observations suggest that different types of neurons have different molecular requirements for regeneration. This is true even for neurons with highly similar structure and functions, such as ALMs and PLMs, both of which extend long processes along the nematode body and are required for mechanosensation (Fig. 5.3 B and C).

Table 5.1. Effects of kinase modulators on the regeneration of C. elegans PLM neurons

<table>
<thead>
<tr>
<th>Small molecule</th>
<th>Molecular target</th>
<th>Regeneration effect vs. control (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>db-cAMP</td>
<td>cAMP-dependent protein kinase (PKA)</td>
<td>-0.388</td>
<td>0.973</td>
</tr>
<tr>
<td>H-89</td>
<td>PKA</td>
<td>-0.680</td>
<td>0.781</td>
</tr>
<tr>
<td>LY-294,002</td>
<td>Phosphatidylinositol 3-kinase (PI3K)</td>
<td>+1.271</td>
<td>0.8941</td>
</tr>
<tr>
<td>PD 98,059</td>
<td>MAPKK</td>
<td>-30.549</td>
<td>0.765</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>PKC*</td>
<td>-60.796</td>
<td>1.623 x 10^-7</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>PI3K'</td>
<td>+7.258</td>
<td>0.606</td>
</tr>
<tr>
<td>Y-27632</td>
<td>ROCK</td>
<td>-12.790</td>
<td>0.367</td>
</tr>
</tbody>
</table>

The commercially available kinase modulators included in our chemical library are listed along with their known targets. The percent differences in mean neurite regrowth with respect to the controls following 48 h exposure of axotomized L4 animals to the chemicals are shown with corresponding P-values.

*At higher concentrations, staurosporine also inhibits Myosin light chain kinase (MLCK), PKA, PKG, and CaMKII.

†At higher concentrations, wortmannin also inhibits mitogen-activated protein kinase (MAPK) and MLCK.

Regenerating neurites in animals treated with staurosporine often exhibited swellings along the axon and big terminal retraction bulbs (Fig. 5.3C). Such structures were observed mainly in the axotomized neurites of treated animals and only rarely in nontreated animals. Because staurosporine is known to induce apoptosis at high concentrations [31-34], we investigated whether staurosporine's effect on regeneration is related to the activation of apoptotic pathways. Classical apoptotic pathways in C. elegans require CED-3, a cysteine protease of the interleukin-1β-converting enzyme
(ICE) family [35, 36]. In vitro mammalian cell culture assays have demonstrated the requirement of this family of caspases (ICE/CED-3 protease family) for the induction and execution of early apoptotic events following exposure to staurosporine [37–39]. We tested staurosporine on a ced-3 mutant genetic background and observed no significant difference in regeneration compared with wild-type animals (Fig. 5.S3A). In addition, we used Hoffman microscopy to perform a time course image analysis of the axotomized neurons in staurosporine-treated nematodes from 24 to 120 h after laser microsurgery. No apoptotic body morphology was detected at any time point within 120 h following axotomy, and the injured neurons persistently expressed GFP (Fig. 5.S3B). Our observations suggest that impairment of neurite regeneration by staurosporine in our experimental system does not involve activation of classical apoptotic pathways.
Figure 5.3 Effect of staurosporine on neurite regrowth is dependent on staurosporine concentration and neuronal type. (A) PLM neurons of L4 nematodes were axotomized, and regeneration was measured after 48 h. Staurosporine inhibited regrowth at concentrations of 5 μM or higher. Toxicity was observed at concentrations higher than 10 μM. (B) Laser microsurgeries were performed on different types of neurons in young adult nematodes, and regeneration was measured 48 h later. Staurosporine had a significant effect only in PLM neurons (**, P ≤ 0.01). (C) Regeneration phenotypes observed 48 h after axotomy of PLM (i and ii) or ALM (iii and iv) neurons in staurosporine-treated and control animals. Arrows and asterisks indicate start and end points of regenerated axons, respectively. Arrowheads in (ii) indicate terminal retraction bulb and axonal swell-
ings formed in PLM neurons after staurosporine treatment. Scale bars: 30 μm. (D) Effect of staurosporine on GABAergic motor neurons at different parts of the nematode body. GABAergic neurons were axotomized in L4 animals and regeneration was measured after 48 h. Treatment of nematodes with 10 μM staurosporine did not significantly alter the regrowth of posterior GABAergic neurons when compared to nontreated animals. The regeneration response was similar among anterior, midbody, and posterior GABAergic neurons after exposure to staurosporine. Error bars in A, B, and D denote the SEM, and n indicates the total number of animals used.

While staurosporine can induce apoptosis only at high concentrations, at lower concentrations it has been shown to inhibit the protein kinases PKC, PKA, PKG, CAMKII, and MLCK, as well as other kinases in a concentration-dependent manner, and to stimulate K-Cl cotransport in red blood cells [30, 40–42]. Because staurosporine’s strongest inhibitory effect is on PKC kinases, and in C. elegans it has been shown to inhibit PKC activity [43], we tested whether it exerts its effects on regrowing neurites via the inhibition of this particular kinase. To this end, subsequent to laser microsurgery of PLM neurites, we incubated nematodes in the presence of the two distinct structural classes of specific PKC inhibitors, chelerythrine or Gö 6983 [44, 45]. Treatment of axotomized animals with either chelerythrine or Gö 6983 at concentrations between 10 and 100 μM also significantly reduced regeneration (70.40 ± 8.20 vs. 123.35 ± 7.86 μm, P = 2.65 E-05 for chelerythrine, and 99.83 ± 8.28 vs. 128.65 ± 9.10 μm, P = 0.023 for Gö 6983) (Fig. 5.4). Furthermore, the PKC activator prostratin significantly increased regeneration of PLM neurites (145.25 ± 7.46 vs. 114.68 ± 8.88 μm, P = 0.01) (Fig. 5.4). In combination, the above results strongly suggest that PKC kinases are involved in the regeneration of C. elegans mechanosensory neurons. Although we cannot exclude contribution of other pathways to the inhibitory effect of staurosporine, these pathways likely do not involve PKA signalling (also targeted by staurosporine), because we did not observe any effect on regeneration after treatment with PKA modulators db-cAMP and H89 (Fig. 5.2 and Table 5.1).
Figure 5.4 Enhancement and inhibition of regeneration by structurally different chemical modulators of PKC activity. (A) PLM neurons of L4 nematodes were axotomized, and animals were incubated for 48 h in the presence of staurosporine, G6 6983, chelerythrine, or prostratin. The lengths of the longest regrowing neurites were compared (*, P ≤ 0.05; **, P ≤ 0.01). Error bars indicate the SEM, and n indicates the total number of animals used. (B) Representative images of regenerating PLM neurites as observed 48 h after laser microsurgery in nontreated (I), G6 6983- (ii), chelerythrine- (iii), or prostratin-treated (iv) animals. Arrows and asterisks indicate start and end points of regenerated axons respectively. Scale bars: 30 μm.
Discussion

We demonstrated here the use of laser microsurgery and microfluidic technologies for \textit{in vivo} screening of chemicals affecting neuronal regeneration. We developed a simple and robust technique to load nematodes from and dispense to standard multiwell plates. This allowed us to use standard technologies for incubation of large numbers of animals in chemicals, while utilizing the manipulation capabilities of our microfluidic chips. In combination with software we developed, we were able to load, image, and perform single-axon-precision surgeries within approximately 20 s.

Screening a chemical library of small molecules indicated the involvement of specific kinase pathways in neurite regrowth after injury in \textit{C. elegans}. We found that the kinase inhibitor staurosporine suppresses regeneration in a neuronal type-specific manner. In addition, we showed that axonal regeneration is significantly enhanced after administration of a PKC activator. Our results are consistent with in vitro studies on goldfish retina explants [46], on adult frog sciatic sensory axons [47], on postnatal mice retinal ganglion cells [48], and on adult mice sensory ganglia [49], wherein the administration of PKC inhibitors following mechanical lesion affects neurite outgrowth. This indicates the existence of conserved neuronal regeneration mechanisms between nematodes and vertebrate organisms. However, in vitro studies have yielded conflicting data regarding the involvement of PKC kinase in inhibitory effects of staurosporine on neuronal regeneration. By performing regeneration studies on whole organisms, we showed \textit{in vivo} that staurosporine blocks regeneration at least partially by inhibiting PKC. We also showed that other kinase inhibitors of PI3K, PKA, MAPKKK, and ROCK did not affect regeneration in a neuron type that is otherwise strongly affected by staurosporine. However, these kinase inhibitors affect neurite growth in other experimental models [50–54]. The lack of response to these other types of kinase inhibitors could be due to the differences among neuronal types, the ineffectiveness of these chemicals in our experimental system, or the absence of inhibitory myelin sheath in \textit{C. elegans}.

Many chemicals have been found to modulate neurite growth in vitro. However, validation of these effects \textit{in vivo} and identification of their mechanisms of action have remained elusive due to the lack of large-scale screening technologies for genetically amenable animal models. The advantages of \textit{C. elegans} genetics and our high-throughput screening technology should allow future discovery of novel molecular pathways required for neuronal regeneration. Further experiments on higher organisms
will show which of these mechanisms are conserved in mammals and may provide means for pharmaceutical or genetic interventions to combat human diseases and injuries.

**Materials and Methods**

Nematode Handling: Nematodes were grown at 15°C in NGM agar plates, unless otherwise mentioned. Standard procedures were followed for *C. elegans* strain maintenance and genetic crosses [55]. Nematode strains used in this study included BZ555: *egls1[pdat-1GFP]*, CX3553: *lin-15B(n765)ky ls104*pstr-1GFP*X, EG1285: *lin-15B(n765)oxls12[punc-47GFP*X, MT1522: *ced-3(n717)IV*, SK4005: *zds5[pmecc4GFP]*I, and *zds5[pmecc4GFP];ced-3(n717)IV*.

Chemical Treatments: The small-molecule library used in the chemical screening was prepared from initial compound stock plates with small-molecule concentrations ranging from 5–10 mM. By consecutive dilutions in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich) and transfers using the Cybi*-Well vario 384/35 μl Head, daughter plates were created, heat sealed, and stored at -20°C, in order to be used on the day of the screening. Compound concentrations in the daughter plates ranged from 2–4 mM. One day prior to laser microsurgeries Escherichia coli OP50 bacteria were inoculated in Luria-Bertani (LB) media and grown overnight. Subsequently the E. coli culture was washed with M9 buffer and bacteria were resuspended in liquid nematode growth media (NGM). For the compound library screens, 55 μl of the NGM resuspended bacterial culture was added to 0.55 μl of DMSO-dissolved compound library. After thorough mixing, 50 μl of the compound-containing NGM culture was further diluted, by adding 50 μl of NGM resuspended bacterial culture, so that the final DMSO concentration was 0.5% and the small-molecule concentrations ranged from 10–20 μM. Control cases contained either 0.5% DMSO or bacterial culture alone. For each library compound, a total of 10–20 animals were tested during the preliminary screen.

For the kinase effector treatments, axotomized animals were incubated at 20°C with 10–100 μM dibutyryl-cAMP, H-89, LY-294,002, PD 98,059, staurosporine, wortmannin, Y-27632, chelerythrine, Gö 6983, or prostratin (Sigma Aldrich) in liquid NGM cultures supplemented with E. coli OP50 bacteria and transferred into fresh cultures every 24 h. For control experiments in treatments with LY-294,002, PD 98,059, staurosporine, wortmannin, chelerythrine, Gö 6983, and prostratin, which were dissolved in DMSO,
liquid NGM was also supplemented with the respective amount of DMSO. Each experiment was repeated at least three times.

Femtosecond Laser Microsurgery: Synchronized L4 nematodes were brought into the chip and immobilized Mai-Tai® HP (Spectra-Physics) femtosecond laser beam with 800 nm wavelength and 80 MHz repetition rate was delivered to a Nikon Ti microscope. ALM axons were axotomized by pulses with 10 nJ energy for 3.2 ms using an objective lens with NA = 0.75. For the control kinase modulator assays, synchronized L3, L4, or young adult nematodes were immobilized in 2% agarose pads with 0.1–1% 1-phenoxy-2-propanol. Neurites of mechanosensory, GABAergic, and AWB or CEP neurons were axotomized by 7 nJ, 9.5 nJ and 11 nJ energy pulses respectively for 1.5 ms, with a 780 nm laser beam at 80 MHz repetition rate.

Data Collection and Analysis: 48–120 h following axotomy, animals were imaged at the area of surgery with a Nikon Ti microscope. The length of the longest regrowing neurite and morphology of regrowing neurites were scored using a MATLAB program. The percent regeneration versus control is used to present the percent difference of regrowth from the mean value of the control. Statistical analysis was performed using a two-tailed Student’s t test.
Figure. 5.51 Preliminary screen of a hand-curated chemical library for effects on regeneration of *C. elegans* mechanosensory neurons. ALM neurons of L4 animals were...
axotomized approximately 200 μm away from the cell body and incubated for 72 h in the presence of chemical library compounds. Maximum regrowth lengths were measured and the percent differences to the respective controls were calculated for each experiment. Bars represent the total mean values from all experiments (*, P ≤ 0.05; **, P ≤ 0.01), error bars denote SEM, and n indicates the total number of animals used in each case.

Figure. 5.52 Effect of staurosporine on regeneration at different developmental stages of *C. elegans*. PLM neurons of L4 or young adult zdIs5 nematodes were axotomized 50 μm or 60 μm away from the cell body respectively, and regrowth was scored after 48 h (**, P ≤ 0.01). Error bars indicate the SEM, and n indicates the number of animals used in each case. Nematodes were incubated in the presence of either 0.47% DMSO (control) or 10 μM staurosporine in 0.47% DMSO, both of which caused significant toxicity and death in L3 or younger animals (hence, data is not provided for these stages).
Figure 5.53 Suppression of neurite regeneration by staurosporine is not due to induction of apoptosis. (A) PLM neurons of L4 nematodes were axotomized, and animals were incubated for 48 h with or without staurosporine. The apoptosis-defective *ced-*
3(n717) genetic background affects neither regeneration nor the effect of staurosporine on the regrowth of PLM neurites after laser surgery. Error bars denote the SEM, and n indicates the total number of animals used. (B) Axotomized L4 nematodes were incubated for 5 d in the presence of 10 μM staurosporine, and the injured neurons were imaged with Hoffman microscopy every 24 h. We did not observe formation of apoptotic bodies in either non-treated or in staurosporine-treated animals at any time points. Persistent expression of GFP in both cases indicated that neurons survive and are functional. Arrows and asterisks indicate start and end points of regenerated axons respectively. The empty arrowhead points to the GFP-expressing cell body of the axotomized neuron in an animal that was exposed to staurosporine for 5 d. Scale bars: 30 μm.
Table 5.51 Chemical library of small molecules screened for effects on neuronal regeneration in *C. elegans* (continued on next page)
<table>
<thead>
<tr>
<th>Target Name</th>
<th>Molecular Structure</th>
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<tr>
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<tr>
<td>JY-37</td>
<td>NC1tCcNcC(N)(C)CeCcC</td>
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</table>

The names of the small molecules used in our preliminary chemical library screen are listed along with their respective target category and molecular structure. Chemical structures are represented by SMILES (Simplified Molecular Input Line Entry Specification).
Figure 5.54 Integrated software controlling robotic, optical and microfluidic platform. The custom software interface controls digital valves that pressurize the microfluidic channels for microfluidic chip operation. A laser is ported into the back of the microscope for femtosecond laser microsurgery.

