Undergraduate Honors Thesis in Physics

Engineering a perfusion-enabled mechanical compressor for long-duration immobilization and microscopy of cells and small organisms

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The study of living specimens is essential to the understanding of organismal behavior. Unfortunately, a major difficulty in the study of live organisms is that many move in and out of the field of view or focal plane during microscopy. The present work seeks to combat this considerable problem by developing a mechanical microcompressor that immobilizes living cells and small organisms for long-duration optical microscopy. The device, dubbed the "Commodore Compressor," features two key innovations: (1) the integration of a perfusion system to keep the trapped specimen alive over several hours, as well as permitting the addition of chemoattractants, drugs, and other chemicals; (2) the incorporation of an optional patterned PDMS platform to improve the efficacy of immobilization in a targeted, organism-specific manner. One application of the Commodore Compressor is in monitoring the change in protein bioluminescence intensity in many trapped *Saccharomyces cerevisiae* cells during synchronized cell cycles. The experiment's feasibility and key techniques have been well demonstrated, although bioluminescence cannot currently be visualized. A second application involves fluorescence imaging of the neural network development of immobilized *Caenorhabditis elegans* over many hours. The development of new patterned PDMS platform designs, aided by the innovative use of established techniques, has driven the present work toward accomplishing the goal, but true long-term viability remains elusive. The Commodore Compressor may be directly used or easily adapted for many other specimen types and experimental scenarios.

1 Introduction

The study of live organisms is a key foundation of the life sciences. As opposed to observing a static, fixed organism, studying living specimens holds many advantages, permitting real-time viewing of dynamic cellular processes as well as movement in response to stimuli. However, the natural motility of many living organisms presents a serious challenge to the microscopist. In liquid medium a specimen may rapidly change its position in all three dimensions, moving in and out of the viewing area and shifting in and out of the focal plane. The emergence of molecular biology techniques and the invention of colored fluorescent proteins have enabled the study of sub-cellular structures down to the level of individual proteins, but when the specimen is moving erratically it becomes practically impossible to image at the sub-cellular level. Consequently, the need to immobilize living specimens while ensuring their viability has been a monumental problem for decades.

There exist many different methods to slow down or immobilize motile specimens while maintaining their viability. These techniques fall into two broad categories, chemical methods and physical, device-based methods [1].

1.1 Chemical methods of immobilization

Chemical methods tend to be the easiest in terms of application. Organism-specific anesthetics are often employed to reduce the overall "liveliness" of a specimen. Alternatively, the surface of microscope slides can be coated with cell-adherent formulas [2]. There also exist multivalent antibodies that bind cilia and/or flagella together to prevent the organism from swimming [3]. Other chemicals, such as the commercially available Protoslo (Carolina Biological), make the liquid medium more viscous to retard specimen movement. These so-called thickening agents have varying degrees of efficacy [4].

A common problem with most chemical techniques is that they tend to adversely affect the specimen, either limiting viability outright or casting doubt on the biological relevance of results obtained using these receptor-altering chemical approaches. As such, physical techniques are sometimes preferred.

1.2 Physical methods of immobilization

Experimenters may devise *ad hoc* methods of various efficiencies for trapping specimens. The simplest method involves making a "wet mount"—placing the specimen on a microscope slide with a minimal amount of liquid medium and placing a coverslip over it—and wicking away liquid using a tissue wipe until the specimen is held. One can then take a quick look at the specimen before the liquid medium dries further, causing the coverslip to crush the specimen [5].

More recently, microfluidic technology, particularly the fabrication of microscale devices using the transparent, bio-inert material poly(dimethylsiloxane) (PDMS) [7][8], has enabled the construction of specialized imaging-friendly devices having specimen-specific traps. For

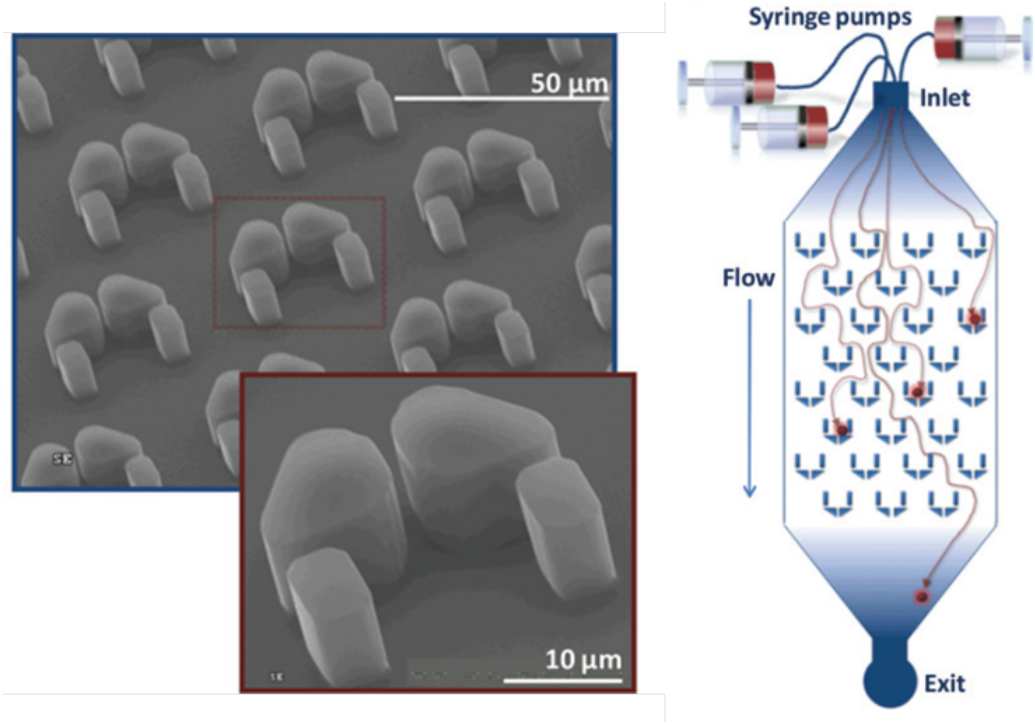


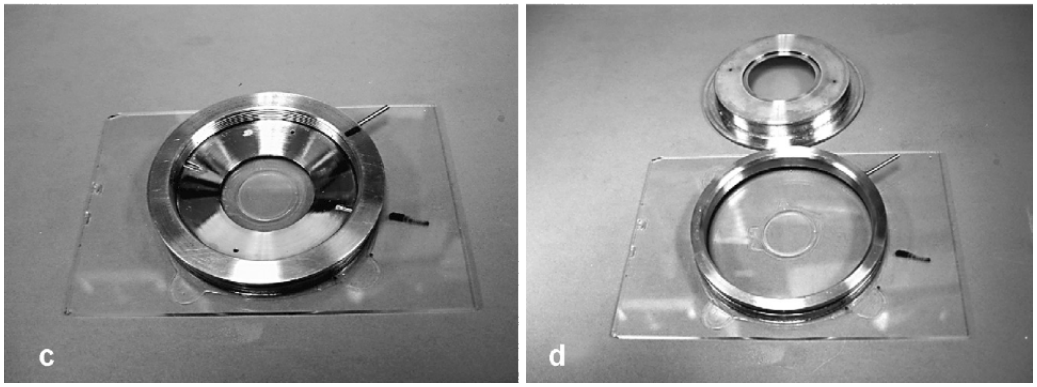
Figure 1: Example of a microfluidic device with bucket-shaped traps, each of which holds a small number of cells. Image from [6].

instance, in a recent investigation on the signaling dynamics of non-adherent T cells, the authors devised a microfluidic chip with bucket-like traps (Figure 1) to contain the cells for the purpose of measuring their secreted metabolites [9]. While microfluidics is an exciting new technology that promises unparalleled flexibility in the design of physical traps, their fabrication complexity presents a deterrent to their widespread use. More significantly, most microfluidic chips are closed devices with a ceiling, which makes loading cells into them less straightforward than the kind of direct placement and manipulation possible with open devices.

1.2.1 Microcompressors

For more precise and direct immobilization, specialized mechanical instruments called microcompressors have been designed, all of which are essentially a coverslip attached to a micrometer that brings the coverslip closer to another glass surface with high precision. While cells may be held with minimal distortion, it has been demonstrated that cells can flatten and become more optically transparent when gently squeezed in a microcompressor; in fact, a new organelle was discovered in conjugating *Tetrahymena thermophilia* under compression [10]. In addition, microcompressors permit the recovery of living specimens after compression for re-culture and further study.