ACKNOWLEDGMENTS

We thank Robert Horvitz's laboratory for discussions and technical support, Charles Jennings and Matthew Angel for valuable comments, and the C. elegans Genetics Center for the strains.
Microfluidic immobilization of physiologically active C. elegans

This chapter details a protocol that enables users to fabricate and manually control a simple microfluidic device for immobilizing C. elegans in a physiologically active state. After the publication of our previous microfluidic screening technologies in the Yanik Lab many biologists requested a simple device that could be operated manually without complex software, electronic valves, and challenging 3 layer fabrication. A single animal can be loaded into the device using a syringe and then immobilized by turning a manual pressurized valve which compresses a flexible membrane around the animal for physical restraint without the use of cooling or physiologically altering substances such as CO₂ or anesthetics. The device has been successfully fabricated by the Stanford Microfluidics Foundry and sent to multiple users. This device was initially designed by Fei Zeng and Christopher Rohde. Cody Gilleland further improved and characterized for the device for manufacturing reliability, developed troubleshooting techniques, engineered for manual use and wrote the protocol. Mehmet Fatih Yanik supervised the entire process.

This first author work was first published in Nature Protocols:


Introduction

We present a protocol for building and operating a microfluidic device for mechanical immobilization of Caenorhabditis elegans in its physiologically active state. The system can be used for in vivo imaging of dynamic cellular processes such as cell division and migration, degeneration, aging and regeneration, as well as for laser microsurgery, Ca²⁺ imaging and three-dimensional microscopy. The device linearly orients C. elegans, and then completely restrains its motion by pressing a flexible membrane against the animal. This technique does not involve any potentially harmful anesthetics, gases or cooling procedures. The system can be installed on any microscope and operated using only
one syringe and one external valve, making it accessible to most laboratories. The device fabrication begins by patterning photoresist structures on silicon wafers, which are then used to mold features in elastomeric layers that are thermally bonded to form the device. The system can be assembled within 3 d.

The nematode *Caenorhabditis elegans* is an extensively studied multicellular model organism, mainly because of its small size, optical transparency, rapid development and amenable genetics. Many assays and manipulations of *C. elegans*, including studies of development, aging and tissue degeneration, laser microsurgery [10] and calcium imaging [56], require reversible immobilization of the animals. The most common and broadly used immobilization methods anesthetize nematodes on agarose pads with water-soluble chemicals [57]. However, anesthesia may affect the physiology of the animals [58], and dynamic cellular processes such as cell division, neurite growth and calcium signaling cannot be studied under anesthesia. In addition, recovery of animals from anesthesia is challenging. Cooling [59] and exposure to CO₂ (ref. [60]) have also been used for immobilizing *C. elegans*, although the physiological effects of these methods on many cellular processes also remain uncharacterized. Therefore, there is significant need for noninvasive and reversible immobilization techniques that enable imaging and manipulation of cellular processes at subcellular resolution.

The physiology of *C. elegans* and its ability to grow in liquid culture make it highly amenable to manipulation in microfluidic devices [17, 18, 20, 22, 23, 24, 61-66]. We recently demonstrated the use of an array of microchannels [23] to orient the animal, in combination with an ultrathin flexible membrane that wraps around the animal to completely constrain its motion without injury [24]. This immobilization method permits even demanding techniques such as two-photon imaging [24] and femtosecond laser microsurgery [11, 17, 24, 56] to be conducted on physiologically active animals at subcellular resolution [24] (Fig. 6.1). We previously detailed a protocol for construction and alignment of a femtosecond laser microsurgery system [12], which can be used on *C. elegans* immobilized by the device [24] described in this protocol.

The technique presented here aligns animals in a linear orientation as we previously demonstrated [23,24], thus simplifying cell tracking, image processing and comparisons between animals. In addition, this device allows the animals to be loaded and unloaded rapidly, and to be recovered quickly and reliably. The devices can immobilize animals at different developmental ages by simply adjusting the pressure levels to accommodate
animal sizes from L4 larval stage to fully grown adult. For smaller animals, the dimensions of the device must be scaled down. Suggestions for this are given in the Experimental design section.

In this paper, we detail a procedure for building and operating such a system. The system can be installed on any inverted microscope and can be operated using only one syringe and one external valve, making it accessible to most laboratories. The device is composed of two PDMS (polydimethylsiloxane) layers, i.e., a flow layer and a compression layer. These PDMS layers are bonded to each other, and the combined layer is bonded to a dish with a cover glass bottom. The animal is loaded to and transported within the flow layer, which contains a narrow channel array that allows fluid to pass while preventing the animals from flowing through. Thus, the animal is linearly oriented by the fluid flowing through the channel array. The compression layer contains a channel above the thin membrane that separates the two layers [67]. When the pressure in the compression layer is raised, the immobilization membrane expands into the flow layer, wrapping around the animal and completely restraining its movement. The animal can then be imaged and optically manipulated through the cover glass at the bottom of the chip through an inverted microscope. Thereafter, the pressure in the compression layer can be released, and the animal can be pushed out of the chip by reversing the flow through the channel array.

Comparison with other methods

Alternatives to anesthetizing *C. elegans* for immobilization include microfluidic methods such as flowing animals into narrow channels [18], cooling them [20] or exposing them to CO₂ (ref. [22]). The approach of using narrow channels allows the use of only one PDMS layer, thus enabling simple fabrication and operation; however, this method has not been shown to provide sufficient immobilization stability for confocal or three-dimensional two-photon imaging. Cooling affects dynamic cellular processes and the effects of CO₂ remain unknown. Guo *et al.* [17] also adapted an immobilization technique using a membrane; however, three-dimensional cellular-resolution confocal or two-photon imaging was not shown (see the high motility of the animal in Supplementary Video 2 of Guo *et al.*[17]). The method [24] described in this protocol allows immobilization of whole animals with subcellular-level stability (see Supplementary Video 1, online), and three-dimensional two-photon images can be acquired even at slow acquisition rates. The stability of the presented technique is due to the extensive optimization
in the thickness, flexibility and roundness of the channels and membranes [24]. In addition, the channel array [23,24] used here allows alignment and positioning of *C. elegans* before immobilization. An immobilization technique using agarose and polystyrene spheres [68] offers simplicity, as it does not require device fabrication. However, the process of rapid immobilization and reliable recovery of animals without loss is challenging and the animals are also held in random orientations, complicating cell tracking and image analysis. In addition, motility of physiologically active animals was not quantified for applications requiring high immobilization stability, such as three-dimensional confocal and two-photon imaging.

**Figure 6.1** Subcellular resolution imaging and optical manipulation of physiologically active *C. elegans*. (A) Immobilized *C. elegans* inside the optically transparent chip. Scale bar, 200 μm. (B) Three-dimensional two-photon image of the GFP-labeled touch neurons (*mec4::GFP*) of the immobilized *C. elegans*. Scale bar, 20 μm. (C) Femtosecond laser microsurgery of touch neuron axons using 200-fs short pulses with 5-nJ energy and 80-MHz repetition rate delivered for 10 ms. Scale bar, 20 μm.

**Experimental design**

The procedure begins with the fabrication of ‘mold-C’ (which is used to mold the aforementioned compression layer) and ‘mold-F’ (which is used to mold the flow layer) by patterning silicon wafers (Fig. 6.2a) with photoresists (using optical masks (Fig. 6.3)), along with tools and materials available in most university
cleanrooms. Following photolithography, a thin layer of PDMS is spun onto mold-F (to form the flow layer in Figs. 6.2b and 6.4a) and a thick layer of PDMS is poured onto mold-C (to form compression layer in Fig. 6.4a and Fig. 6.2b). These PDMS layers are partially cured in an oven. The compression layer is peeled from mold-C and a hole is punched into the compression layer to provide fluidic access to the channels in that layer (Fig. 6.2c). The compression and flow layers are then aligned and bonded to each other by further thermal curing (Fig. 6.2c). Once these layers have bonded and have then been peeled from mold-F, two holes are punched to access the channels in the flow layer (Fig. 6.2d). The layers are then plasma bonded to a cover glass and thermally cured overnight (Fig. 6.2d). Finally, steel pins and tubing interface the completed chip to a manual valve for pressurizing the compression layer and to a syringe for loading and unloading animals (Fig. 6.2e). The steps involving photoresist patterning, PDMS and plasma bonding include time-sensitive sequences as detailed in the TIMING section.

Three optical masks (either negative or positive) are used to make molds by optically patterning a thin layer of a light-sensitive polymer (i.e., photoresist) that is spin coated onto a silicon wafer, as shown in Figure 6.5a–c:

1. Negative ‘flow-1’ mask (red) and ‘compress-1’ mask (blue) are used to pattern negative photoresist (i.e., the areas of photoresist exposed to UV light through the optical masks will be crosslinked. The regions that are not crosslinked are dissolved during the photoresist development step). The negative masks should be transparent in areas in which the photoresist remains on the wafer surface forming the mold.

2. Positive ‘flow-2’ mask (green) is used to pattern positive photoresist (i.e., the regions of photoresist exposed to UV light chemically break and dissolve during the photoresist development step). The positive mask should be dark (opaque) in areas in which the photoresist remains on the wafer surface forming the mold.

Both negative and positive photoresists are used to form structures with square edges (flow-1) and rounded edges (flow-2), respectively, on the same wafer mold. Negative resist retains the square edges during the reflow process, whereas positive resist becomes rounded.
The flow-2 photoresist layer (comprising the regions of the flow layer in which \textit{C. elegans} is transported) is too thick to be reliably coated in a single step using SIPR-7123 photoresist. To create this thick layer (Steps 18–22), two separate layers of 50 μm are spin coated to achieve the desired thickness of 100 μm.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.2.png}
\caption{Procedure overview. (A) Photolithography techniques are used to generate photoresist patterns on silicon wafers creating mold-C and mold-F. (B) Structures are then molded into PDMS layers using wafer molds by pouring a thick layer onto mold-C and spin coating a thin layer onto mold-F, followed by curing in an oven. (C) The PDMS layer is peeled from mold-C and a hole is punched to access the compression layer. This layer is then aligned onto the PDMS layer on mold-F. The PDMS layers are then baked in an oven overnight forming a strong thermal bond. (D) The combined PDMS layers are then peeled from mold-F and two holes are punched to access the flow layer. The combined PDMS structure is then plasma bonded to a dish with a cover glass bottom and baked overnight. (E) The chip is interfaced with steel pins and connected with tubing to a syringe and a manual valve.}
\end{figure}
Figure 6.3 Layout showing the masks used to fabricate the compression and flow layers of the chip. (A) Mask compress-1 (blue) is for the 65-\(\mu\)m-thick compression layer. Mask flow-2 (green) is for the 100-\(\mu\)m-thick region of the flow layer where the animals flow. Mask flow-1 (red) is for the 10-\(\mu\)m-thick region comprising the channel array within the flow layer. (B) Expanded view of the mask regions showing the overlap of Mask flow-1 and Mask flow-2 to form the channel array. See Supplementary Figure 6.1 for the mask drawings in AutoCAD format.
Reflowing the positive flow-2 photoresist layer improves the immobilization stability achieved by these devices. In addition, rounded channels allow incorporation of microfluidic valves [67] to integrate fluidic control directly into the device [23,24].

The masks for photolithography can be ordered from various companies including Fineline Imaging, Micro Lithography Services and Advanced Reproductions. To place a mask order, send the AutoCAD file containing the mask patterns provided in Supplementary Figures 6.1-5 to the company of your choice (Supplementary Fig. 6.1 for the AutoCAD file and Supplementary Figs. 6.2-5 for the corresponding pdf files). Specify in your order whether each mask is negative or positive. These files are available online.

The PDMS layers will be thermally bonded together because plasma bonding is less reliable due to the variability of the plasma treatment process (unless automated) and high sensitivity to particulates and surface defects. In addition, alignment of PDMS layers is critical for the compression layer to function properly. Before thermal bonding, PDMS layers are pressed together and can be aligned multiple times under a stereomicroscope to achieve perfect alignment before the layers are baked to thermally bond them together. Such alignment is not possible with the plasma bonding technique.

We recommend the following design rules for scaling down the chip to accommodate animals in the L1-L3 larval stages:

1. The flow-1 layer thickness and spacing in the channel array should be scaled to one-fifth of the animal's width.

2. The flow-2 layer thickness should be scaled to half the animal width.

3. The thin PDMS layer spin coated on mold-F should be scaled such that the immobilization membrane thickness remains the same for robustness and fabrication reliability.

4. The compress-1 layer thickness should remain the same.

5. The thick PDMS layer formed on mold-C should remain at the same thickness to provide rigidity to support interfacing with steel pins and tubing.

For those looking for more information on soft lithography techniques, a recent protocol by Qin et al. [69] also gives a general overview. However, unlike the protocol pre-
sented here, Qin et al. does not discuss fabrication of devices with multiple PDMS layers.

Figure 6.4 Microfluidic chip for mechanical immobilization of *C. elegans*. (A) Diagram of the chip with numbered arrows showing *C. elegans* manipulation steps. (1) Loading of a nematode from the input/output port-B into the flow layer (yellow) and its restraint by a narrow channel array. (2) The animal is immobilized by pressurizing the compression layer (blue) through port-A, causing the immobilization membrane to flex into the flow layer (yellow). (3) Release and unloading from the chip. (B) Cross-section of the chip showing the immobilization method when (i) the compression layer (blue) is not pressurized, allowing the animal to be positioned and (ii) the compression layer is pressurized, restraining the animal's motion within the flow layer (yellow), enabling laser microsurgery through the cover glass.
Figure 6.5 Fabrication of photoresist molds and PDMS layers. The patterning of mold-C (A) and mold-F (b–d). Molding (e–f) and bonding (G) of PDMS layers. Bonding of the released PDMS layer to glass (h–i). Actuation of the immobilization membrane when pressurized (J). These illustrations are referenced throughout the fabrication procedure.

MATERIALS

EQUIPMENT

General equipment and reagents
- Inverted fluorescence microscope (Nikon Eclipse TI (Nikon) or similar model) including a stage plate capable of holding a standard 50-mm dish with a cover glass bottom
- Image acquisition software (NIS-Elements 2.0, Nikon) and compatible computer
- High-resolution CCD camera (Photometrics Coolsnap HQ2, Photometrics)
- Powder-free gloves (VWR)
- Microscope ruler (Ted Pella, cat. no. 6085)
- Equipment and reagents for culturing C. elegans animals
- KH₂PO₄
- Na₂HPO₄
- NaCl
- MgSO₄
- Distilled water

**Master mold fabrication**
- Silicon wafers (4-inch diameter; University Wafer)
- Photomasks. The procedure for ordering a photomask is detailed in the Experimental design section
- Alignment and UV exposure tool. This is an expensive piece of equipment and is typically shared by multiple laboratories at the microfabrication facilities available at most universities (Lamp power rating 200 mW cm⁻²). UV exposure time is dependent on source intensity. Adjust exposure time to accommodate differences in source intensity if not using the recommended source! **Caution** Use protective eyewear with UV light.
- Spinner with vacuum chuck (Headway Research)
- Negative photoresist SU-8 2075 (Microchem)
- Negative photoresist SU-8 2025 (Microchem)
- Negative photoresist developer (PM acetate (propylene glycol monomethyl ether acetate); Eastman)
- Isopropyl alcohol (IPA; VWR, cat. no. BDH1133-1LP)
- Positive photoresist SPIR-7123 (Micro-Si)
- Positive photoresist developer AZ440 (AZ Photoresists Group of Hoechst Celanese)
- Hexamethyldisilazane (HMDS; SPI-Chem) ! **Caution** It is toxic, corrosive and highly flammable.
- F13-TCS (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane; United Chemical Technologies, cat. no. T2492-KG) ! **Caution** It is toxic and corrosive.
- Hot plates set to 65, 95 and 140 °C with temperature ramp capability (2) (PMC, cat. no. Dataplate 730)
- Petri dishes (150 mm × 15 mm; Falcon, cat. no. 35-1058)
Glass Pasteur pipette and rubber bulb (VWR, cat. nos. 14673-010 and 56310-002)
- Cotton swabs (VWR, cat. no. 10806-005)
- Eye-dropper bulbs (5 ml, VWR, cat. no. 56335-022) !Caution! Photoresists, developers, HMDS and F13-TCS are toxic chemicals. Proper protective equipment should be worn according to institutional regulations.

**PDMS device fabrication**
- PDMS RTV615A base, RTV615B curing agent (GE Silicones)
- Vacuum desiccators (230 mm; Bel-Art, cat. no. 420250000) connected to a vacuum line
- Chemical fume hood
- Oven set to 80 °C (Yamato, cat. no. DX-400)
- PDMS mixer/degasser machine (Thinky, cat. no. ARE-250) (optional)
- Plastic mixing cups (Thinky, matched to ARE-250 mixer) (optional)
- Hole punchers with 0.75-mm and 0.5-mm diameters (1 of each) and cutting mat (Ted Pella, cat. nos. 15071, 15072 and 15097)
- Plasma chamber (Harrick, cat. no. PDC-001) and its vacuum pump (Leybold Vacuum Products, cat. no. Trivac D2.5E)
- Clear adhesive tape (Scotch brand, 3M)
- Glass slide (75 mm × 50 mm × 1 mm; Fisher Scientific, cat. no. 12-550C)
- Aluminum foil
- Lint-free fabrication wipes (VWR, cat. no. HP-9310-4828)
- Laboratory scale (Ohaus, cat. no. sp202)
- Dish with cover glass bottom (50 mm outer diameter × 7 mm high, glass area: 40 mm diameter; Ted Pella, cat. no. 14026-20 or Electron Microscopy Sciences, cat. no. 70674-52)

**Off-chip system**
- Pressure gauge (1/8 inch, NPT 30 inches; Noshok, cat. no. 15-100)
- Pressure regulator (Minuteman controls, cat. no. R-800-300-W/K)
- Right-angle threaded tee (nylon, 1/8 inch NPT(F) × 1/8 inch NPT(F) × 1/8 inch NPT(M); Cole-Parmer, cat. no. C-06349-20)
- Female Luer locks to 1/8 inch NPT (Cole-Parmer, cat. no. C-31200-60)
• Male Luer lock rings to 200 series barb (Value Plastics, cat. no. MTLL230-1)
• Male Luer plugs (Value Plastics, cat. no. LP4-1)
• Dispensing needle tips (McMaster-Carr; 23 G, 0.5 inch long, type 304, inner diameter 0.017 inch, outer diameter 0.025 inch)
• Tygon tubing (VWR; 0.02 inch inner diameter, 0.06 inch outer diameter, 50 ft, 0.02 inch wall)
• Steel pins for chip-to-tube interface (New England Small Tube; 0.025 inch outer diameter × 0.017 inch inner diameter, 0.500 inch length, stainless steel tube, type 304, cut, deburred, passivated)
• Manual three-way valve: stopcock with two female Luer connections and male lock (Cole Parmer, cat. no. C-30600-02)
• Syringe (3 ml Luer-Lok tip; BD, cat. no. REF 309585)
• Teflon thread tape (1.3 cm × 7.6 m; VWR, cat. no. 60490-100)

Microscope optics
• High numerical aperture (NA) objective lens for high-resolution imaging and laser microsurgery. NA > 0.7 recommended. (For example, magnification: ×20, NA: 0.75, air lens; Nikon, cat. no. MRD00200)
• Fluorescence filter cube for use with the fluorescent reporter protein expressed by the organism. (For GFP, Nikon HQ:F, Nikon)
• Optical notch filter at the laser wavelength (Thorlabs, cat. no. FES0700)
• Standard square cage plate, SM1 threaded inner bore (Thorlabs, cat. no. CP02)
• Epi- and bright-field shutters (Sutter, cat. nos. LB-SC and IQ25-SA)

Reagent Setup
Experimental animals (C. elegans): The cellular process to be visualized should be labeled with a fluorescent dye or reporter protein for imaging. The animals can be cultured on and transferred from either agar plates or liquid [57]. For imaging mechanosensory neurons for femtosecond laser microsurgery, the SK4005 strain (which expresses GFP in the touch neurons) can be used. I Caution All animal experiments must comply with relevant institutional and national animal care guidelines.
M9 Buffer solution *C. elegans*: M9 buffer solution (as used in ref. 3) is composed of the following: 3 g of KH$_2$PO$_4$, 6 g of Na$_2$HPO$_4$, 5 g of NaCl, and 1 ml of 1 M MgSO$_4$; add H$_2$O to adjust to 1 liter. Sterilize by autoclaving.

**PROCEDURE**

Fabrication of molds • TIMING ~4 h plus baking overnight (time sensitive)

1| Dehydrate a silicon wafer by placing it on a hot plate at 135 °C for 10 min. Allow the wafer to cool for 2 min before proceeding to the next step (see Fig. 6.5a).

2| Center the wafer on the spinner chuck and apply vacuum. Spin wafer at 1,000 r.p.m. and place 2 ml of HMDS onto the spinning water using an eye-dropper bulb and continue spinning for 10 s.

3| Coat a 65-μm layer of SU-8 2075-negative photoresist onto the wafer (Fig. 6.5a). Center the wafer on the spinner chuck, and turn on the spinner-chuck vacuum to hold the wafer. Pour sufficient photoresist onto the wafer to cover a 5-cm circle. Turn on the spinner at 500 r.p.m. for 5 s, ramp the spin rate from 500 to 3,500 r.p.m. in 5 s and then spin for 30 s at 3,500 r.p.m.

4| Place aluminum foil on top of the hot plate. Bake the wafer for 3 min at 65 °C, followed by 8 min at 95 °C; this will make the photoresist more rigid for mask alignment and UV exposure.

5| Edge bead removal: Center the wafer on the spinner chuck and apply vacuum. Dip a cotton-tipped swab into PM acetate (developer) and apply gently but firmly to the edge of the wafer while spinning at 50 r.p.m. to remove the excess material from the edge of the wafer.

! **Caution** If the edges are raised because of the edge bead, the mask will not make proper contact with the photoresist, resulting in a misaligned pattern.

6| UV light exposure: Load the compress-1 mask (blue features in Fig. 4) into the mask aligner and place the wafer on the base of the alignment tool. Position the compress-1 mask on the wafer to pattern the negative photoresist. Ensure that the mask is flat against the photoresist. Remove the UV block. Expose to UV light for 40 s when using the recommended 200 mW cm$^{-2}$ source. Reinsert the UV block.
CRITICAL STEP: UV exposure time is dependent on source intensity. Adjust exposure time to accommodate differences in source intensity if not using the recommended source.

7| Bake the wafer on hot plates for 1.5 min at 65 °C, followed by 6.5 min at 95 °C. Allow the wafer to cool for 5 min.

8| Center the wafer on the spinner chuck and apply vacuum. Develop the negative photoresist by applying PM acetate onto the spinning wafer using an eye-dropper at 500 r.p.m.; follow this by rinsing the wafer with IPA at 1,000 r.p.m. and drying it with nitrogen air gun. Ensure that the pattern is fully developed.

9| Cover the wafer with a Petri dish or place in a wafer carrier to protect from dust.

10| Fabrication of mold-F for the flow layer containing two layers fabricated sequentially (Steps 10–25; see Fig. 6.5b–d):

Repeat Steps 1 and 2 to pre-treat a new wafer.

11| Coat a 10-μm-thick layer of SU-8 2025–negative photoresist onto the wafer. Center the wafer on the chuck and turn on the vacuum. Set the spinner to start at 500 r.p.m. Pour sufficient photoresist onto the wafer to cover a 5-cm circle. Turn on the spinner at 500 r.p.m. for 5 s, ramp from 500 to 4,000 r.p.m. in 5 s and then allow wafer to spin for 30 s at 4,000 r.p.m.

CRITICAL STEP: Remain close to the surface while pouring the photoresist to prevent bubbles from forming; bubbles may result in fabrication failure.

12| Bake the wafer on hot plates for 1 min at 65 °C, followed by 5 min at 95 °C, to make the photoresist more rigid for mask alignment during UV exposure.

13| Remove the edge bead as in Step 5.

14| UV exposure: Load the flow-1 mask (red features in Fig. 3) into the mask aligner and place the wafer on the base of the alignment tool. Position the flow-1 mask on the wafer to pattern the negative resist. Ensure that the mask is flat against the photoresist. Remove UV block. Expose to UV light for 40 s when using the recommended 200 mW cm⁻² source. Reinsert the UV block.
CRITICAL STEP: UV exposure time is dependent on source intensity. Adjust exposure time to accommodate differences in source intensity if not using the recommended source.

15| Bake the wafer on hot plates for 1 min at 65 °C, followed by 5 min at 95 °C.

16| Develop the negative photoresist with PM acetate as in Step 8.

17| Bake the wafer at 140 °C for 10 min.

CRITICAL STEP: Allow the wafer to cool for 5 min before proceeding to the next step. If the wafer is not cooled before proceeding to the next step, bubbles will form in the photoresist, causing fabrication failure.

18| Center the wafer on the spinner chuck and apply vacuum. Coat the first 50-μm-thick layer onto the wafer; center the wafer on the spinner chuck and set speed to 600 r.p.m. Pour sufficient positive photoresist (SIPR-7123) onto the wafer to cover a 5-cm circle. Spin at 600 r.p.m. for 100 s.

CRITICAL STEP: The flow-2 photoresist layer (comprising the regions of the flow layer where C. elegans is transported) is too thick to be coated in a single step. This is the first of two separate layers of 50 μm that will be spin coated to achieve the desired thickness of 100 μm.

19| Bake the wafer on the hot plates while ramping the temperature from 40°C to 100°C at a rate of 360 °C h⁻¹.

CRITICAL STEP: The temperature ramping is key to ensure thorough and even hardening of the thick layer.

20| When the temperature on the first hot plate reaches 100°C, transfer the wafer to the other hot plate at 140°C to bake for 10 min.

CRITICAL STEP: Allow the wafer to cool for 10 min before proceeding to the next step.

Caution If the wafer and photoresist are not cooled before proceeding to the next step, air bubbles will form because of heat in the newly poured photoresist, causing fabrication failure.
21 | Center the wafer on the spinner chuck and apply vacuum. Remove the edge bead with IPA while using the same method as in Step 5.

22 | By repeating Steps 18–21, coat an additional 50-μm-thick layer of photoresist onto the wafer to achieve a coating that is 100 μm in total thickness.

23 | UV light exposure: Load the flow-2 mask (green features in Fig. 4) into the mask aligner and place the wafer on the base of the alignment tool. Position the flow-2 mask on the wafer. Ensure that the photoresist pattern of flow-1 layer on the wafer is properly aligned with the flow-2 mask using fiducial crosses. Ensure that the mask is flat against the photoresist.

Remove the UV block. Expose to UV light for 4.5 min when using the recommended 200 mW cm$^{-2}$ source. Reinsert the UV block.

**CRITICAL STEP:** UV exposure time is dependent on source intensity. Adjust exposure time to accommodate differences in source intensity if not using the recommended source.

24 | Develop the positive photoresist: Place in a beaker with AZ440 developer for 30 min. Swirl the beaker gently to allow fresh developer to reach the photoresist.

**CRITICAL STEP:** If the photoresist is overdeveloped, features will not have the correct dimensions. This will result in immobilization failure.

25 | Reflow the flow-2 layer by ramping the temperature from 20°C to 150°C at a rate of 10 °C h$^{-1}$ using a hot plate. This process can take place overnight.

**CRITICAL STEP:** The reflow process rounds the edges of the photoresist (Fig. 6.5d) and results in proper roundness of the immobilization membrane, which is necessary for stable immobilization. **TROUBLESHOOTING**

**Fabrication of PDMS devices • TIMING ~2 h plus curing overnight (time sensitive)**

26 | Cleaning and pretreatment of mold-C and mold-F (Steps 26–27): Use a dry nitrogen gun to remove any dust particles from mold-C and mold-F (Fig. 5.6a). Place wafers in Petri dishes to protect them from dust particles when not in use. **TROUBLESHOOTING**
27| Place wafer in vacuum desiccators beside a folded aluminum foil reservoir with a 5-cm diameter and 1.5-cm raised edges. Place two drops of F13-TCS on the aluminum foil using a glass Pasteur pipette with a rubber bulb. Close the desiccator and turn on the vacuum for 3 min.

**CRITICAL STEP:** F13-TCS is used on the newly fabricated molds, which can then be used at least 20 times before this F13-TCS coating step is repeated.

**CAUTION:** F13-TCS is toxic and corrosive. This step should be carried out with the desiccator placed inside a chemical hood. Use a separate desiccator in later PDMS fabrication steps to avoid cross-contamination. Latex gloves should be worn when dispensing F13-TCS and should be discarded after use.

28| Molding of the PDMS layers (Steps 28–37; see Figs. 6.5e–h and 6.6b–m): Prepare 5:1 ratio PDMS by combining 50 g of RTV615A base with 10 g of RTV615B curing agent in a mixing cup labeled ‘C’ (‘mixing cup–C’); the label indicates that the PDMS is for constructing the compression layer.
Figure 6.6 Images of PDMS fabrication, hole punching, plasma bonding and interfacing with steel pins and tubing as detailed in Steps 26–60. The corresponding step is labeled in each image.

29| Prepare 20:1 ratio PDMS by combining 15 g of RTV615A base with 0.75 g of RTV615B curing agent in a mixing cup labeled ‘F’, indicating that the PDMS is for constructing the flow layer.
30| Mix the contents of mixing cup–C using the recommended mixer/degasser machine (Thinky, cat. no. ARE-250; option A) or by stirring manually as a more cost-effective method (option B).