Several variants of microcompressors have been designed over the years. Until the present work, the most advanced microcompressor available had been the unit constructed by Karl J. Aufderheide. His compressor, known as a "rotocompressor," permitted the microscopist to view specimens under Köhler illumination on an upright microscope and subsequently employ high-resolution light microscopy. Briefly, the Aufderheide unit (Figure 2) consisted of a 2-inch by 3-inch glass base with a 12 mm diameter, #2 thickness coverslip cemented in the center. A circular screw-down mount held a 25 mm diameter, #1 thickness coverslip and applied a slight bowing to the coverslip, which mitigated problems caused by not strictly parallel glass surfaces. Both the top and bottom glass surfaces could be silanized to minimize shearing of the trapped specimen during operation. The coverslip mount fitted into an inner ring, which was engaged to an outer ring (cemented to the glass base) via fine threads that permitted precise control over the compression process. The fact that the coverslip mount could be lifted up to open the device while leaving the threads engaged meant that the experimenter could achieve remarkable consistency by pre-setting the desired height of compression, lifting the coverslip mount to load the specimen, and replacing the coverslip mount to immediately arrive at a the predetermined level of compression. Although the usefulness of the Aufderheide unit cannot be understated, his device had the main disadvantage of being a closed system. The lack of a flow system supplying nutrients to the trapped specimen made the device unsuitable for imaging some organisms over several hours. As a consequence, it was impossible to chemically alter the specimen environment once compression was applied—a characteristic that precluded experiments such as those that required chemoattractant stimulation to the trapped specimen. As such, the implementation of a perfusion system that allows the addition of nutrients or chemoattrac-



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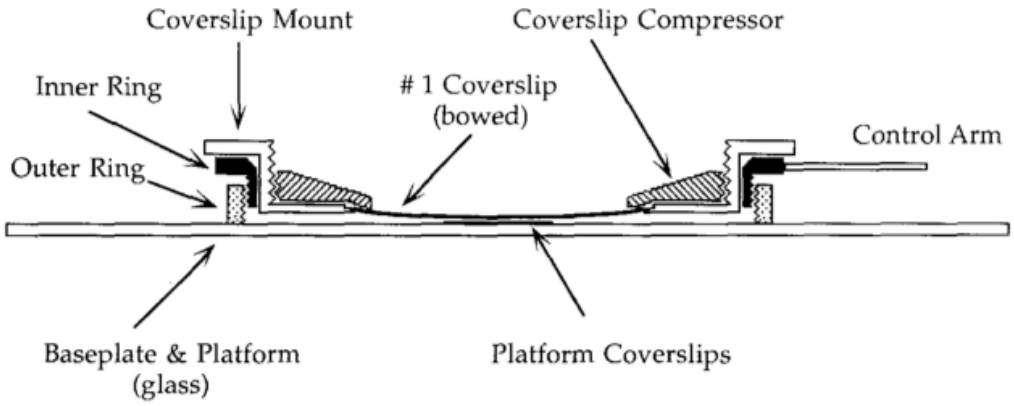


(b)

Figure 2: The Aufderheide rotocompressor. **(a)** Photograph. **(b)** Schematic. For scale, the glass baseplate measures 2 in. × 3 in. Images from [1] and [11], respectively.

tants to the trapped specimen would be of great value to the study of cell and organismal behavior. Another important limitation to the Aufderheide unit was that it had been designed for upright microscopes, not the inverted microscopes commonly used in modern cell biology. Since the coverslip mount fitted into the inner ring by gravity, inverting the Aufderheide compressor for use on an inverted microscope would likely cause the coverslip mount to fall off. In all, even though the Aufderheide unit had been the most advanced mechanical instrument for live specimen immobilization, its design drawbacks limited its practical applications.

1.3 The need for a better compressor

The present work has been focused on heavily redesigning the Aufderheide compressor to expand its applicability to more specimen types and experimental scenarios. The desired applications of the compressor dictated its development paths. In particular, two laboratories at Vanderbilt University wanted to employ the compressor for their experiments. the Carl Johnson laboratory sought to trap at once many cells of the budding yeast *Saccharomyces cerevisiae* to monitor the change in bulk bioluminescence intensity of a number of targeted proteins during the cell cycle. Since the experiment depended on the availability of fresh, oxygen-rich liquid medium over several hours, the compressor as designed by Aufderheide could not be used since it was a closed system.

Meanwhile, the David Miller laboratory hoped to immobilize the nematode *Caenorhabditis elegans* to observe the worm's neural network development, specifically the branching of PVD neuron dendrites [12], over about eight hours. Previously, the worm had to be periodically anesthetized to suppress movement during 3D imaging. Not only did the worm display a different orientation for each time point, the anesthesia could have caused unknown developmental defects in the worm. Immobilization approaches involving microfluidic devices [13] could have been a possibility, but the difficulties in fabrication and in loading worms into these closed-ceiling devices led the Miller lab to seek a more procedurally direct approach promised by the compressor. As with the experimental demands of the yeast experiment, the compressor needed to have a perfusion system to supply fresh liquid medium to the trapped specimen.

Given the challenges laid out by the above ambitious experimental goals, the present work seeks to engineer a compressor capable of meeting these demands. Shortly after the work was commenced, it was realized that the modern techniques of PDMS microfabrication held much greater possibilities for improving the compressor than just modifications to the metal and glass parts. Within the microfabrication facilities of the Vanderbilt Institute for Integrative Biosystems Research and Education (VIIBRE), PDMS could be inexpensively made into varying shapes for a range of purposes. For instance, it would be possible to make channels out of PDMS in creating a perfusion system for the compressor. Of course, PDMS microfabrication could not cure all deficiencies in the Aufderheide compressor, particularly those arising from the design of the metal pieces themselves. As such it was decided that the metal pieces fundamental to the operation of the compressor must be overhauled. In

all, it was the *sum* of PDMS microfabrication and the traditional machining modifications that resulted in the most innovative and practical compressor to date.

2 Methods and results

2.1 Overview of microfabrication techniques

Microfabrication was carried out following standard protocols established by the Vanderbilt Institute for Integrative Biosystems Research and Education (VIIBRE). The technique is divided into two main procedures, photolithography (Figure 3, A–D) and PDMS replica molding (Figure 3, E–G). In photolithography, positive relief structures that serve as a mold template or master are created on a silicon wafer from a photoresist called SU-8, a material that becomes polymerized when exposed to UV light. Selective UV exposure is controlled by a patterned photomask, typically created in Mylar film or chrome. In essence, the photoresist is spin-coated onto a silicon wafer. The photomask is then laid on top of the photoresist, and UV light is shone onto the wafer, crosslinking exposed SU-8. In a developing step, unexposed SU-8 is washed away while exposed SU-8 remain on the wafer. In PDMS replica molding (Figure 3, E–G), PDMS pre-polymer and linking agent is mixed in a 10:1 ratio and cast over the positive relief structures on the silicon wafer. Once the PDMS is cured, it is cut and peeled away. Structures on the PDMS are in opposite polarity to those on the silicon wafer. Finally, PDMS is bonded to a glass surface, forming enclosed channel structures.

For the purposes of my research, microfabrication has presented an easy and relatively inexpensive way to create flow channels and open-faced relief structures to enhance the compressor's capabilities and adapt it to various specimen types.

2.2 Incorporation of perfusion

2.2.1 Drilling holes

As a first step toward trapping specimens for an extended period of time, a perfusion system was incorporated into the Aufderheide compressor. Two holes, each 1.0 mm in diameter with center-to-center separation of 5.0 mm, were drilled into the center of the 2 inch × 3 inch microscope slide that constituted the base of the compressor. The holes had to be sufficiently close together to facilitate flow from one hole to another. The act of drilling holes through a glass slide sounds trivial, but there was an exceptional complication. Recall that a 12 mm diameter, #2 thickness platform coverslip was present in the center of the base glass, as it was needed to raise the surface by a sufficient height to meet the bowed top cover-glass (Figure 2). As such, holes had to be drilled through both the platform coverslip and the glass base, a feat that required many tries to master due to the change in the resistance encountered by the drill bit at the interface between the two glass pieces. In the drilling process, the drill bit was being pressed down at a constant force. The inconsistency at the glass-glass interface caused the downward force to unexpectedly