(A) Using a mixer/degasser machine

(1) Weigh the mixing cup–C and holder. Place mixing cup–C in a mixer/degasser machine and set for 2 min of mixing, followed by 3 min of degassing at 2,000 r.p.m. (or 400g) at room temperature (20–25 °C). Set the weight counterbalance and start the mixer.

(B) Stirring manually

(1) Stir the contents of mixing cup–C using a plastic stirring stick for 3 min while scraping the sides of the container to ensure that all contents are integrated. Degas mixing cup–C for 60 min in a vacuum desiccator and remove any bubbles using a paper clip.

31| Repeat Step 30 to mix and degas mixing cup–F.

32| Wrap the bottom of mold–C with aluminum foil, sealing firmly around the edges of the wafer with the foil. Curl up the remaining foil, providing a 1-cm-high reservoir for pouring PDMS onto the wafer (Fig. 6.6b).

33| Pour the contents of mixing cup–C onto mold–C (Figs. 6.5e and 6.6b). Ensure that the mixing cup is close to the wafer surface to prevent unwanted bubbles from being trapped in the poured PDMS (Fig. 6.6b).

34| Place mold–C in the desiccator for 10 min under vacuum to remove air from the PDMS (Fig. 6.6c).

35| Coating of a 120-μm layer (Fig. 6.3f) of 20:1 ratio PDMS onto mold–F: Center mold–F on the vacuum chuck, turn on the spinner-chuck vacuum and set spin speed to 710 r.p.m. (Fig. 6.6d). Pour the contents of mixing cup–F onto the center of mold–F in a 5-cm circle (Fig. 6.6e). Spin for 60 s.

CRITICAL STEP: Avoid introducing bubbles by keeping the mixing cup close to the wafer surface while pouring.
36| Remove bubbles from the PDMS on mold-C. Carefully vent the desiccator and remove the lid. Use a paperclip to move the remaining bubbles on the surface of the PDMS to the edge of the wafer. Ensure that there are no bubbles on the wafer surface (Fig. 6.6f). TROUBLESHOOTING

37| Bake both mold-C (Fig. 5e) and mold-F (Fig. 5f) in an oven for 30 min at 80 °C to provide rigidity to the PDMS layers (Fig. 6.6g).

38| Thermal bonding of PDMS compression and flow layers (Steps 38–46; see Figs. 6.5e–h, 6.6h–n): Remove mold-C (Fig. 6.5e) and mold-F (Fig. 5f) from the 80 °C oven and allow both to cool for 10 min. Touch the PDMS surface of each wafer at the edge to verify that the surface is relatively sticky for adhesion and not completely rigid from overcuring to enable strong thermal bond (see Figs. 6.5e–h, 6.6h–n). TROUBLESHOOTING

39| Use a razor blade to trim the foil from the edge of mold-C (Fig. 6.6h) and peel the foil from the backside of the wafer (Fig. 6.6i). Carefully and slowly peel the PDMS layer from the wafer surface (Fig. 6.6j–k) and place it on a piece of clean foil with the molded side down.

**CRITICAL STEP:** Avoid touching the molded surface of the layer by handling only from the sides of the layer, as any residue from gloves can interfere with the thermal bonding in subsequent steps.

40| Use a razor blade to cut out the device components from the PDMS compression layer. Cut around the outermost box surrounding each device (Fig. 6.6i).

41| Place the device components cut out from the compression layer on a hole-punching mat, with the molded feature side of the PDMS facing up. Use clear adhesive tape to remove any debris that may be on the PDMS surface. Punch the access point (labeled 'A' in Fig. 6.3a) to the compression layer starting from the molded side using a 0.5-mm hole puncher. Ensure that the hole puncher is sharp and free of debris. Press firmly and punch through the PDMS until contact is made with the mat (Fig. 6.6m). Thereafter, lift up the device and depress the plunger to eject the PDMS. Repeat for each device.

TROUBLESHOOTING
42| Inspect each device component thoroughly under a stereomicroscope. Press the tape firmly on the molded side of the PDMS to remove any debris that can result in device failure. **TROUBLESHOOTING**

43| Clean the top surface of the PDMS layer on mold-F using clear adhesive tape. Ensure that the device is free of residues from accidental fingerprints.

44| Using a stereomicroscope, align the PDMS device components from the compression layer over the PDMS device components in the flow layer. Ensure that the molded side of the compression layer is aligned to the fiducial crosses (Fig. 6.3). Press firmly on top to remove any air bubbles trapped between the two layers (Figs. 6.5g and 6.6n).

45| Bake the combined compression and flow layers by placing them in an 80 °C oven overnight to achieve the thermal bond (Fig. 6.5g).

46| Gently peel the combined PDMS layers from the wafer surface (Fig. 6.5h). Begin by rolling the edge of the thin flow layer (Fig. 6.6o) to the base of the thick compression layer and lift both layers up together (Fig. 6.6p). Use a razor blade to trim the edges around the combined PDMS layers (Fig. 6.6q). If this is the first time that the molds are being used to fabricate a PDMS device, proceed to Step 47 (PDMS device characterization) to ensure that the molds are properly fabricated. If the mold fabrication has already been characterized, proceed to Step 49.

**CRITICAL STEP:** The thin PDMS layer may stretch or tear if it is not lifted at the same time with the thick layer.

**Characterization of PDMS device fabrication • TIMING ~20 min**

47| Cut a cross-section of the PDMS device vertically along port-B and port-C using a razor blade (as in the cross-section shown in Fig. 6.5h). Place the cross-section onto a microscope slide to measure the thickness of each layer (Fig. 6.7 and Table 6.1).

48| Record an image of the cross-section. Measure the dimensions of each layer as well as membrane thicknesses, using a standard microscope ruler (Fig. 6.7).

**Hole punching and plasma bonding of the device to cover glass • TIMING ~20 min plus baking overnight**
49| Punch two holes (labeled 'B' and 'C' in Fig. 3a) to create port-B and port-C (Fig. 6.3a) for off-chip access to the flow layer as in Step 41, but using a 0.75-mm hole puncher rather than the 0.5-mm hole puncher used in Step 41 (Fig. 6.6m).

50| Wrap a double-width glass slide (1 mm x 50 mm x 75 mm) with transparent adhesive tape to form a support base (Fig. 6.6q).

▷ CRITICAL STEP: This tape will also protect the surface of these glass slides from plasma treatment in Steps 53–55, and prevent the PDMS device or dish from bonding to them. Ensure that the glass surface is completely covered with tape.

51| Place lint-free fabrication wipes on the table to provide a padded surface (Fig. 6.6s).

▷ CRITICAL STEP: This padded surface will protect the delicate cover glass bottom of the dish during the bonding process in Step 56 and when inserting steel pins in Step 60.

52| Inspect the PDMS device and the dish (with cover glass bottom) for particles and use clear adhesive tape to remove debris.

53| Place the PDMS device and the dish (with cover glass bottom) onto the support base (created in Step 50) inside the plasma chamber, with the molded side of the flow layer facing up and the raised edge of the cover glass dish also facing up (Fig. 6.6q).

Figure 6.7 Cross-section of the microfluidic device showing the immobilization region. The device is vertically cut along the line from port-B to port-C. The thermally bonded compression layer and flow layer are visible. A PDMS channel array is molded from the flow-1 layer with 10 µm thickness. A 15- to 20-µm-thick PDMS membrane is formed between the compress-1 and flow-2 layers. This device will be plasma bonded to a cover glass. The fabrication tolerances are listed in Table 6.1. Scale bar, 50 µm.
CRITICAL STEP: The dish and PDMS device should not be in contact with each other inside the plasma chamber.

54| Turn on the vacuum for 2 min. Turn on the plasma source and slightly lower the vacuum in the plasma chamber until the purple glow in the chamber turns light pink in color (panel r of Fig. 6.6) and continue plasma exposure for 1 min.

55| Turn off the plasma source, followed by the vacuum pump. Gently vent the chamber to release the vacuum and avoid disturbing the items in the chamber. Remove the treated samples from the chamber using the support base without touching the treated surfaces that are to be bonded together.

56| Place the dish with the cover glass bottom (with the raised edge facing up) on top of the fabrication wipes (from Step 51) to provide cushioning. Immediately place the device with the molded side of the flow layer facing down and center it on the dish. Press firmly but gently on the device to remove air bubbles but avoid cracking the cover glass (Fig. 6.6s). Continue pressing for 20 s to ensure good contact between the PDMS channel array and cover glass.

57| Inspect the completed device (Fig. 6.5i) under a stereomicroscope to ensure that the device is bonded to the cover glass with no air bubbles trapped between the PDMS layers. Air bubbles may disrupt the fluidic channels or the channel array. 

TROUBLESHOOTING

58| Bake the device in an 80 °C oven for 24 h to complete thermal bonding.

Assembly and connection of off-chip components • TIMING ~20 min

59| Assemble the pressure gauge and regulator (panel b of Fig. 6.8). Use Teflon thread tape to ensure an air-tight seal around screw connections. Connect the tubing from the regulator to the air pressure supply (Fig. 6.8 and Table 6.2).
TABLE 6.1 | PDMS device fabrication tolerances

<table>
<thead>
<tr>
<th>Compression Layer thickness</th>
<th>Flow layer thickness</th>
<th>Immobilization membrane thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>~6 mm thickness</td>
<td>~120 µm</td>
<td>~15–20 µm</td>
</tr>
<tr>
<td>&gt;6 mm will not allow hole puncher to punch through the device</td>
<td>&lt; 120 µm will result in an extremely thin membrane structure that will likely tear apart or stretch during fabrication</td>
<td>&lt; 15 µm will cause fabrication failure</td>
</tr>
<tr>
<td>&lt; 6 mm will not provide the mechanical stability to support the steel pins inserted to interface with tygon tubing</td>
<td>&gt; 120 µm will result in a membrane that will be too thick for proper immobilization</td>
<td>&gt; 20 µm will require high pressures for immobilization that may injure the animals and/or delaminate the PDMS layers</td>
</tr>
</tbody>
</table>

Figure 6.8 Microfluidic device integrated with off-chip components. (A, B) Components for manual chip operation (A) include the syringe (attached to port-B for loading the animal or port-C for unloading the animal) and manual valve-A (attached to port-A for pressurizing the compression layer). The large plastic tubing is labeled ‘8’ in both a and b, showing the connection between panels. (B) Construction of the air pressure regulator and gauge using connectors and Teflon thread tape. The ‘flow arrow’ indicates the direction of the flow from the pressure source to the chip. (C) Placement of an animal in the M9 buffer of the dispensing needle tip of port-A using an eyelash glued to a toothpick. The numerically labeled components are listed in Table 6.2.
Table 6.2 | List of components for the microfluidic system.

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Syringe</td>
</tr>
<tr>
<td>2</td>
<td>Dispensing needle tip, 23 G</td>
</tr>
<tr>
<td>3</td>
<td>Tygon tubing</td>
</tr>
<tr>
<td>4</td>
<td>Steel pin</td>
</tr>
<tr>
<td>5</td>
<td>Microfluidic device</td>
</tr>
<tr>
<td>6</td>
<td>Manual three-way valve: stopcock with Luer connections</td>
</tr>
<tr>
<td>7</td>
<td>Male Luer lock to barb connector</td>
</tr>
<tr>
<td>8</td>
<td>Large flexible plastic tubing</td>
</tr>
<tr>
<td>9</td>
<td>Screw to female Luer lock connector</td>
</tr>
<tr>
<td>10</td>
<td>Teflon thread tape</td>
</tr>
<tr>
<td>11</td>
<td>Right-angle threaded tee</td>
</tr>
<tr>
<td>12</td>
<td>Pressure gauge</td>
</tr>
<tr>
<td>13</td>
<td>Pressure regulator</td>
</tr>
<tr>
<td>14</td>
<td>Pressure source</td>
</tr>
<tr>
<td>15</td>
<td>Toothpick with glued eyelash</td>
</tr>
</tbody>
</table>

60] Insert a dispensing needle tip into a 15 cm piece of Tygon tubing and insert a steel pin into the opposite end of the Tygon. Gently insert the steel pin into the PDMS hole to access port-A (Figs. 6.6t and 6.8a). Avoid tearing the PDMS to prevent creation of unwanted particles. Repeat for both port-B and port-C.

CRITICAL STEP: The dish should be placed on a flat surface padded with lint-free fabrication wipes when inserting the steel pins to avoid cracking the delicate cover glass.

61] Connect one of the three-way manual valve-A ports to the pressurized air from the regulator and gauge (Fig. 6.8a). Leave one of the ports open to atmospheric pressure. Connect one of the ports to the dispensing needle tip (which should be connected to
port-A by means of Tygon tubing). Ensure that the arrow on the regulator is pointing toward the manual valve-A, directing flow into the chip.

**Manual operation • TIMING ~20 min for initial priming; thereafter, ~1 min per animal**

62 | Inspect the device and prime to remove air bubbles before operation (Steps 62–63): Pressurize port-A (Fig. 6.9) to inspect the immobilization layer and channel array for defects (Fig. 6.10). Disconnect the tubing and steel pin from device port-A. Completely fill the 15-cm-long inlet tube with deionized water (using a syringe) and reconnect the tube to the device port-A. Set the pressure regulator to ~3 p.s.i. Turn on the manual valve-A connected to port-A to pressurize the layer with low pressure. Slowly increase the pressure regulator to ~15 p.s.i. and wait 10–15 min for air bubbles to permeate into the surrounding PDMS to fill the compression layer with water. Turn valve-A such that the input to port-A is set to atmospheric pressure. \(? TROUBLESHOOTING\)

63 | Aspirate M9 buffer solution into the syringe. Fill the flow layer using low pressure from the M9-filled syringe attached to port-B and gently remove any trapped bubbles.

64 | Use a worm pick to transfer a single animal from either liquid or an agar plate into a small volume of M9 in the dispensing needle tip connected to port-B (Fig. 6.8c). Hold the M9-filled syringe with the tip facing downward and connect it to the dispensing needle tip of port-B (Fig. 6.8a). Gently flick the syringe to remove any air bubbles that may be trapped.

65 | Pressurize the syringe connected to port-B to gently flow liquid through the channel array in order to position the animal in a linear orientation along the channel array (Fig. 6.9a). \(? TROUBLESHOOTING\)

66 | Pressurize the compression layer (Fig. 5j) by turning manual valve-A to the pressurized source (Figs. 6.8b and 6.9b). Optimize the immobilization pressure by adjusting the pressure regulator to account for differences in animal size and/or fabrication parameters. \(? TROUBLESHOOTING\)
Figure 6.9 Manual operation of the microfluidic device. The table indicates the state of each port during operation. (A) Loading of the animal from syringe as in Step 65. (B) Immobilization of the animal in linear orientation as in Step 66. (C) Unloading of the animal to the agar plate as in Step 68.
Figure 6.10 Comparison of failed and successful fabrication of channel array. (A) The immobilization membrane is too thin and has collapsed or sagged (denoted by ‘*’). The membrane has bonded either to the cover glass below during plasma bonding or to the compression layer above during thermal bonding. The boxed region shows the pillars of the channel array that did not plasma bond properly to the glass. Scale bar, 100 μm. (B) Magnified image of the boxed area in a that shows the region (between the dashed white lines) in which the pillars did not properly bond to the cover glass. Scale bar, 25 μm. (C) Proper bonding while the immobilization membrane is not pressurized. Scale bar, 25 μm. (D) Proper bonding when pressurized. Scale bar, 25 μm.