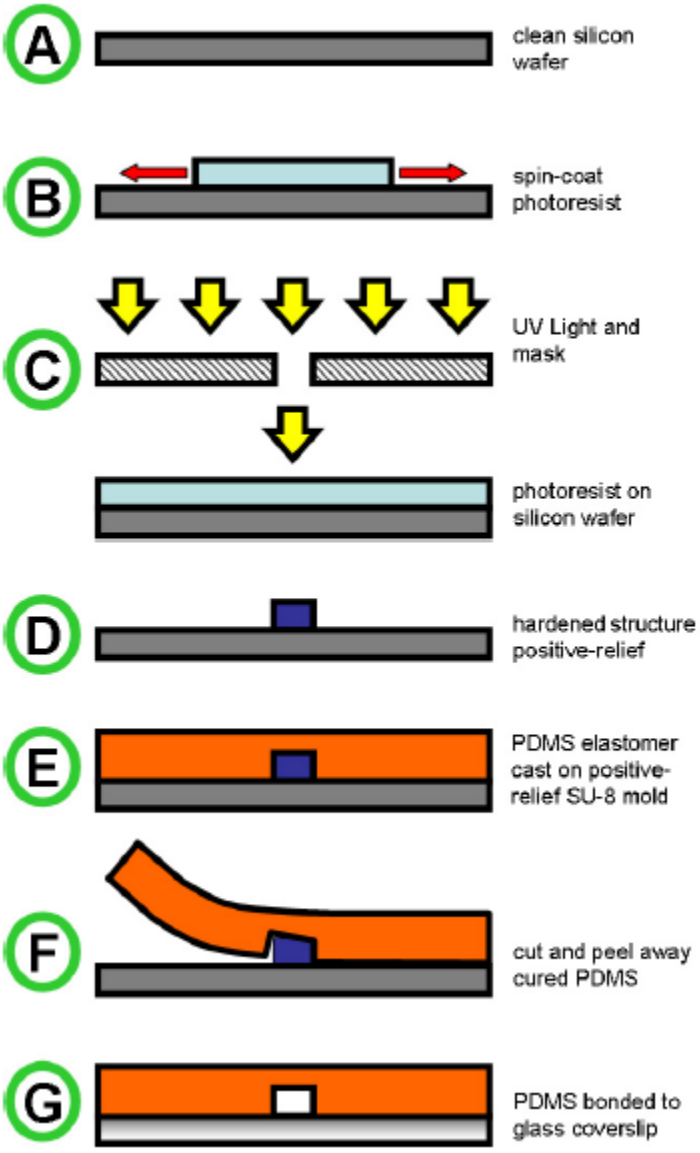


Figure 3: Schematic of the overall microfabrication procedure. Details are described in the main text. Image by Kevin Seale, VIIBRE.

change in magnitude, resulting in either a conical-shaped "blow-out" in the glass base or the shattering of the platform coverslip. The fact that the platform coverslip was glued onto the glass base did little to help. This challenging drilling technique was finally mastered by employing a diamond drill tip, a fast rotational speed, a slow downward drilling rate, and plenty of cooling. Drilling was first carried out by John Fellenstein at the physics machine shop but was later done by myself using a manual drill press, after I had sufficient practice to ensure a good success rate.

2.2.2 PDMS channels

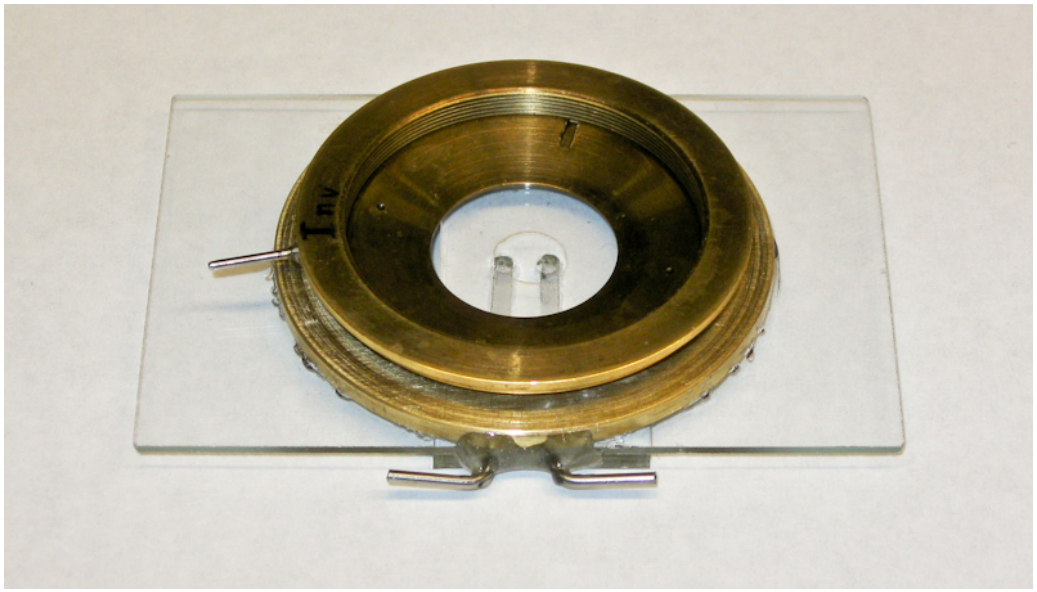
Once the holes were drilled, it was necessary to create support structures that would allow one to connect tubing to the ports. One approach taken was to employ a simplified microfabrication process to create PDMS channels underneath the glass base that led from the drilled holes to the edge of the device (Figure 4). Instead of pursuing the full photolithography process, a mold for channels was constructed using two toothpicks secured onto a clean microscope slide (e.g. 1 inch × 3 inch or 2 inch × 3 inch) with Elmer's School Glue. As channels were designed to align with the drilled holes in the glass base, the separation between the ends of the toothpicks in the middle of the slide was roughly the same as that between the holes (5.0 mm center to center). After the glue dried, any length of toothpick beyond the edge of the microscope slide was cut off with a razor blade. The mold was then placed in a Petri dish, ready for PDMS casting. Following standard replica molding techniques, PDMS ($\sim$ 10 g) was then poured onto the mold and cured at 60°. Subsequently, a rectangular slab of PDMS around the toothpicks was cut out and plasma bonded to the underside of the drilled glass base. Care was taken to ensure that the holes in the glass base were aligned with the ends of the PDMS channels. Any PDMS overhang from the edge of the base after bonding was cut off.

At first it was attempted to insert tubing directly into the PDMS channels, but the small size of the toothpicks used in the mold made the task nearly impossible. The solution was to create narrow metal tubing connectors. Two syringe needles (18G1½, for example) were cut into tubes, bent 90° in the middle, and inserted into the channels. They were secured with epoxy (Araldite brand).

2.2.3 Direct tubing insertion into PDMS

Although the first iteration of perfusion structures worked reliably, it had some drawbacks. Although the process of making the perfusion structures was simpler than typical microfabrication by bypassing photolithography, the procedure remained laborious. Furthermore, the long perfusion channels required that the device be primed prior to trapping a specimen, which would add complexity to the experiment. Liquid that remain in the channels may be suctioned into the compressor chamber during device operation, ruining compression experiments that are sensitive to the starting volume of liquid inside the chamber.

To avoid these problems, the PDMS perfusion channels were replaced by two small blocks



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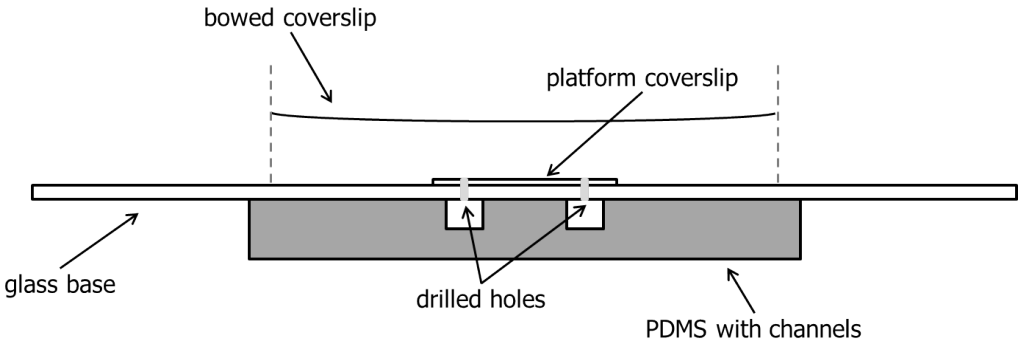

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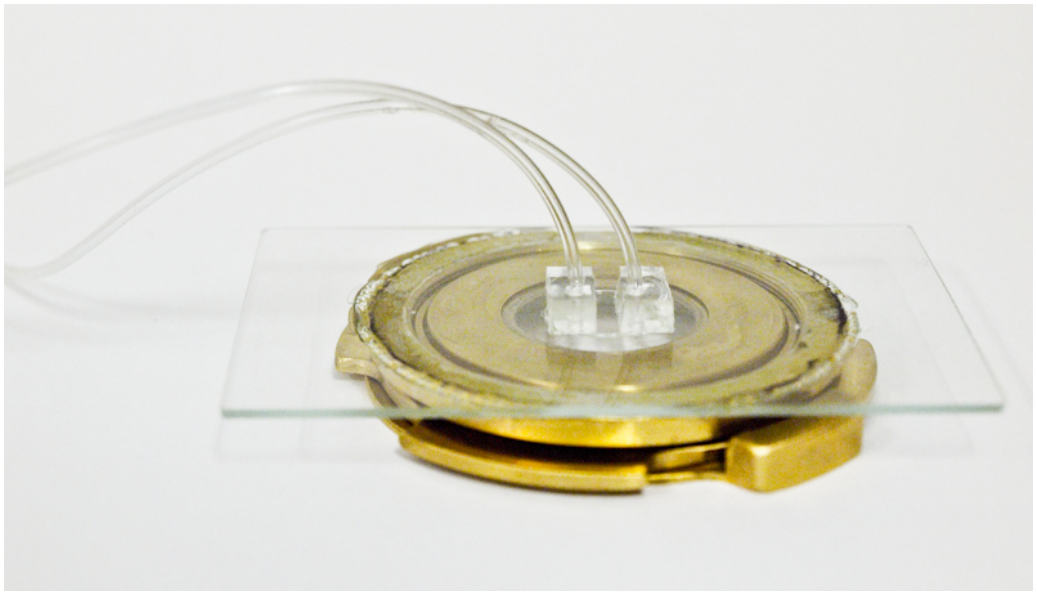
Figure 4: A slightly modified Aufderheide compressor that includes an elaborate perfusion system made out of PDMS channels and metal tubing connectors. **(a)** Photograph. **(b)** Schematic, shown without the mechanical parts of the compressor.