67) Place the chip on the inverted microscope. Perform the desired imaging and optical manipulations such as fluorescence, confocal and two-photon imaging (Fig. 6.1b), laser microsurgery, calcium imaging or photostimulation.

68) Remove the dispensing needle tip of port-B from the Tygon tubing and direct the end of the tubing onto an agar pad or reservoir to dispense the animal. Turn valve-A to atmospheric pressure to release the pressure in the compression layer. Attach the syringe to the dispensing needle tip of port-C. Gently pressurize the syringe in port-C to flow the animal out of the device through port-B. Replace the dispensing needle tip to the Tygon tubing in port-A (Fig. 6.9c).

69) Repeat Steps 65–68 to immobilize additional animals (Fig. 6.9). Remove debris (Fig. 6.11) by gently increasing the flow through the channel array or use the bleaching procedure (Table 6.3).
To store the device, flush the microfluidic channels with deionized water and place male Luer caps on each of the three dispensing needle tips. Cover the device with a Petri dish to avoid dust particles.

Figure 6.11 Device contamination by debris and PDMS particulates. (A) Micrograph of an animal immobilized against the channel array. The buildup of debris, which occurs after thousands of cycles, is indicated by the arrow within the channel array. Such contamination can be removed by following the bleaching procedure described in Table 6.3. Scale bar, 15 μm. (B) Large PDMS debris caused during PDMS hole punching. Scale bar, 100 μm.
Table 6.3 Bleaching procedure.

<table>
<thead>
<tr>
<th>Step</th>
<th>Bleaching procedure for removal of biological material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Remove all the pins and tubing from the microfluidic device</td>
</tr>
<tr>
<td>2</td>
<td>Prepare a 2% (vol/vol) bleach solution in deionized water (1 ml bleach:50 ml deionized water)</td>
</tr>
<tr>
<td>3</td>
<td>Use a syringe to gently inject the 2% bleach solution into each port-B and port-C of the device, thoroughly filling the entire device. Deliver gentle pulses to dislodge debris. Avoid high pressures to prevent damage to the device</td>
</tr>
<tr>
<td>4</td>
<td>Soak the device for 4–12 h to break down the biological material. Soak time is dependent on the level of contamination</td>
</tr>
<tr>
<td>5</td>
<td>Use the syringe to gently inject additional 2% bleach solution. Gently force the loosened debris out of the chip without delaminating thermally bonded layers</td>
</tr>
<tr>
<td>6</td>
<td>Use a separate syringe filled with deionized water to thoroughly rinse the device to remove all of the bleaching solution</td>
</tr>
<tr>
<td>7</td>
<td>Flush the chip with M9 buffer solution and reconnect the ports of the chip</td>
</tr>
</tbody>
</table>

Bleaching procedure is adapted from Lee et al. [70].

**TROUBLESHOOTING**

Troubleshooting advice can be found in Table 6.4.
### TABLE 6.4 Troubleshooting table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>The photore sist on wafer is not rounded after refl ow</td>
<td>Ramping of temperature is not uniform</td>
<td>Ensure that the cover on hot plate allows adequate heat transfer. Try a new hot plate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Photoresist has expired or has been overexposed to air/light</td>
<td>Use new photoresist</td>
</tr>
<tr>
<td>26</td>
<td>Particles on wafer cannot be removed with nitrogen gun</td>
<td>PDMS and dust particles adhere to the photore sist on wafer surface</td>
<td>Do not use tape to avoid damaging photore sist structures. Pour a layer of PDMS over the wafer, bake for 1 h and peel off to remove dust particles, as described in Steps 28-39 for mold-C</td>
</tr>
<tr>
<td>36</td>
<td>Bubbles trapped in wet PDMS near the wafer surface</td>
<td>Air is trapped while pouring PDMS</td>
<td>Use the rounded edge of a paper clip to drag the bubble to dislodge it. Do not touch wafer surface or photore sist structures</td>
</tr>
<tr>
<td>38</td>
<td>Surface of PDMS layers is too rigid and not sticky enough for bonding</td>
<td>Surface is thermally undercured</td>
<td>Ensure that the oven temperature and bake time are correct. Otherwise, reduce the bake time by 5 min in Step 37 or try new oven</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDMS is thermally overcured</td>
<td>After checking temperature as suggested above, increase the bake time by 5 min in Step 37</td>
</tr>
<tr>
<td>41</td>
<td>Failure in punching holes into PDMS</td>
<td>Particles are generated by tearing of PDMS because of dullness of punching tip</td>
<td>Replace with new hole puncher</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDMS is too rigid</td>
<td>Check PDMS curing procedure and base/curing agent ratios</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metal tip retracts in plastic shaft</td>
<td>Replace with a new hole puncher</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDMS layer is too thick to completely punch through</td>
<td>Decrease amount of PDMS used to reduce thickness</td>
</tr>
<tr>
<td>42</td>
<td>PDMS particulates cause chip failure</td>
<td>Particulates during fabrication or hole punching block sections of the device (see Fig. 11b)</td>
<td>Wipe down bench surfaces and use fresh aluminum foil after each sequence of fabrication step. Use additional tape to clean PDMS surfaces</td>
</tr>
<tr>
<td>57</td>
<td>Delamination of PDMS thermal bond</td>
<td>Overcuring of PDMS layers before thermal bonding reduces adhesion properties</td>
<td>Check oven temperature. Reduce the initial curing time in Steps 37-25 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residue from gloves or external source</td>
<td>Avoid touching PDMS surfaces that are to be bonded together. Ensure that surface of benches are clean. Change gloves after handling wet PDMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Channel array is distorted (Fig. 10)</td>
<td>Check oven temperature and curing time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immobilization membrane is collapsed/stacked (Fig. 10)</td>
<td>See TROUBLESHOOTING Step 26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The PDMS flow layer is not properly cured before peeling from mold-F</td>
<td>Use a syringe attached at port-A to slowly and gently pressurize the channel to separate the membrane. Be careful to avoid stretching or tearing the membrane to prevent device failure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wafer surface is not free of debris before spinning PDMS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane has bonded to the PDMS compression layer</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 6.4 Troubleshooting table. (continued)

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>Failure in filling of compression layer from port-A with water</td>
<td>Membrane is bonded to the cover glass</td>
<td>Plug port-C with male Luer cap and gently pressurize port-B to slowly lift the membrane; additionally, a second syringe can be attached to port-A to apply a vacuum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane is too thin or has torn or stretched, preventing operation</td>
<td>PDMS flow layer may be too thin or photoresist on mold-F may be too tall. Refer to Figure 5 for device characterization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surfaces are not clean</td>
<td>Clean the PDMS surface with tape and avoid touching the surfaces</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cover glass is cracked</td>
<td>Increase the amount of fab wipes for pad layering on the table in Step 51 and use less force when pressing on the device in Step 56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasma chamber is not clean</td>
<td>Air filters on the vacuum line may not be working properly. Check filters and have plasma chamber cleaned</td>
</tr>
<tr>
<td>65</td>
<td>Sufficient flow cannot be achieved</td>
<td>Water leak at the steel pin connector to port-A of the chip</td>
<td>The punched hole may be torn or not seating properly. Reseal around the pin and tubing by applying wet 20:1 PDMS and by baking for 1 h. Leave the pin and tubing in place to prevent seating of the hole</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blockage of external fluidic lines due to possible pinched tubing or dried M9 buffer. Blockage is often hidden in steel connector tubes</td>
<td>Disconnect each line separately and check for continuity from pressure source. Rush lines with fresh M9/deionized water using syringe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blockage inside the chip or in the channel array (Fig. 11)</td>
<td>Use a syringe filled with M9 to apply gentle pulses to dislodge the debris. Do not apply high pressures to avoid damaging the device. Bleach the chip using the procedure in Table 3</td>
</tr>
<tr>
<td>66</td>
<td>Cannot achieve sufficient immobilization</td>
<td>Debris is blocking compression layer from compressing uniformly</td>
<td>See TROUBLESHOOTING Step 65 to remove debris</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 p.s.i. is insufficient to immobilize the animal</td>
<td>Slowly increase pressure to 20 p.s.i.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 p.s.i. is insufficient to immobilize the animal</td>
<td>The tubing is blocked; see TROUBLESHOOTING Step 65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The fabrication is not within the tolerance in Figure 7 and Table 1</td>
<td></td>
</tr>
</tbody>
</table>

**TIMING**

Steps 1–25, Fabrication of molds: ~4 h plus baking overnight (time sensitive)

Steps 26–46, Fabrication of PDMS devices: ~2 h plus curing overnight (time sensitive)
Steps 47 and 48, Characterization of PDMS devices: ~20 min

Steps 49–58, Hole punching and plasma bonding: ~20 min plus baking overnight

Steps 59–61, Assembly and connection of off-chip components: ~20 min

Steps 62–70, Manual operation: ~20 min of initial priming and optimization; thereafter, ~1 min per animal

ANTICIPATED RESULTS

This protocol results in a fully functional microfluidic system capable of studying dynamic cellular processes at subcellular resolution in physiologically active *C. elegans*. The immobilization stability achievable is comparable to that induced by strong anesthetics such as NaN₃ (Fig. 6.12) and is sufficient for tasks such as two-photon imaging and femtosecond laser microsurgery, as demonstrated in Supplementary Video 1 (online). Once fabricated, the device can be operated by a single syringe and one manual valve.

To assess the viability of the immobilized animals, their health was measured by conducting a standard lifespan assay [24]. Animals were loaded into the chip and immobilized by the microfluidic membrane for 1 min at 15 p.s.i., and then dispensed onto an agar pad. Microfluidic immobilization did not affect the lifespan of animals with respect to control animals that remained on agar pads throughout the experiment (Fig. 6.13). The immobilized population (12 animals) had a mean lifespan of 17.3 d (s.d., 5.0 d) and the control population (9 animals) had a mean lifespan of 16.9 d (s.d., 4.0 d). Both populations were monitored each day to check for dead animals (i.e., those that did not respond to prodding with platinum pick). Surviving animals were transferred to a fresh plate to be monitored the next day.
Figure 6.12 Assessment of immobilization stability. (A) Time-lapse image of an immobilized animal showing subcellular features. Three images of the anterior ventral mechanosensory neuron and its axon taken at 5-s intervals, colored with red (earliest), blue and green (latest) and overlaid on top of each other to assess any movement. The overlap of all three images results in white regions. The inset shows a magnification of the outlined region. Scale bar, 20 μm. (B,C) Cell body position and displacement. The movement of cell bodies using the microfluidic immobilization technique (12 animals) is compared with the 10 mM NaN₃ anesthetic technique (9 animals). The movement of cell bodies was tracked using a computer algorithm (see Supplementary Methods of Zeng et al. [24]). The frames were acquired at 20–30 Hz and ×50 magnification. (B) The average displacement of cell bodies divided by the time between frames is shown in a histogram. (C) Drift from origin as a function of time is shown to compare the stability of microfluidic immobilization with respect to 10 mM NaN₃.
Figure 6.13 Lifespan analysis of the immobilized population and control population. Animals were brought into the chip and immobilized for 1 min each using 15 p.s.i. and then recovered on agar pads. The mean lifespan of the immobilized population (17.3 d) was consistent with that of controls (16.9 d).
Mehmet Fatih Yanik developed the femtosecond laser technique to sever single axons in *C. elegans* during his graduate studies and we developed new platform technologies to enable this technique to be employed on large scales for nerve regeneration in *C. elegans*. In combination with the microfluidic technologies we performed the first large scale demonstration of nerve regeneration with single axon precision which revealed a new class of nerve regenerating drugs. During this time we developed extensive troubleshooting skills and formulated this protocol to share them with the research community. The troubleshooting techniques were developed by Cody Gilleland, Joseph Steinmeyer, and Christopher Rohde. The beam expander structure was developed by Mark Scott and Joseph Steinmeyer. Mark Scott developed the laser alignment technique. Cody Gilleland, Joseph Steinmeyer, and Carlos Pardo wrote the manuscript. Mehmet Fatih Yanik supervised the entire process. Cody Gilleland also performed laser surgery on the zebrafish screening platform that was published in Nature Methods [Pardo-Martin et al. (2010) Nature Methods].

This co-first author work has been published in Nature Protocols:


Femtosecond laser microsurgery is a powerful method for studying cellular function, neural circuits, neuronal injury and neuronal regeneration because of its capability to selectively ablate sub-micron targets in vitro and *in vivo* with minimal damage to the surrounding tissue. Here, we present a step-by-step protocol for constructing a femtosecond laser microsurgery setup for use with a widely available compound fluorescence microscope. The protocol begins with the assembly and alignment of beam-conditioning optics at the output of a femtosecond laser. Then a dichroic mount is assembled and installed to direct the laser beam into the objective lens of a standard inverted microscope. Finally, the laser is focused on the image plane of the microscope to allow simultaneous surgery and fluorescence imaging. We illustrate the use of this setup by pre-
senting axotomy in *Caenorhabditis elegans* as an example. This protocol can be completed in 2 d.

Introduction

The non-linear absorption that results from focusing a train of near-infrared (NIR) femtosecond laser pulses through a transparent specimen enables the specific ablation of submicron-scale features with minimal collateral damage [71]. This technique has been used to study the function of microtubules [72], mitochondria [73] and other organelles [74] in cultured cells, tissues and whole organisms, as well as for optotransfection [75] and laser microdissection [76,77]. In addition, femtosecond laser microsurgery has been used to investigate the effects of neural injury and other biological processes in model organisms such as *Caenorhabditis elegans* [10,11,13-15,78-81], *Drosophila melanogaster* [82] and rodents [83]. We previously used this technique to make the first observation of regeneration in *Caenorhabditis elegans* by cutting neurites of motor neurons *in vivo* using 100 pulses of NIR light at a repetition rate of 1 kHz with low energies (10–40 nJ per pulse) and ultra-short pulse durations (200 fs) [10]. Full neurite regrowth and recovery of locomotive response upon touch stimulus was observable within 24 h.

We had also previously demonstrated the first microfluidic system to capture and mechanically immobilize whole organisms (*C. elegans*) for the purpose of sub-cellular resolution femtosecond laser microsurgery [17,24], two-photon imaging [24] and high-throughput screening [18,20,23]. UV lasers [84] and pulled glass pipettes [85] have also been used for microsurgery in cultured cells and tissues; however, the inaccuracy of these techniques and the damage done to surrounding tissue limits their use *in vivo*. Although femtosecond laser systems are complex pieces of equipment, their costs have come down significantly in recent years as the technology has matured.