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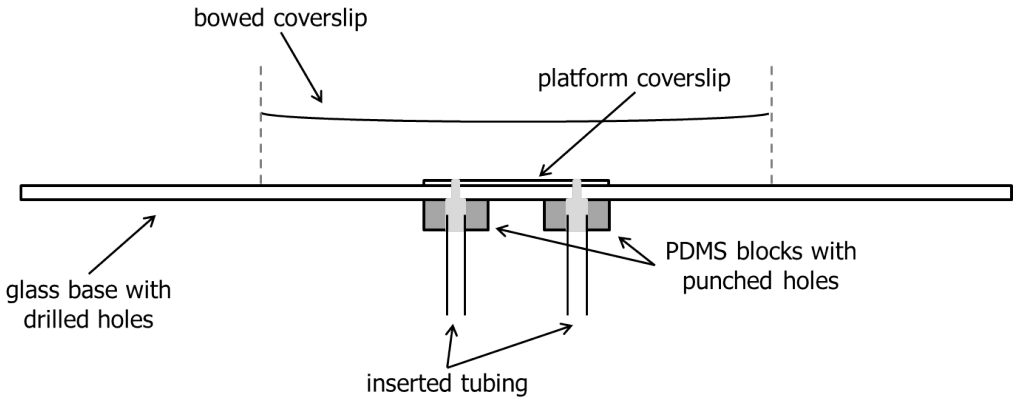


(b)

Figure 5: Compressor, modified so that perfusion tubing is directly inserted into attached PDMS blocks. **(a)** Photograph of the bottom of the compressor. **(b)** Schematic.

of plain PDMS bonded directly underneath the perfusion holes (Figure 5). Ports aligned with the holes in the glass base were punched through the PDMS blocks. To perfuse fluid, tubing was inserted directly into the ports. Alternatively, to reduce stress on the PDMS blocks from repeated insertion and removal of tubing, simple secondary connectors were made from 10 μL micropipette tips. This approach has the advantage of sheer simplicity, as well as making perfusion priming largely unnecessary. However, the fact that tubing is connected perpendicularly to the glass base poses a significant disadvantage. On some microscopes, particular certain upright microscopes used in my laboratory, the condenser must be brought very close to the specimen plane for proper Köhler illumination. There is simply not enough room to accommodate the tubing protruding from the base of the compressor. For that reason, the method of directly inserting tubing into PDMS cannot yet completely replace the approach of fabricating PDMS channels as described in Section 2.2.2.

2.2.4 Operation of a perfusion-enabled compressor

Perfusion was driven by a syringe pump (Harvard Apparatus PHD 2000). Two 10-mL plastic syringes (BD Biosciences) were mounted on the syringe pump, one on either side of the moving mechanical bar, to create a reciprocal infusion/refill system in which the two identical syringes were moved in opposite directions at equal rates. Tubing extended from each syringe and was fitted onto the steel tubing connectors on the base of the device. If priming the channels was necessary (Section 2.2.2), the pump was run at a high rate ($\sim$ 10 mL/hr) until the input channel was filled. The coverslip mount was removed, and excess liquid that leaked out of the input channel was dried using a tissue wipe. A specimen was then placed in a small droplet (typically $\sim$ 1 mm diameter) onto the center of the viewing area, and the coverslip mount replaced. Under a stereoscope, compression was applied by turning the inner ring, which rotated and lowered the coverslip mount, until the specimen was barely flattened. Further compression was carefully applied under 4x or 10x objective magnification on a full microscope, either upright or inverted, until the desired level of flattening was reached. Perfusion was turned on at a rate of 0.5 to 2 mL/hr, depending on the specimen.

2.3 Mechanical improvements: The Commodore Compressor

In parallel with the incorporation of perfusion into the compressor, modifications, largely carried out by my adviser, were made to the mechanical parts of the original Aufderheide compressor to make a more robust, precise and flexible device. The new device, now termed the Commodore Compressor, is depicted in Figure 6 and represents a significant advance from the Aufderheide unit in the following ways:

1. The coverslip mount is able to bow the top coverslip to an even greater degree, which improves trapping consistency.

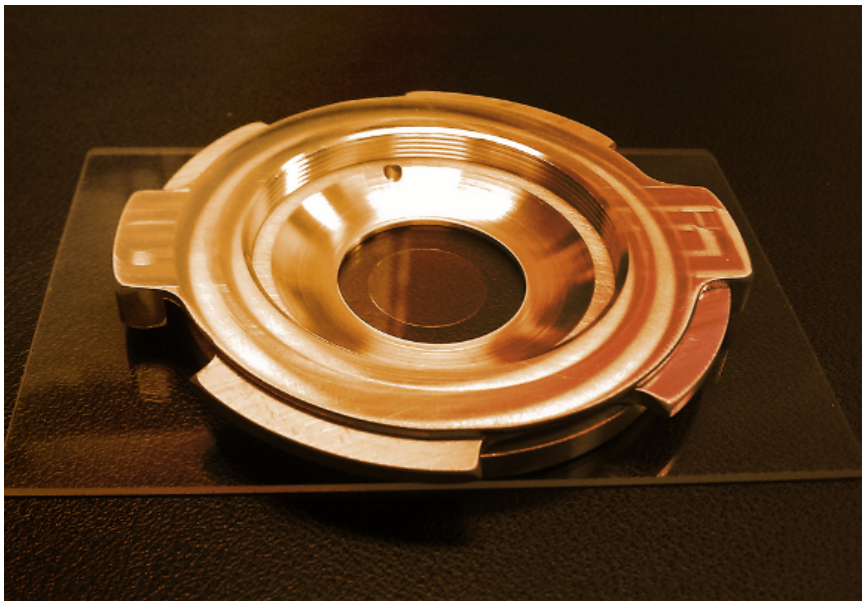


Figure 6: The COMMODORE COMPRESSOR.

2. The coverslip mount and the inner ring can now be locked into each other, ensuring a tight fight. More importantly, the COMMODORE COMPRESSOR may be used upside down on an inverted microscope without risking the coverslip mount falling.

3. Every metal piece is thicker to increase the success rate of machining and to better resist abuse.

4. All pieces are mass produced on a computer-controlled milling machine. The ability to have many prototype devices on hand has become incredibly useful during the research phase.

The basic COMMODORE COMPRESSOR without a perfusion system is now available from Vanderbilt University[1].

2.4 Immobilization of *S. cerevisiae*

2.4.1 Methods

The COMMODORE COMPRESSOR was employed to immobilize the budding yeast *Saccharomyces cerevisiae*. Using a bioluminescent yeast strain (CEN.PK113-7D[14], Carl Johnson laboratory), the preliminary goal was to trap many yeast cells in the compressor and visualize the luminescence of constitutively expressed firefly luciferase. If successful, then the