In this protocol, we detail a procedure to add a femtosecond laser microsurgery capability to a standard fluorescence microscope using commercially available components. The femtosecond laser microsurgery system (Fig. 7.1 and corresponding component descriptions in Table 7.1) is designed to take up minimal space while allowing access to facilitate easy and reproducible alignment. The design provides flexibility in the choice of objectives, filter cubes, laser wavelengths and laser–pulse repetition rates while requiring no modification of the fluorescence microscope. The protocol begins with the installation of major components including the optical table, the microscope and the laser. Next, beam-conditioning optics (optical isolator, electro–optic modulator (EOM),
Glan–Thompson polarizer and half-wave plate) are assembled and mounted. The construction and installation of a dichroic mounting adapter, an assembly that brings the laser beam in line with the optical axis of the objective, is then described. The protocol then details the installation of a periscope to raise the beam from the table to the microscope input port and a laser beam expander to resize the beam to fill the back aperture of the objective. The protocol concludes with step-by-step instructions for axotomy in C. elegans, which is included as an example application of the system. Adapting the system for other microsurgical applications, such as those mentioned above, involves modifications only to the standard sample preparation steps2–7 and use of different objective lenses\[71\]. For example, although an air objective lens with a numerical aperture (NA) of 0.75 is sufficient for axonal ablation in C. elegans, for axotomy in zebrafish larvae, researchers have used water-immersion objective lenses with 0.8 or higher NAs\[26\]. For ablation of cytoskeletal filaments of cells in vivo, oil-immersion objective lenses with higher NAs of 1.4 are used\[77\]. In addition, different laser–pulse repetition rates have been used in the literature\[71, 77, 13\], where the lower pulse repetition rates yield reduced heat accumulation in specimen\[1\], and therefore less collateral damage. The high speed EOM and the high pulse-repetition rate laser used in this protocol provide the ability to choose any pulse repetition rate ranging from sub-KHz to 80 MHz, thus providing sufficient flexibility.

The system described in this protocol does not include a two-photon imaging capability. However, this capability can be easily added by replacing the top periscope mirror with scanning mirrors and by adding a photomultiplier tube to one of the camera ports of the microscope\[87\]. To adapt the laser system for simultaneous use by multiple microscopes simply requires the addition of a beam splitter, EOM, periscope, beam expander and dichroic mounting adapter for each additional microscope. If necessary, a regenerative amplifier or a higher-power laser can be used to ensure that sufficient power is delivered to each setup.

**MATERIALS**

**REAGENTS**

- Experimental animals. Although transgenic strains of cells/animals expressing fluorescent reporter proteins are not required for successful surgery, they do ease in the identification and targeting procedures. (C. elegans; the zdIs5 strain
can be used for GFP-expressing mechanosensory neurons.) Caution All animal experiments must comply with the relevant institutional and national animal care guidelines.

- Cover glass (0.175 mm x 25 mm x 50 mm)
- Microscope cover slides (1 mm x 25 mm x 50 mm)
- Agarose gel 1.5%
- Sodium azide 10 mM

EQUIPMENT

General equipment

- Femtosecond laser (Spectra Physics Mai Tai HP Ti:Sapphire, tunable wavelength Class IV laser). More cost-effective femtosecond lasers with a fixed wavelength can also be used including: Polarynox femtosecond fiber lasers, Del Mar Photonics Ti:Sapphire custom made kit, model TISSA100 and HighQLaser FemtoTrain, model IC-1045-3000. Minimum recommended pulse energy is ~20 nJ (i.e., average power 1.6 W for a laser with 80 MHz pulse repetition rate)
- Laser warning sign and safety equipment which meet requirements set by institutional as well as local and national safety standards. Laser goggles with minimal safety rating of OD7+ are recommended (Diode/Nd:YAG 42F goggle, Kentek, cat. no. KGG-42F)
- Optical table with sufficient surface area (1) (3.0 m x 1.5 m) and set of four air-damped legs (Newport, cat. nos. RS-4000-510-12 and I-2000-413.5, respectively)
- Inverted fluorescence microscope system (1) (Nikon Eclipse Ti or similar model, Nikon) including a 70-mm stage-up kit to provide sufficient clearance for the rear-entry of the laser beam and a stage plate capable of holding a standard glass slide
- Image acquisition software (1) (NIS-Elements 2.0) and compatible computer.
- High-resolution CCD camera (1) (Photometrics Coolsnap HQ2)
- RMS-threaded IR and VIS alignment disk (400–640 nm and 800–1700 nm) (Thorlabs, cat. no. RMSIR)
- RMS 45 to CFI 60 objective adapter (1) (Nikon, cat. no. MXA20750)
- IR card (1) (Newport, model no. F-IRC4)
- Infrared viewer (1) (Newport, cat. no. IRV1-2000)
• Optical power meter and detector (1) (Newport, part nos. 1918-C and 818P-010-12, respectively)
• Ø1/2” Post (2”) (1) (Thorlabs, cat. no. TR2)
• Ø1/2” Post holder (2”) (1) (Thorlabs, cat. no. PH2-ST)
• Standard base (1) (Thorlabs, cat. no. BA2)
• Function generator with single-pulse generation capability (1) (BK Precision, model no. 4030) CRITICAL STEP If another model or device is used, ensure that the function generator, or any source in general driving the high-voltage (HV) amplifier, is capable of driving the input load of the high voltage amplifier. For example, if the input impedance of the HV amplifier is 50 Ω and the maximum input voltage is 1 V, the function generator must be able to supply at least 20 mA to have access to the full input range.
• Oscilloscope (Tektronix, TDS2024)
• 3/8” corded drill driver (1) (RiDGID, model# R70002)
• Drill set (Ryobi, model# AR2074)
• 1/4”-20 cap and set screws and #8-32 cap and screws (ThorLabs, cat. nos. HW-KIT2 and HW-KIT3, respectively)

Laser setup

• Optical isolator (1) (Conoptics, cat. no. 712TGG)
• Half-wave plate for 600–1050 nm high power applications (1) (Thorlabs, cat. no. AHWP05M-950)
• High-precision rotation mount for Ø1” Optics (1) (Thorlabs, cat. no. PRM1)
• Glan–Thompson polarizer with 600–1050 nm anti-reflection coating (1) (Thorlabs, cat. no. GL10-B)
• Polarizing prism mount (1) (Thorlabs, cat. no. SM1PM10)
• Kinematic mount for thin Ø1” Optics (1) (Thorlabs, cat. no. KM100T)
• EOM (1) and requisite HV amplifier (Conoptics, cat. no. 350-160 EOM with amplifier) ! CAUTION The cables connecting EOM to the HV amplifier carry high voltage. Only the appropriately rated cables from the EOM manufacturer should be used. (Optional, see Steps 10–12) A high-speed mechanical shutter capable of providing a 2.2 ms pulse can be substituted for the EOM; however, the EOM enables the added benefit of two-photon excitation targeting (see Step 93).
- Optical isolator mount (1) (Conoptics, model M102, modified to hold the isolator)
- EOM mount-labjack (1) (Conoptics, model M102) (optional, see Steps 10–12)
- Safety shutter (1) (Electro-Optical Products SH-10 Interlock safety shutter with silver coated mirror, for NIR and DSH-10-110 V controller)
- Beam block (includes TR3 post) (7) (Thorlabs, cat. no. LB1)
- 2” High universal post holder (for use with beam blocks) (7) (Thorlabs, cat. no. UPH2)
- Ø1/2” post (2”) (3) (Thorlabs, cat. no. TR2)
- Ø1/2” post holder (2”) (3) (Thorlabs, cat. no. PH2-ST)
- Standard base (3) (Thorlabs, cat. no. BA2)
- 50-Ω BNC cables of sufficient length (2) to connect the function generator to the oscilloscope and to the HV amplifier (or high-speed shutter controller if that option is used). T-joint BNC-cable connector (http://Cablesnmore.com, cat. nos. N23713 and X15305, respectively)

Dichroic mounting adapter
- IR dichroic mirror (1) (Chroma, cat. no. 670dcspxr)
- Nikon adapter plate (‘dust cover’) (70 mm stage-up kit, Nikon)
- Ø1” (1”) pedestal pillar post (1) (Thorlabs, cat. no. RS1P)
- Compact kinematic mount (1) (Thorlabs, cat. no KMS)
- BA1 Standard base (1) (Thorlabs, cat. no. BA1)
- Dichroic cube holder (1) (large aluminum filter cube, Nikon)
- High-performance epoxy (1) (Loctite Fixmaster, Loctite, part no. L08FA12920)
- #8–32 screw × 1/4’ with low-profile head (1) (McMaster–Carr, cat. no. 91770A190)

Periscope and beam expander
- 40 mm focal length, Ø1”, NIR achromatic doublet lens (1) (Thorlabs, cat. no. AC254-040) (dependent on back aperture of objective lens; see Step 43)
- 200 mm focal length, Ø2”, NIR achromatic doublet lens (1) (Thorlabs, cat. no. AC508-200) (dependent on back aperture of objective lens; see Step 43)
- Single-axis position stage with micrometer (1) (Newport, cat. no. 460P-X)
• Ø1.5" post clamp adapter plate (1) (Thorlabs, cat. no. C1520)
• 14" tall Ø1.5" mounting post (3) (Thorlabs, cat. no. P14)
• Ø1.5" post mounting clamp (4) (Thorlabs, cat. no. C1501)
• Right-angle kinematic cage mount (2) (Thorlabs, cat. no. KCB1)
• Ø1" gold mirror (3) (Thorlabs, cat. no. PF10-03-M01)
• 12" optical rail (1) (Thorlabs, cat. no. RLA1200)
• Ø2" lens mount (1) (Thorlabs, cat. no. LMR2)
• Ø1" optics, translating lens mount (1) (Thorlabs, cat. no. LM1XY)
• Ø1" iris (4) (Thorlabs, cat. no. ID25)
• Rail carrier (4) (Thorlabs, cat. no. RC1)
• Ø1/2" post (2") (7) (Thorlabs, cat. no. TR2)
• Ø1/2" post holder (2") (6) (Thorlabs, cat. no. PH2-ST)
• Standard base (5) (Thorlabs, cat. no. BA2)
• Ultra-stable kinematic Ø1" mirror mount (1) (Thorlabs, cat. no KS1)

Microscope optics

• High-NA objective lens for laser surgery (1). NA greater than 0.7 recommended. Examples: Mag: 20x, NA: 0.75, air lens (Nikon, cat. no. MRD00200). For work in C. elegans and Danio rerio axotomy, a 20x, NA: 0.75 objective provides a suitable balance between field of view and NA for rapid and reliable axotomy. If a different objective is used, the focal lengths of lenses L1 and L2 may need to be altered as discussed in Step 43 of the protocol below.
• Fluorescence filter cube for use with the fluorescent reporter protein expressed by the organism (1) (For GFP, Nikon HQ:F, Nikon)
• Optical notch filter at the laser wavelength (1) (Thorlabs, cat. no. FES0700)
• Standard square cage plate, SM1 threaded inner bore (1) (Thorlabs, cat. no. CP02)
• Epi and bright-field shutters (1) (Sutter, cat. no. LB-SC and IQ25-SA)
Figure 7.1 Optical system layout. See Table 7.1 for component list.
Table 7.1 | Component list for the optical system layout of Figure 1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Femtosecond laser</td>
</tr>
<tr>
<td>B</td>
<td>Optical isolator</td>
</tr>
<tr>
<td>C</td>
<td>Half-wave plate</td>
</tr>
<tr>
<td>D</td>
<td>Glan–Thompson polarizer</td>
</tr>
<tr>
<td>E</td>
<td>Electro–optic modulator (optional)</td>
</tr>
<tr>
<td>F</td>
<td>Safety shutter and beam block</td>
</tr>
<tr>
<td>G</td>
<td>Kinematically mounted mirror</td>
</tr>
<tr>
<td>H</td>
<td>Ø1” Iris</td>
</tr>
<tr>
<td>I</td>
<td>Periscope</td>
</tr>
<tr>
<td>J</td>
<td>Ø1” Lens L1 on z-adjustable stage</td>
</tr>
<tr>
<td>K</td>
<td>Two Ø1” irises</td>
</tr>
<tr>
<td>L</td>
<td>Ø2” Lens L2</td>
</tr>
<tr>
<td>M</td>
<td>Beam expander</td>
</tr>
<tr>
<td>N</td>
<td>Dichroic mounting adapter</td>
</tr>
<tr>
<td>O</td>
<td>20× 0.75 Numerical aperture (NA) objective</td>
</tr>
<tr>
<td>P</td>
<td>Epifluorescence filter turret</td>
</tr>
<tr>
<td>Q</td>
<td>Lower half of microscope body</td>
</tr>
<tr>
<td>R</td>
<td>Camera</td>
</tr>
</tbody>
</table>
PROCEDURE

Laser safety section

Caution Before initiating the protocol, ensure that the appropriate ‘Laser in Use’ safety signs are installed at the proper locations. In addition, the laser warning system as well as the laser system itself must comply with the relevant institutional, local and national laser safety guidelines. It is important to observe good laser safety practice, including not wearing reflective items such as jewelry and wristwatches when working near the laser. Do not look through the microscope eyepieces when the laser is on and when either the internal laser shutter or the safety shutter is open. Use beam blocks to safely terminate the laser beam while aligning. The laser should be shuttered while inserting components into the beam path. When installing a component into the beam path, use the IR card and/or IR viewer to visualize the location of the beam. Close either the internal laser shutter or the safety shutter (depending on the component location) and then place the component in the path. Following this, open the shutter and view the location of the beam in relation to the component being aligned. Close the necessary shutter and make more adjustments. If necessary, repeat this on/view-off/adjust cycle multiple times to obtain satisfactory alignment.

CRITICAL STEP: These steps are time independent and may be stopped and started when necessary; however, it is imperative that the laser is either properly shuttered or turned off when not in use.

Setting up the optical table, laser, power meter, microscope and optical isolator • TIMING 6 h

1 | Install the optical table (this should be done by the manufacturer).

2 | Install the femtosecond laser system such that the beam is aligned down the center of the optical table (this should be done by the manufacturer).

3 | Install the microscope body and supporting equipment at least 1.5 m from the output of the laser to allow sufficient room for the beam-conditioning optics (installation of the microscope should be done by the manufacturer).

4 | Attach the optical power detector to the power meter. Mount the detector on one of the Ø1/2″ posts and insert the post into a Ø1/2″ post holder mounted on a standard
base (BA2) for easy insertion and positioning of the detector in the beam path. Turn on the attenuator in front of the optical power detector to avoid damage (see manufacturer’s manual for details). The optical power meter should be brought to zero to calibrate it for ambient radiation.

**Caution** It is critical both for user and equipment safety that the power meter is accurately calibrated.

5. Turn on the laser, leaving the internal shutter closed and allow it to warm up. Set the laser wavelength to 800 nm.

6. Set the output power of the laser to a low yet stable level (~150 mW) and ensure that the laser is not pulsing.

This state of the laser is used for aligning optical components in steps below.