[1] `http://compressor.vueinnovations.com`

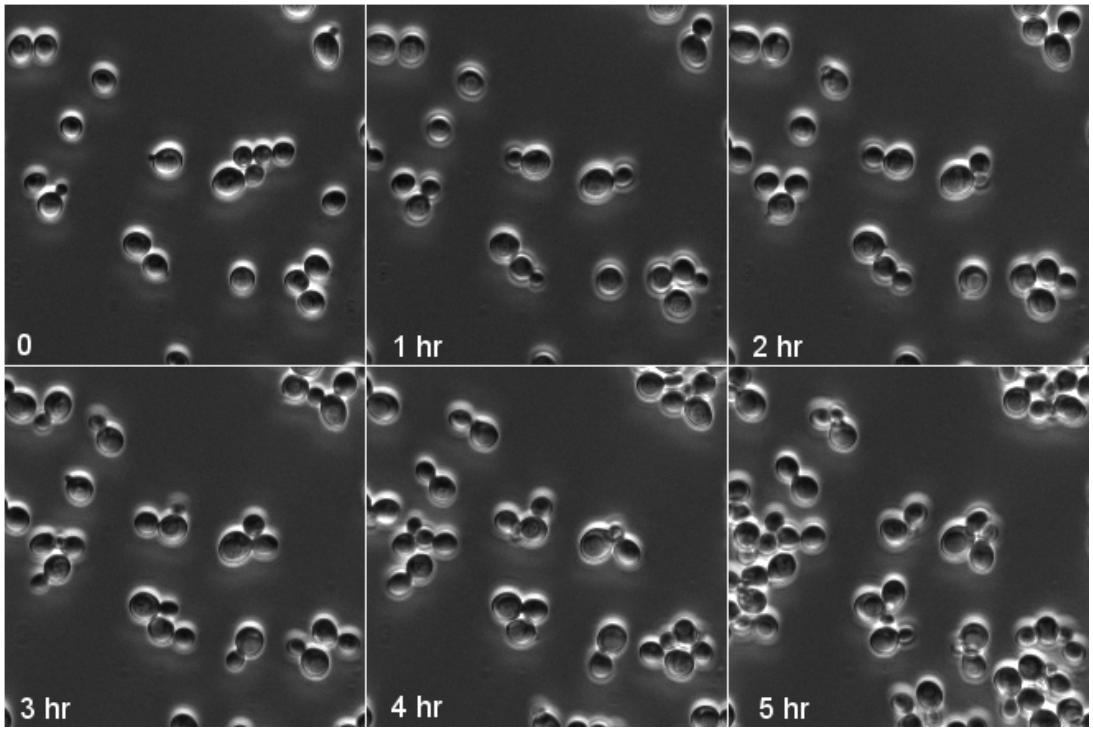


Figure 7: Trapped *S. cerevisiae* cells growing inside the compressor in room temperature. YPD medium was perfused at 0.5 mL/hr.

luciferase could be attached to various promoters to examine the bulk activity of specific proteins during the cell cycle of synchronized yeast.

To carry out an experiment, a tiny drop of somewhat concentrated yeast solution in YPD was placed onto the center of the compressor's viewing area and compressed according to Section 2.2.4. YPD medium was perfused at a rate of typically 0.5 mL/hr. Some yeast cells would be swept away by the perfusion, since not all cells could be trapped equally given the bowed shape of the top coverslip. The remaining cells were monitored over time, with the compression adjusted as necessary.

2.4.2 Results

Prior to visualizing bioluminescence, a precursor experiment was carried out to confirm that cells would continue to grow while trapped inside the Commodore Compressor. Indeed, budding was observed in immobilized yeast cells (Figure 7). After a budding event both mother and daughter cells generally remained trapped, although in rare instances either the daughter cell or both cells were swept away by the perfusion stream after budding. Occasionally, dislodged cells upstream of the viewing area entered the region of interest and became stuck, causing what appeared to be a sudden increase in cell count (e.g. the 4-

hr to 5-hr transition in Figure 7). This phenomenon could easily be reduced by decreasing the initial concentration of cells loaded into the compressor.

To visualize the bioluminescence, the experiment was carried out with the setup in the Johnson laboratory, where light-blocking panels around the microscope blocked out stray light and enhanced sensitivity. However, although spectrophotometric readings on the yeast cells verified their light output, no luminescence whatsoever could be observed with the microscope. This was true with both cells trapped in the compressor and cells on a plain coverslip.

2.5 Immobilization of *C. elegans*

The procedure for trapping *C. elegans* was similar to that for yeast, with small differences. The particular strain of *C. elegans* used (*wdIs52*, *F49H12.4*::gfp + unc-119 [15], David Miller laboratory) contained GFP integrated into its genome. Worms around the L3 stage were picked from NGM Petri plates into a well of M9 buffer, the standard buffer for *C. elegans* [16]. A worm was then transferred from the well onto the viewing area of the compressor in a small droplet no more than 2 mm in diameter. The rest of the procedures remained the same.

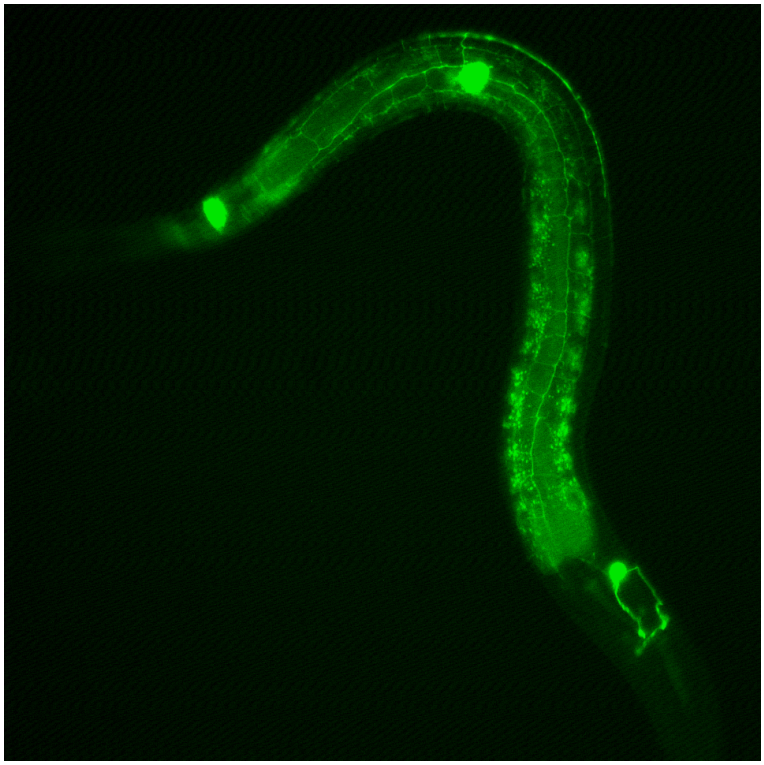
2.5.1 Stock Commodore Compressor (glass-glass)

Experiments quickly revealed that the greater strength of the worms compared to single cell organisms made them more difficult to immobilize. In fact, a worm in the compressor tended to struggle until it was fully overcome by the compression. Predictably, in these circumstances the viability of the worm was not very long. Neuron death, visualized by the formation of bright "beads" along the axons accompanied by the general loss of fluorescence throughout the entire neural network (Figure 8), was observed in trapped worms. It was reasoned that a worm could live longer if it was more gently compressed, but then the worm would not be well immobilized.

After serious thinking, it was hypothesized that the worm could be better trapped if the smooth, silanized glass surfaces were replaced by PDMS, which was less slippery and more elastic. Another solution would be to introduce PDMS microstructures into the compressor chamber such that a worm would be constrained laterally in addition to the vertical pressure. Thus began the search for the optimal design of PDMS structures that would best confine worms.

2.5.2 Circular PDMS mound

In the earliest and most basic attempt, a circular PDMS "mound" was placed inside the compressor chamber in lieu of the 12 mm platform coverslip (Figure 9). The design was in fact a set of two mounds, with a larger one on the bottom about 1 cm in diameter and a smaller one on top sized to fit between the two perfusion holes. During PDMS fabrication, the larger mound was created by simply pouring PDMS directly on the glass base, after



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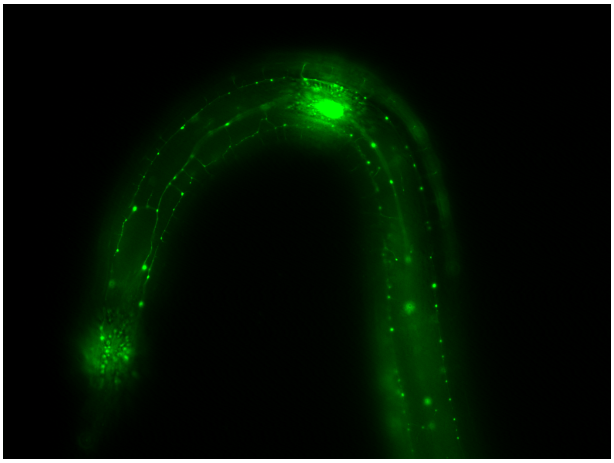

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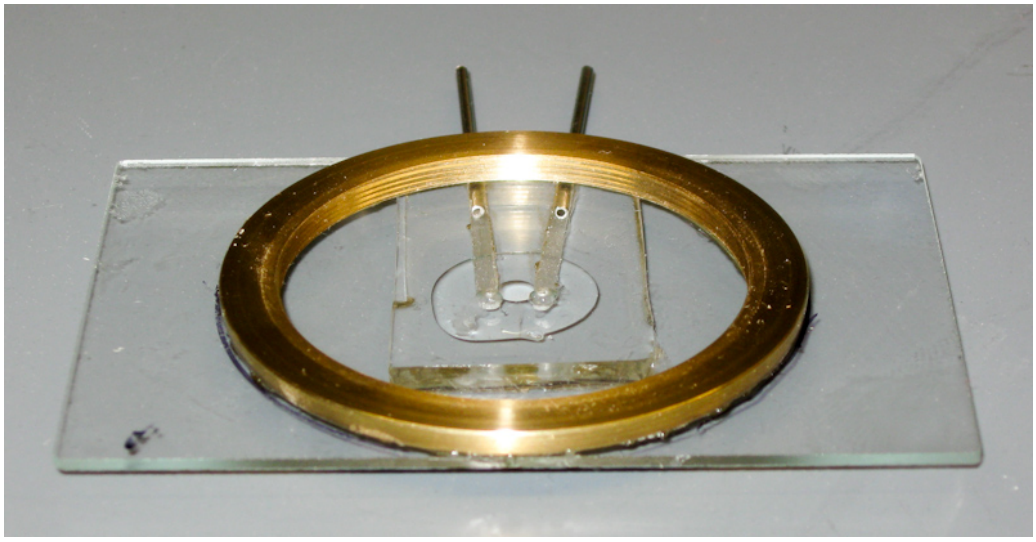
Figure 8: Comparison of healthy and beading neurons in the same worm, (a) shortly following compression and (b) after 1 hour and 9 minutes of continuous compression in the stock Commodore Compressor. The differences in the outlines of the axons and dendrites are striking.



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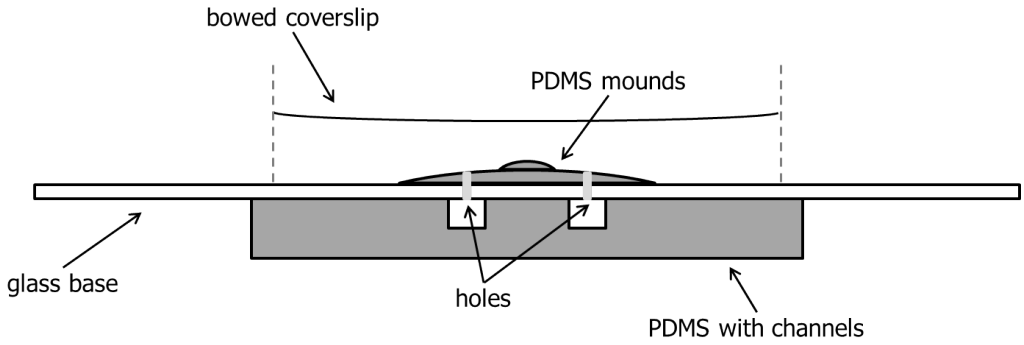


(b)

Figure 9: A set of PDMS mounds for trapping *C. elegans*. Note the presence of a smaller inner mound, on which the worm was immobilized, and the larger outer mound, which served to raise the inner mound as well as increase its contact angle. The perfusion system shown was of the PDMS channels type described in Section 2.2.2, except that the tubing connectors had not yet been bent. **(a)** Photograph. **(b)** Schematic. The curvature of the mounds is exaggerated for clarity.

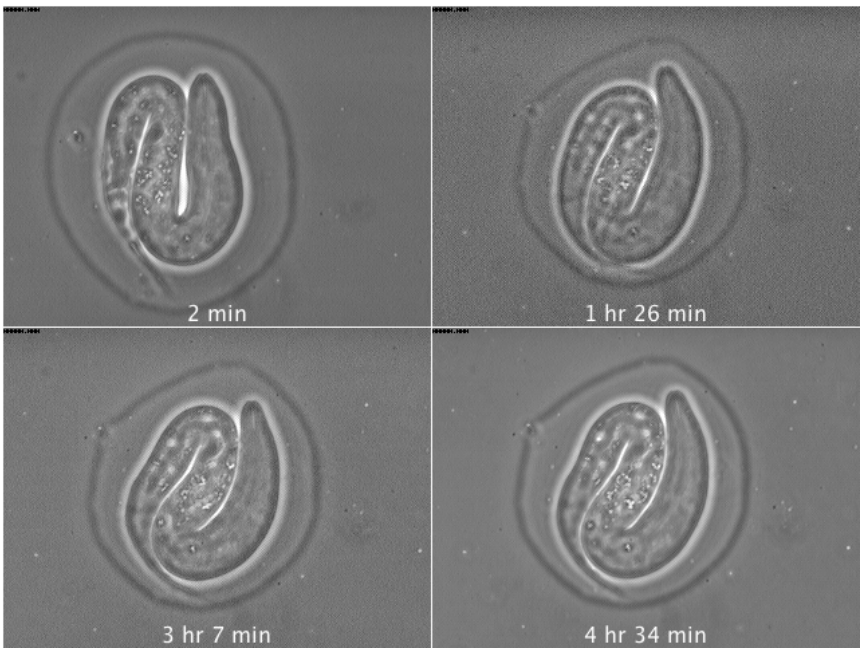


Figure 10: *C. elegans* trapped in the perfusion-enabled compressor with a set of PDMS mounds. M9 buffer was perfused through the chamber at 0.5 mL/hr. The dark ring around the worm in each image was a pocket that isolated the worm from the perfusion stream. Images were taken under 40x objective with phase contrast.

the perfusion holes had been sealed using transparent tape. After curing, a drop of PDMS was placed on top to create the smaller mound using a sharp tool such as a pipette tip. Once the second round of curing was completed, holes were punched through the larger mound and the transparent tape to meet with the perfusion holes.

There were several reasons for having two mounds instead of one. The bigger mound by itself would have created a large contact area with the top coverslip; the friction between PDMS and glass would have prevented further application of compression once the two surfaces touched. As for the smaller mound by itself, a PDMS drop of that size placed on the glass base would have cured almost completely flat and thus unable to touch the top coverslip of the compressor. As such the larger mound not only served to raise the smaller mound but also to increase its contact angle, since PDMS is much less hydrophilic than glass. Indeed, the implausibility of having either platform by itself had been confirmed by experiment.

The results of a typical experiment (Figure 10) demonstrated that the worm could be sufficiently immobilized. However, the PDMS mound was considered a failure because it failed to allow the perfusion stream to reach the worm. As compression was applied, the worm was pushed into the PDMS due to the polymer's high elasticity, effectively burying

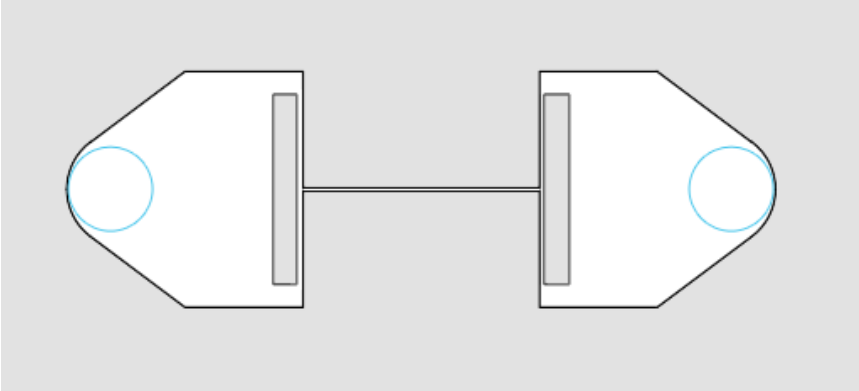


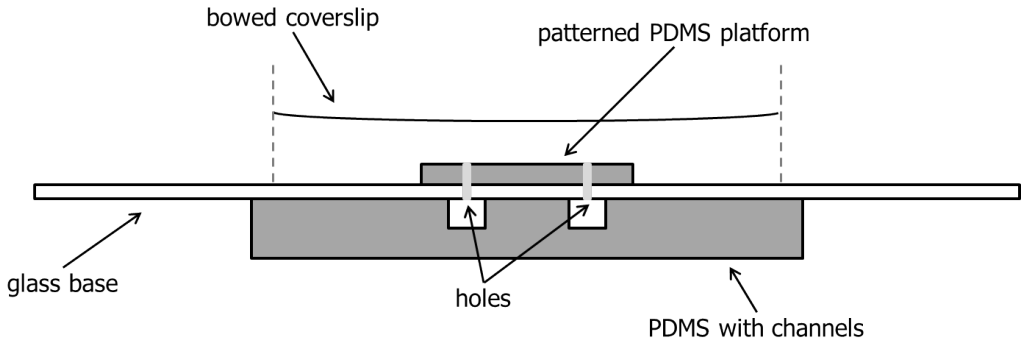
Figure 11: Channel structures on the surface of the single-channel PDMS platform. The central channel measured 2 mm long by 30 μm wide. The structure height was typically between 10 and 20 μm. Entrance and exit ports for perfusion are marked with blue circles. Fluid would flow out of one blue circle through the areas marked in white into the other blue circle. The center-to-center separation between the ports was 5.0 mm, same as the separation between the drilled perfusion holes in the glass base.

the worm in as the PDMS surrounding the worm subsequently created a tight seal. The dark circle around the worm at each time point in Figure 10 in fact marked where the PDMS had isolated the worm. Given the lack of fresh liquid medium, each worm trapped this way eventually died.