**Caution** Do not allow the output power of the laser to exceed 200 mW.

7. Install the optical isolator (Component B in Fig. 7.1) with its mounting hardware in front of the laser to eliminate back reflections (follow the instructions of the manufacturer for alignment). The optical isolator prevents reflection back into the femtosecond laser, which can result in instabilities in the output power and disrupt mode-locking.

**Caution** The optical isolator contains a powerful magnet that can attract metallic tools such as screwdrivers during installation, thus causing damage. It is also imperative that sufficient beam block assemblies, composed of one beam block with post (LB1) and one 2” high universal post holder, are placed around the isolator to absorb any beams reflected from the crystal surfaces of the isolator as shown in Figure 7.1 by the dashed orange lines. The placement and number of beam blocks is dependent to the alignment of the optical isolator.

Installing the equipment to control the laser power • TIMING 2 h

8. Install the half-wave plate (Component C in Fig. 7.1) using the high precision rotation mount, one Ø1/2” post, one Ø1/2” post holder and a standard base (BA2) at the output of the optical isolator. Rotate the half-wave plate mount to the 0° position.
9] Install the Glan-Thompson polarizer (Component D in Fig. 7.1) using the polarizing prism mount, the kinematic mount for thin \( \phi 1" \) optics, one \( \phi 1/2" \) post, one \( \phi 1/2" \) post holder and a standard base (BA2). Place a beam block assembly to absorb the rejected beam.

10] Install the EOM (Component E in Fig. 7.1) (or high-speed shutter; see equipment list), and place a beam block assembly to absorb the rejected beam. Follow the installation instructions of the manufacturer.

**Caution** When aligning the EOM, 200 mW or less average power must be used to avoid damage.

11] Install the HV amplifier (if using EOM), but do not turn it on. Connect the HV amplifier to the EOM using the included HV-rated cables.

**Caution** The HV-rated cables look similar to standard low-voltage-rated BNC cables. Using improperly rated cables increases the risk of electrocution and may damage equipment.

12] Use a T-joint connector and two 50-\( \Omega \) BNC cables to connect the output of the function generator both to the input port of the HV amplifier (or high-speed shutter controller) and also to the input of the oscilloscope. To visualize single electrical pulses on the oscilloscope screen, set the oscilloscope to trigger and hold on the rising edge at 0.5 V. Consult the oscilloscope manufacturer’s manual for detailed operation instructions.

13] Set the function generator to produce a square pulse with a 2.2 ms duration and 1.0 V amplitude (or the voltage required by the high-speed shutter control). Consult the function generator manufacturer’s manual for detailed operation instructions.

14] Mount the safety shutter using one \( \phi 1/2" \) post, one \( \phi 1/2" \) post holder and a standard base (BA2) as well as a beam block assembly to serve as a rejected beam dump (Components F in Fig. 7.1). Using the IR card and IR viewer, adjust the position of the safety shutter and the beam block such that the beam is not clipped when the safety shutter is open and the beam is reflected into the center of the beam block when the safety shutter is closed.

15] Close both the internal laser shutter and the safety shutter.
16| Turn the laser to its maximum emission power and ensure that it is pulsing. For the Mai Tai HP laser the expected maximum emission power is approximately 3.0 watts at 800 nm.

? TROUBLESHOOTING

17| Place the optical power detector at the output of the EOM. Turn on the HV amplifier. Open the internal laser shutter and adjust the position of the detector to maximize the power displayed on the meter.

18| Adjust the bias voltage of the HV amplifier until the measured power reaches its minimum.
Figure 7.2 An exploded view of the dichroic mounting adapter. (A-B) (A) The dichroic mounting adapter is composed of a metal filter cube, which contains an infrared (IR) dichroic mirror (B). (C) The filter cube is attached to a BA1 standard base using glue. (D) The opposite face of the BA1 standard base attaches to a two-axis compact kinematic mount also using glue. (E) The compact kinematic mount is attached to a $\varnothing 1$" (1") pedestal pillar post with a $\#8-32 \times 1/4$" set screw. (F) The assembly comprising components A–E mounts to a Nikon adapter plate from a 70 mm stage-up kit so that the dichroic mirror sits in the beam path. (G-H) (G) This is accomplished by drilling a hole and using a $\#8-32 \times 1/4$" screw (H) to position the dichroic mirror over the opening in the adapter plate. (I) The entire assembly is affixed to the microscope using screws included in the
70 mm stage-up kit. Figure 7.1 shows the location of the dichroic mounting adapter on the microscope.

19| Turn off the HV supply.

20| Rotate the half-wave plate (Component C in Fig. 7.1) until the power measured is appropriate for surgery: for axotomy in *C. elegans*, 1.2 W is sufficient when using a 20x objective lens with NA = 0.75 and a 2.2 ms exposure time. For higher NA objectives or longer exposure times, lower power levels are sufficient [71].

21| Turn on the HV amplifier and record the value on the power meter. This is the minimum amount of transmitted laser power. The ratio of the power set in Step 20 to this value gives the contrast ratio of the laser power at the sample. A ratio of at least 50 is desirable.  

**TROUBLESHOOTING**

22| Close the internal laser shutter.

23| Remove the power detector from the beam path.

**Assembly of the dichroic mounting adapter • TIMING 1 h and a 24-h pause**

24| The dichroic mounting adapter, shown in detail in Figure 7.2, is composed of the dichroic mirror, the Nikon adapter plate from the 70 mm stage-up kit for a Nikon Eclipse TI, one Ø1" (1") pedestal pillar post, one compact kinematic mount, one standard base (BA1), one dichroic cube holder. The dichroic mounting adapter directs the laser beam into the objective lens without interfering with the normal optical paths of the microscope.

25| Mount the dichroic mirror in the dichroic cube holder.

- CRITICAL STEP: Ensure that the coated side of the dichroic mirror is facing the laser.

26| Glue the dichroic cube holder to the standard base (BA1) using two-part epoxy following the diagram in Figure 7.2.

27| Glue the compact kinematic mount to the standard base using two-part epoxy following the diagram in Figure 7.2.
CRITICAL STEP: To ensure that the components are glued properly, place the assembly upside-down on a flat surface overnight while the epoxy cures.

28| Attach the pedestal pillar post to the compact kinematic mount using one #8–32 x 1/4” set screw.

29| Hold the dichroic cube holder so that the dichroic mirror is centered above the large hole in the Nikon adapter plate. Use a marker to draw a circle on the Nikon adapter plate around the bottom of the pedestal pillar post.

30| Using a hand-held power drill, make a 0.25” hole in the Nikon adapter plate at the center of the marked circle as shown in Figure 7.2.

31| Use a low-profile #8–32 x 1/4” screw to attach the pedestal post to the dust cover through the hole drilled in Step 30.

32| Attach the completed dichroic mounting adapter (Component N in Fig. 7.1) to the top of the microscope’s fluorescent filter turret (Component P in Fig. 7.1) using the screws and hardware included in the 70-mm stage-up kit.

**Assembly of the periscope • TIMING 1 h**

33| Construct a periscope (Component I in Fig. 7.1) using one Ø1.5”. Mounting post, one BA2 standard base, two right-angle kinematic cage mounts, two Ø1” gold mirrors and two Ø1.5” post mounting clamps. Place the periscope assembly on the optical table as shown in Figure 7.1 so that it will be able to direct the beam into the laser entry port of the microscope.

34| Install a Ø1” gold mirror in the ultra-stable kinematic Ø1” mirror mount.

35| Repeat Step 7 to lower the laser output power.

36| Turn off the HV amplifier.

37| Mount the ultra-stable kinematic Ø1” mirror mount using a one Ø1/2” post, one Ø1/2” post holder and a standard base (BA2) (Component G in Fig. 1) on the optical table. Open the internal laser shutter and the safety shutter. Coarsely align the kinematic mirror to center the laser on the periscope’s lower mirror. Close the safety shutter and secure the kinematic mirror to the optical table.
38| Place a Ø1” iris at the input of the periscope (Component H in Fig. 7.1). This iris will ease the process of realignment when needed.

39| Coarsely align the periscope to direct the laser horizontally into the back port of the microscope.

40| Adjust the angle of the lower periscope mirror to center the laser on the upper periscope mirror.

41| It may be necessary to repeat Steps 37–40 several times iteratively to obtain a satisfactory alignment.

42| Close both the safety shutter and the internal laser shutter.

**Assembly of the laser beam expander • TIMING 1 h**

43| Determine the focal lengths of lenses L1 and L2 needed to fill the back aperture of the objective used for axotomy. In this protocol, the laser beam diameter of 3 mm must be expanded to fill the 15-mm-in-diameter back aperture of the objective lens, and therefore the beam must be expanded fivefold. The amount of expansion is determined by the ratio of the focal length of lens L2 to that of lens L1, which in this protocol is 200 mm/40 mm = 5. The distance between the lenses is the sum of their focal lengths f1 and f2 as shown in Figure 7.3, which in this protocol is 40 mm + 200 mm = 240 mm.

44| Construct the beam expander following the diagram in Figure 7.4 using two Ø1.5” mounting posts, two standard bases (BA2), two Ø1.5” post mounting clamps, one 12” optical rail, three rail carriers, one single-axis position stage with micrometer, one Ø1” translating lens mount, one Ø2” lens mount and four Ø1/2” post holders.

45| Place the beam expander on the optical table next to the microscope as shown in Figure 7.1. Positioning of the beam expander optics close to the microscope in this manner provides two benefits: first, it allows for a single person to (re)align the setup, thus facilitating quick troubleshooting. Second, it reduces the amount of real estate on the optical table consumed by the optics and supporting hardware.
Figure 7.3 Optical path for simultaneous epifluorescence imaging and laser axotomy. The femtosecond laser, indicated by the red line, passes through beam conditioning optics before being directed up by the near infrared (NIR) dichroic mirror into the back aperture of the objective lens. The epifluorescence excitation, indicated by the blue line, is simultaneously directed into the back aperture of the objective lens by the filter cube. The fluorescence emission, indicated by the green line, passes through multiple filters and is captured by the camera.
Figure 7.4 An exploded view of the beam expander. Lenses L1 and L2 (a and b, respectively) sit in their mounts that are attached to Ø1/2" posts. Two Ø1" irises (C) are also attached to Ø1/2" posts. All four posts sit securely in Ø1/2" post holders (D), one of which is attached to a single-axis stage (E) with rotatable micrometer and Ø1.5" post clamp adapter plate (F), whereas the remaining three are attached to rail carriers (G). These four assemblies firmly attach to the 12" optical rail (H) which is mounted to the two Ø1.5" posts by two Ø1.5" post mounting clamps (I). The entire assembly is mounted using BA2 standard bases.

46 | Use a bubble level to ensure the 12" optical rail is level. The rail carriers slide onto the optical rail and are hand-tightened with the included screws. The single-axis position stage with micrometer is mounted using cap screws.

47 | Space the outer Ø1/2" post holders by a distance equal to f1 + f2. The distance between these two Ø1/2" post holders is finely adjusted below in the protocol.

48 | Lower the laser power as in Step 6.
49| Completely close the iris closest to the objective. Close the remaining two irises leaving an aperture of approximately 5 mm.

50| With the aid of the IR viewer and IR card, adjust both the height and the angle of the upper periscope mirror until the laser beam passes through the two open irises.

51| Open all three irises.

52| Center the beam on the dichroic mirror by adjusting only the height of the upper periscope mirror and the position of the periscope assembly on the optical table. If the periscope assembly is displaced, repeat Steps 37–40 to re-center the beam on the lower periscope mirror.

53| Close all three irises leaving an aperture of approximately 5 mm. Adjust the height of the 12” optical rail until the laser passes through all three irises. Use a bubble level to ensure the 12” optical rail is level. TROUBLESHOOTING

54| Close both the safety shutter and the internal laser shutter.

Coarse alignment of the laser • TIMING 30 min

55| Construct the IR alignment tool by drilling a 1/8” diameter hole through the center of the RMS-threaded IR alignment disk and then insert the modified IR alignment disk into an RMS 45 to CFI 60 objective adapter.

! Caution The alignment disk is built from brittle plastic so drilling must be done with care.

56| Thread the completed IR alignment tool into the microscope nosepiece.

57| Place a cover glass on the stage plate to act as a reflecting surface for laser alignment.

58| Lower the laser power for alignment as in Step 6.

59| Tilt the condenser arm away from the stage to facilitate viewing of the IR alignment tool.

60| Open the internal laser shutter and the safety shutter.
Using the IR viewer, observe the top of the IR alignment tool as shown in Figure 7.5. 

**Caution** Do not hold the IR viewer directly in the beam path as this could result in damage to the IR viewer.

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**Figure 7.5 Use of the infrared (IR) alignment tool.** The IR alignment tool is composed of an RMS IR-aligning disk and an RMS 45 to CFI 60 objective adapter. (A) Without lenses L1 and L2, the transmitted beam is directed to the center of the dichroic mirror, thus resulting in a glowing spot on the field of the disk. (B) Adjusting the dichroic mirror causes the transmitted beam to pass through the center hole and an additional spot caused by the reflection of the beam from the cover glass appears on the field of the disk. The reflected spot is caused by the non-normal incidence of the transmitted beam on the cover glass. Moving the transmitted spot half-way towards the initial location of the reflected spot by adjusting the upper periscope mirror and then moving the transmitted spot back to the center hole by adjusting the angle of the dichroic mirror,
achieves normal incidence of the beam on the cover glass. (C) Normal incidence is indicated by both reflected and transmitted beams passing through the center. (D) Inserting both lenses L1 and L2 into the beam path (Steps 68–70), results in a large, symmetric circular illumination on the IR alignment tool.

62| If the laser is not passing through the center hole then a glowing dot will appear on the surface of the IR alignment tool as shown in Figure 7.5a. Use the compact kinematic mount of the dichroic mounting adapter to center the laser on the IR alignment tool. ? TROUBLESHOOTING

63| If the laser beam is not normal to the cover glass two spots will appear on the surface of the IR alignment tool as shown in Figure 7.5b: a glowing ring around the center hole (the ‘transmitted beam spot’) and a spot off to the side (the ‘reflected beam spot’).

64| Note the location of the reflected beam spot.

65| Adjust the angle of the upper periscope mirror to move the transmitted beam spot approximately half the distance towards the initial location of the reflected beam spot (from the previous step). In doing this, the reflected beam spot will itself move and may disappear, as the transmitted beam is no longer passing through to the center hole. ? TROUBLESHOOTING

66| Adjust the angle of the dichroic mirror using the compact kinematic mount of the dichroic mounting adapter to bring the transmitted beam spot back to the center hole. The reflected beam spot should move back towards the center hole at approximately twice the rate as the transmitted beam spot, and they will meet in the middle as shown in Figure 7.5c. ? TROUBLESHOOTING

67| Close the safety shutter. Insert L2 making sure that the curved surface of the lens faces away from the objective.