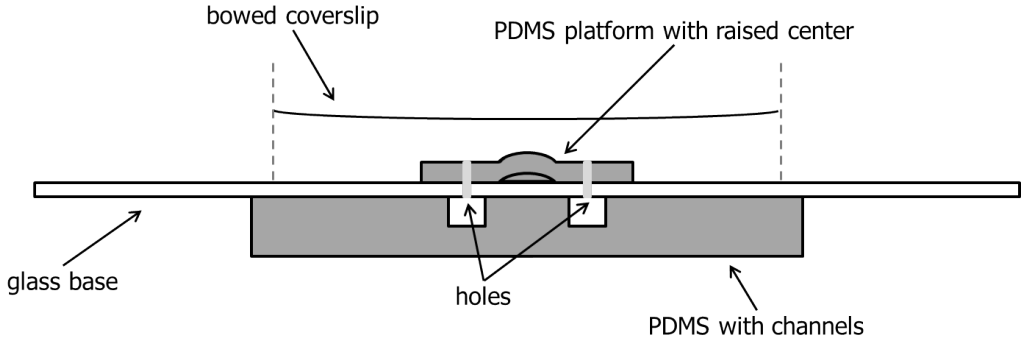
2.5.3 Single-channel PDMS platform

After the failure of the simple PDMS mound, the next PDMS platform (Figure 11) tried to actively shape perfusion to flow past the trapped worm. The design consisted of a single channel, 2 mm long by 30 μm wide, into which a worm could be manipulated during loading using a sharp tool (such as a wire or a glass pipette tip) and subsequently trapped. The two vertical "bars" (shown in gray in Figure 11), placed 30 μm from the junction, prevented the worm from escaping the channel (unless it was too small) it could not wiggle its body in such a way to fully turn the corner. Prior to loading, a buffer-filled well containing the worm to be trapped was placed on ice for 5 minutes to retard the worm's movement.

To fabricate this PDMS platform, the full photolithography and microfabrication techniques were pursued (Section 2.1), with some differences in procedure necessitated by the geometry of the compressor itself. Because the patterns were designed to face up, instead of plasma bonding PDMS in the conventional manner with the channels structures down, bonding had to be done in the reverse orientation. Furthermore, as with the circular mounds when the larger mound was tried by itself, the PDMS surface tended to contact a large area of the top coverslip of the compressor, causing high friction that prevented fur-



(a)



(b)

Figure 12: Perfusion-enabled compressor with integrated PDMS platform: **(a)** flat profile, **(b)** with raised center. The curvature is exaggerated for clarity.

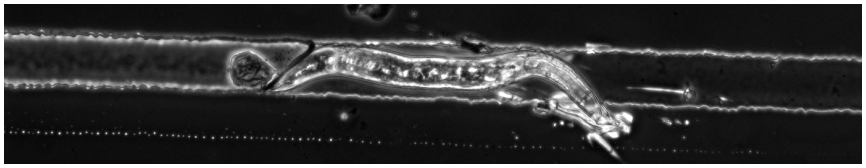
ther compression by rotation. To combat this problem, a small drop of PDMS (~2–3 mm in diameter) was placed onto the glass base in between the perfusion holes and cured, before the subsequent bonding of the PDMS platform itself. The small drop served to elevate the center of the PDMS platform so that the top coverslip could make contact with the center first. Figure 12 compares the two methods of bonding the PDMS platform, with and without the raised center. The downside of raising the middle of the PDMS platform was that the perfusion stream must be forced through a narrower region with greater resistance as it enters the central channel. If the resistance was high enough, flow could not be obtained. Achieving a delicate balance between good top coverslip contact and ease of perfusion had been a persistent issue for this and subsequent PDMS platform designs, until the method to hydrophilize PDMS channels to help flow was employed (Section 2.5.6).

Experiments revealed much about the advantages and shortcomings of the single-channel PDMS platform. In particular, manipulating a worm into a channel proved to be a difficult task, even with the use of ice to slow the worm's movement. No matter how long a worm was left on ice, it usually recovered within 30 seconds in room temperature. Chilling the compressor also did little to help. In addition, an important limitation was that the worm must constantly be immersed in liquid; if the drop of buffer dried out while the worm was on the PDMS platform, the worm died. Nevertheless, for the times that the worm was successfully manipulated into the channel, the subsequent trapping was easy and efficient. The side walls of the channel largely restricted the worm's wiggling (Figure 13a). The junction also worked well in preventing the trapped worm from escaping (Figure 13b). To demonstrate that the worm could be held completely still, a vertical stack of 8 fluorescent images, sometimes called a "Z-stack," of the worm was taken, with a Z-axis step size of 1 μm. Following image acquisition, the 8 slices were flattened into a 2D projection image (Figure 13c) using the *Wavelet-transform based focussing* stack-projection algorithm present in the MBF ImageJ bundle. The resulting image showed the worm's neural network as clear, sharp lines, implying that the worm did not move during the entirety of image acquisition. Had the worm wiggled during the Z-stack exposure, the axons or dendrites would have shifted in position across the image slices, giving rise to blurs and/or double images in the projection.

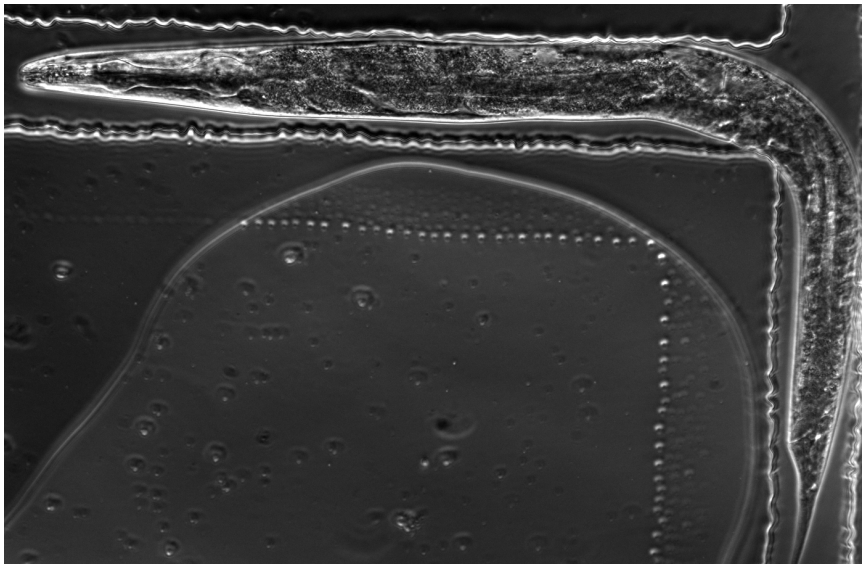
Despite the occasional experimental success in trapping *C. elegans* using the single-channel PDMS platform, a more serious setback was encountered: like compressing a worm against glass surfaces in the stock Commodore Compressor (Section 2.5.1), beading of the neurons was observed in most experiments. Viability of the trapped worm could not be maintained beyond a few hours at most. Thus, for both procedural and viability reasons, a better PDMS platform design was sought.

2.5.4 Agar variant

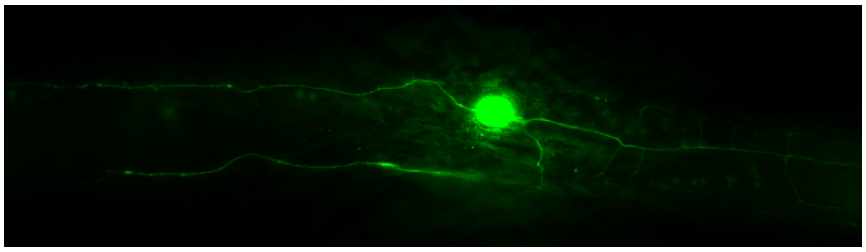
As mentioned in the context of the single-channel PDMS platform, experimental evidence suggested that worms placed onto a PDMS platform must remain immersed in liquid buffer to remain viable. Yet given that worms grow on agar NGM plates without being constantly



(a)



(b)



(c)

Figure 13: Representative images of a *C. elegans* trapped in the perfusion-enabled compressor with the single-channel PDMS platform. **(a)** 10x objective, phase contrast. **(b)** 20x objective, phase contrast. The worm was situated at the end of the channel but could not escape. **(c)** 40x objective, green fluorescence; a Z-stack of 8 images (step size of 1 μm) was collapsed into one image. The lack of blur in the collapsed image demonstrates good immobilization.