68| Open the safety shutter. Using the IR viewer, observe the IR alignment tool and center the transmitted beam spot by adjusting the position and pitch of L2. ? TROUBLESHOOTING

69| Close the safety shutter. Insert L1 making sure that the curved surface of the lens faces L2.
70| Open the safety shutter. Using the IR viewer, observe the IR alignment tool and center the transmitted beam spot by adjusting the position and pitch of L1 using both the \( \varnothing 1/2'' \) post and the \( \varnothing 1'' \) translating lens mount. Note that the transmitted beam spot will be larger and diffuse after inserting L1 as shown in Figure 7.5d. Ensure that the beam is not clipped (i.e., the transmitted beam spot should be a full, symmetric and circular disk on the IR alignment tool). ? TROUBLESHOOTING

71| Close the safety shutter and internal laser shutter.

**Fine alignment of the laser and image focal planes • TIMING 1 h**

72| Insert the objective to be used for surgery. Make sure that no filter cube is in the active slot of the epifluorescence filter turret. Turn on the HV amplifier and adjust the half-wave plate as discussed in Steps 17–21.

![Figure 7.6 Ablated patterns in permanent marker on cover glass under different alignment conditions.](image)

(A,B) When the image plane is focused on the boundary of the marked and unmarked glass surfaces, and when the system is properly aligned, the resulting cutting pattern is narrow and symmetric (A), whereas the firing pattern is relatively small and also symmetric (B). If the beam is clipped and/or lenses L1 and L2 are misaligned, the cutting pattern is wider in one direction than the orthogonal direction (C). In addition, the firing pattern becomes asymmetric. If the laser is out of focus, the cutting pattern is blurred and the firing pattern is larger (D). Scale bar is 50 μm.
73 | Close the internal laser shutter and the safety shutter.

74 | Draw lines on a clean cover glass using a non-water-based permanent marker (e.g., Sharpie). Place the cover glass on the stage plate.

CRITICAL STEP: Use only a single pass with the marker, as a thick coating will not allow the laser to produce thin cuts.

75 | Turn on the camera and image acquisition software.

76 | Open the internal laser shutter and the safety shutter.

77 | Locate the image of the laser in the preview window of the image acquisition software. Center the beam in the preview window by adjusting the angle of the dichroic mirror using the compact kinematic mount of the dichroic mounting adapter. TROUBLESHOOTING

78 | Mark the position of the laser on the computer monitor with a piece of tape.

CRITICAL STEP: Do not move the image acquisition software preview window after marking the beam location.

79 | Close the internal laser shutter and the safety shutter.

80 | Insert the laser notch filter into a 30 mm-standard square cage plate.

81 | Place the laser notch filter between the dichroic mirror and the microscope epifluorescence filter turret.

82 | Tilt the condenser arm to its original upright position. Turn on the bright field source, adjust the exposure of the camera and focus on the edge of a marker line. Move the stage to position the edge of a marker line under the piece of tape on the computer monitor.

83 | Open the safety shutter. Depending on the locations of L1 and L2 set in Steps 67–70 the region of marker underneath the tape will be ablated to varying degrees of sharpness, symmetry and size. While staying focused on the edge of the marker line, adjust the position of L1 along the optical rail using the micrometer to adjust the focus of the laser beam to increase the sharpness of the ablation. In addition, adjust L1 using its
translating mount axes to further improve the shape of the ablation. TROUBLESHOOTING

84| Characterize the laser alignment by using the single-pulse button on the function generator to generate spots and compare the result to the image in Figure 7.6. When the system is properly aligned, the cutting patterns shown in Figure 7.6 should be ~3 µm wide and the firing patterns should be ~9 µm across. Note that these values have been found to be suitable for axotomy in C. elegans using a 20x, NA: 0.75 objective. TROUBLESHOOTING

85| Close the safety shutter and the internal laser shutter.

Example application: in vivo laser axotomy on C. elegans TIMING 25 min

86| Prepare the microscope for fluorescence imaging and remove the laser notch filter (1 min).

- TIMING: These steps are strongly time dependent and must be completed within 2 h once the anesthetic has been administered to ensure minimal toxic effects (C. elegans). The rapid development cycle of the animals may influence the biological process under study, so care should be taken to ensure that the animal is in the proper developmental stage.

87| Turn on the HV amplifier and adjust the half-wave plate as discussed in Steps 17–21 (3 min).

88| Open the internal laser shutter and the safety shutter (2 min).

89| Locate the image of the laser in the preview window of the image acquisition software and adjust the piece of tape on the monitor if necessary (2 min). TROUBLESHOOTING

90| Close the safety shutter and return the laser notch filter to its position above the microscope filter turret (2 min).

91| Immobilize an animal on a cover glass using standard procedures [57]. Place the cover glass on the stage plate. Ensure that the animal is as close to the cover glass as possible (5 min).
Using fluorescence imaging, locate the animal and move the stage to bring the axon or cell to be ablated directly under the piece of tape. Bring the target axon or cell into focus ( < 30 s).

Open the safety shutter and adjust the focus to achieve two-photon fluorescence of the target axon, indicative of proper focusing. Adjust stage position and focus if necessary ( < 30 s).

Press the single-pulse button on the function generator to ablate the target axon or cell. Immediately close the safety shutter to avoid damaging the organism/cell ( < 10 s).

TROUBLESHOOTING

Recover the animal using standard procedures [57] (5 min).

At the end of the experiment, close the safety shutter and internal laser shutter. Turn off the laser and the fluorescence and bright field sources (3 min).
• TIMING

Steps 1–7, Setting up the optical table, laser, power meter, microscope and optical isolator: 6 h

Steps 8–23, Installing the equipment to control the laser power: 2 h

Steps 24–32, Assembly of the dichroic mounting adapter: 1 h + 24 h pause

Steps 33–42, Assembly of the periscope: 1 h

Steps 43–54, Assembly of the laser beam expander: 1 h

Steps 55–71, Coarse alignment of the laser: 30 min

Steps 72–85, Fine alignment of the laser and image focal planes: 1 h

Steps 86–96, In-vivo laser axotomy on C. elegans: 25 min

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 7.2.

Table 7.2 Troubleshooting table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Laser Is not pulsing</td>
<td>Optical isolator is not functioning properly, allowing back reflections into the laser</td>
<td>Check optical isolator manual and ensure the component is aligned correctly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laser power is set too low</td>
<td>Check the power output of the laser</td>
</tr>
<tr>
<td>21</td>
<td>Power ratio at electro-optic modulator (EOM) is insufficient</td>
<td>Bias voltage for high-voltage (HV) amplifier is incorrect</td>
<td>Repeat Step 18</td>
</tr>
<tr>
<td>53</td>
<td>The laser beam will not pass through all three irises</td>
<td>The 12” optical rail is not parallel to the beam path</td>
<td>Carefully rotate the entire beam expander structure to bring the 12” optical rail parallel to the beam path</td>
</tr>
<tr>
<td>62</td>
<td>Cannot find laser spot on the infrared (IR) alignment tool</td>
<td>Dichroic mirror is severely misaligned</td>
<td>Adjust the rotation of the 81” pedestal post holding dichroic and/or compact kinematic mount on the adapter plate and realign</td>
</tr>
</tbody>
</table>

(continued)
Table 2 Troubleshooting table (continued).

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>65, 66</td>
<td>Transmitted beam spot disappears as it is moved</td>
<td>The beam is being clipped</td>
<td>Adjust the position and/or rotation of the 81&quot; pedestal pillar post; if insufficient, realign the periscope, beam expander and dichroic mounting adapter</td>
</tr>
<tr>
<td>68</td>
<td>The beam spot cannot be properly centered using only L2</td>
<td>The laser beam is not passing through the center of L2</td>
<td>Adjust the beam expander hardware, in particular the height of the 12&quot; optical rail from the table</td>
</tr>
<tr>
<td>70</td>
<td>Expanded beam shape is asymmetric or irregular in shape</td>
<td>Clipping of the beam and/or poor orientation of the lenses</td>
<td>Check alignment using IR viewer and IR card and correct the misaligned components</td>
</tr>
<tr>
<td>77</td>
<td>Cannot find laser in preview window</td>
<td>Beam may not be falling on the CCD of the camera</td>
<td>Rotate the camera in its mount if the sensitive area is particularly small</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Filter cube may be in the active slot of the epifluorescence filter turret and may be blocking the laser beam</td>
<td>Remove the filter cube</td>
</tr>
<tr>
<td>83</td>
<td>Cannot obtain sharp cuts on the permanent marker</td>
<td>Imaging and laser plane of focus are mismatched</td>
<td>Find both planes of focus: if the laser plane of focus is below the imaging plane, move L1 closer to L2 with the micrometer; if laser focus is above, move lenses apart</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laser is not pulsing</td>
<td>Change the settings of the laser to pulsing mode or go to troubleshooting of Step 16</td>
</tr>
<tr>
<td>84</td>
<td>Firing pattern is asymmetric or large</td>
<td>Clipping of laser beam</td>
<td>Trace the beam path using the IR card to determine the point of clipping and correct the misalignment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Misalignment of lenses</td>
<td>Realign the lenses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laser is not focused on the image plane</td>
<td>Adjust the position of L1 along the 12&quot; optical rail using the micrometer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Power level is too high</td>
<td>Verify the high-power level using Steps 17–21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laser is not centered on or overfilling the back aperture of the objective lens</td>
<td>Redo Steps 63–71 to correctly align the incoming beam on the back aperture of the objective</td>
</tr>
<tr>
<td></td>
<td>The position of cuts shifts when focusing</td>
<td>Non-normal incidence of laser beam onto the back aperture of the objective</td>
<td>Redo Steps 62–66 to correctly orient the incoming beam</td>
</tr>
<tr>
<td>89</td>
<td>Laser spot has moved significantly since last usage</td>
<td>The alignment of components have changed</td>
<td>Check alignment using IR viewer and IR card and correct the misaligned components</td>
</tr>
<tr>
<td>94</td>
<td>Failure to successfully ablate the target</td>
<td>Laser is not focused on the image plane</td>
<td>Repeat Steps 72–85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Energy of the laser is too low</td>
<td>Check maximum power of the beam using power detector at the output of the EOM; see Step 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Target is too deep in the tissue for efficient cutting; excessive scattering</td>
<td>Reorient the sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Numerical aperture (NA) of the objective is too low</td>
<td>Use objectives with higher NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laser is not pulsing</td>
<td>Change the settings of the laser to pulsing mode or go to TROUBLESHOOTING of Step 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Significant laser pulse dispersion</td>
<td>Analyze the entire beam path for portions which may be causing significant dispersion and adjust/replace components with ones suited for high-speed near infrared (NIR) lasers</td>
</tr>
<tr>
<td></td>
<td>Specimen is damaged at low power, even when not firing</td>
<td>Minimum transmitted power is too high</td>
<td>Check/correct EOM bias voltage and the rotational angle of the half-wave plate</td>
</tr>
</tbody>
</table>
ANTICIPATED RESULTS

Characterization of the femtosecond laser surgery system

The setup described in this protocol produces a circular laser spot with a full-width at half-maximum (FWHM) of 1.7 μm at the focal plane (see Fig. 7.7). An objective with a higher NA can be used to generate a smaller laser spot size at the expense of the working distance and/or the field of view. Using the components described in this protocol, the total power loss between the output of the EOM and the sample is 47.5%.

![Point spread function of the laser at the focal plane. The system described in this protocol generates a circular laser spot at the sample with a full width at half-maximum of 1.7 μm.](image)

**Figure 7.7** Point spread function of the laser at the focal plane. The system described in this protocol generates a circular laser spot at the sample with a full width at half-maximum of 1.7 μm.

**Axotomy of C. elegans mechanosensory neurons**

With a 20x (NA = 0.75) objective, we use a 1.20 W (measured at the output of the EOM, which corresponds to 0.63 watts at the sample), 2.2 ms-long laser pulse train to carry out axotomy on C. elegans zdis5 strain (GFP-labeled mechanosensory neurons) (see Fig.
Although low-magnification objective lenses have smaller NAs, they allow simultaneous visualization of the cell body and large portions of the axons, enabling surgery of the axons at precisely measured distances from the soma.

Lower laser power and shorter pulse trains can be used; however, the reproducibility of surgery decreases at lower powers in part because of increased sensitivity to laser focus [71]. In addition, it becomes difficult to distinguish photodamage from photobleaching. Photobleaching is often followed by spontaneous recovery of fluorescence in the axon at the site of injury within a few minutes, whereas photodamage leads to permanent non-fluorescent regions. The threshold power levels for photobleaching versus photodamage can be characterized using dye-filling techniques [10,11]. Conversely, higher laser powers and/or longer pulse trains often result in extensive scarring, causing widespread injury to the animal [11,71,88].

Upon ablation, we often briefly observe fluorescent protein leaking from the axon terminals and diffusing into the surrounding area. The brightness of the axon terminals decreases initially, and recovers within several minutes, leaving a non-fluorescent region at the site of surgery. Within several minutes both the proximal and distal axon terminals retract by several microns (arrow 2 in Fig. 7.8b). Within a few hours, the proximal axon terminal starts regrowing (arrow 3 in Fig. 7.8b) [10]. The regrowing proximal axon terminal may also fuse with the distal axon terminal [11].
Figure 7.8 Femtosecond laser microsurgery. (A) A highly localized region (arrow) of a *Caenorhabditis elegans* mechanosensory neuron is ablated using the system described in this protocol. (B) Following surgery at point 1, the ablated process first retracts to point 2 and then regenerates to point 3. Scale bar represents 10 μm.
By enabling the rapid production of transgenics on a large scale we are able to enhance the feedback loop of gene/function assignments that could enable a wide variety of whole animal models of related to human disease. This pipeline is described in Figure 8.1.

![Diagram of C. elegans discovery pipeline enhancement](image)

**Figure 8.1 C. elegans discovery pipeline enhancement.** By starting with a human insight from genomics data we can generate a specific humanized gene variant and quickly introduce it into a humanized worm. We can then validate the phenotype and then produce a certain type of intervention by introducing a drug or a laser surgery manipulation. The result can then be imaged at high resolution and then the results can be analysed with computer vision algorithms and fed back into the iterative learning system.
Once the microinjection process bottleneck is removed it is then shifted to the purification of the transgenic population. Instead of traditional selections that require visually screening for stable lines and maintaining them manually, we will use a recently-developed positive selection scheme based on antibiotic resistance to facilitate establishing and propagating transgenic lines at large scale (Giordano-Santini et al., 2010 [89]). The use of industrial liquid handling technologies could enable complete automation and industrialization of the gel preparation, handling and recovery/feeding of the worms after injection. Using liquid culture large populations of injected P0s could be grown up until the second generation F2 and then purified using the antibiotic technique.
Figure 8.2 Computer vision algorithms for development of assisted injections. The process flow could be developed to run in the following steps:

1. Needle calibration to Z height and XY alignment
2. The yellow line tracks worm perimeter and aligns all boxes for detection
3. Detection and feedback for puncture of gonad
4. Test pulse is detected in grey box
5. Dispensing to fill the gonad is detected in the black boxes
6. Red box analyzes post-injection needle clogging
7. Grey box analyzes overfilling and spilling of contents signifying injection failure

As each of these steps are processed in a semi-automated fashion so that data can be stored to then train sophisticated algorithms how to perform these experiments.
References


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