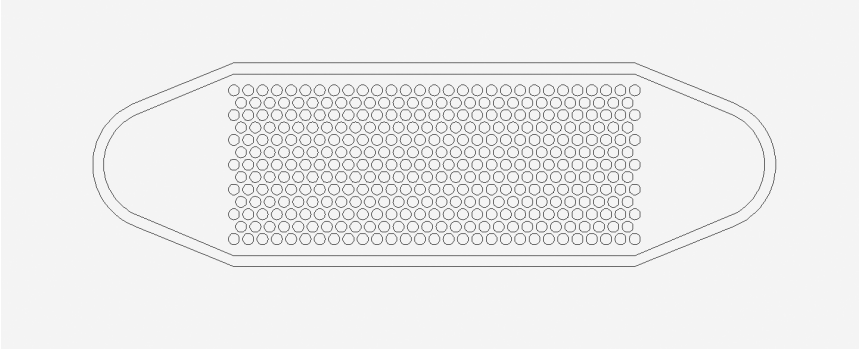


Figure 14: Schematic of the artificial soil design for the patterned PDMS platform. The closest distance between two circular posts was 30 μm, approximately the (uncompressed) diameter of a trapped worm. Structure heights varied between 10 and 20 μm. The platform was bonded onto the glass base of the compressor using the "PDMS drop" technique (Section 2.5.3) to elevate the center. Holes for perfusion were punched in the post-free regions inside the outer wall that surrounded the posts.

surrounded by a droplet (because LB agar has a high liquid content), it was speculated that if patterned platforms could be made out of agar instead of PDMS, then they would be able to maintain the worm's viability without requiring it to be immersed in buffer.

Predictably, procedures for making the single-channel agar platform differed from those of its PDMS counterpart only in that instead of PDMS, LB agar in M9 buffer was cast onto the master. A variety of concentrations were used, from 6% up to 10% weight/volume. More concentrated agar better resisted deformation from the worm's movements, at the cost of becoming less transparent to light for bright-field imaging. Higher concentrations could not be achieved due to physical limits in solubility. Since the agar platform could not be strongly bonded to the glass base, a solution was devised to hold the agar block in place. A rectangular "fence" of PDMS was plasma bonded onto the glass base, and afterward the agar block was fitted inside the PDMS fence, trimming the edges of the agar as necessary. Although the agar adhered to the glass only by conformal contact, the PDMS fence prevented the agar from rotating during compression.

After several trapping experiments employing the single-channel agar platform, it became clear that the agar platforms performed poorly. *C. elegans* could be trapped but not fully immobilized due to the softness of the agar. Furthermore, a trapped worm was strong enough to escape the channel structure by tunneling through the agar, even at the highest possible agar concentration of 10%. The idea of making patterned platforms from agar instead of PDMS was abandoned, at least for immobilizing *C. elegans*.

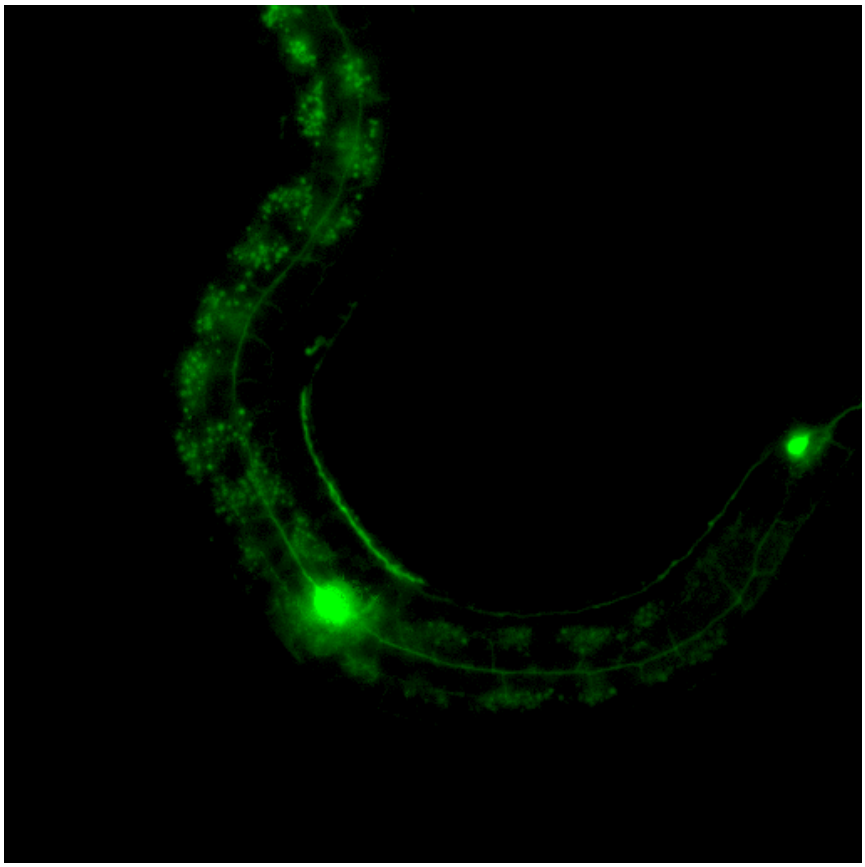


Figure 15: *C. elegans* immobilized in the compressor employing the artificial soil PDMS platform. Depicted is a 2D projection of a 3D stack (same processing technique as Figure 13c). Coherent, unblurred outlines imply that the worm remained still for the duration of the image stack acquisition.

2.5.5 Artificial soil: field of circular PDMS posts

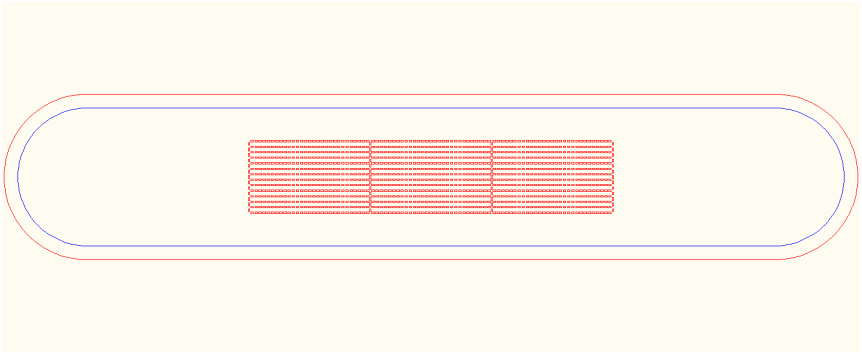
Inspired from a published PDMS device design that used circular patterns to create an artificial soil environment for *C. elegans* [17], a patterned PDMS platform containing structures of similar design was created (Figure 14). Compared to straight channels, the artificial soil design could permit confining a worm in a more natural morphology, leading to reduced stress and possibly increased viability time. Experimentally, it was seen that *C. elegans* did conform well among the posts, adopting a naturally bent shape (Figure 15). However, the trapping success rate was very low; barring the most lucky of circumstances, most worms simply crawled forward and escaped compression. To increase the efficacy of trapping, it became necessary to design another patterned PDMS platform.

2.5.6 PDMS platform: array of rectangular slots

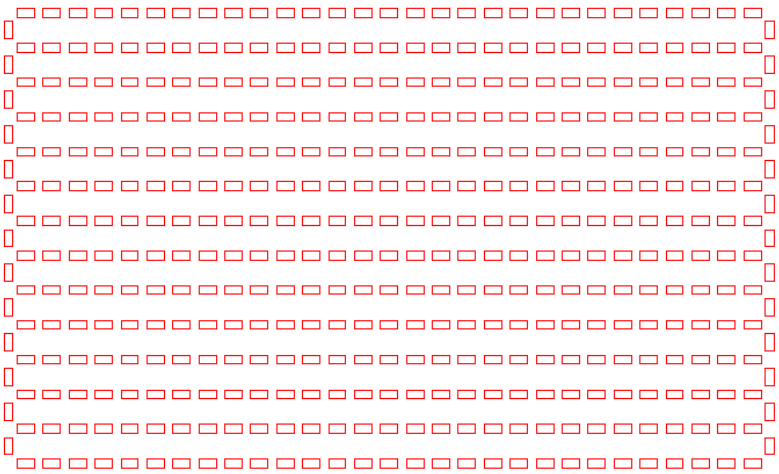
As an advancement from the single-channel design (Section 2.5.3), a PDMS platform featuring an array of rectangular "slots" was designed (Figure 16). Each slot was intended to fit a *C. elegans* lengthwise, and the placement of many slots in an array would maximize trapping ease. In practice, due to the difficulty of physically manipulating a worm into one of the slots, compression was freely applied, and the field of posts served to trap a free-swimming worm at several localized spots. A worm often became pinned at a few places on its body but could still wiggle its head and tail.

During initial testing of the PDMS platform, it was noted that the very dense layout of the posts and the small gaps between them created too much resistance for liquid buffer to easily flow through. Since the center of the platform was slightly elevated due to the presence of the PDMS droplet underneath (a result of the technique discussed in Section 2.5.3), the high flow resistance caused the perfused buffer to flow around the central area where the trapped worm would be located. To remedy this serious setback, it was found in literature that PDMS's surface, normally hydrophobic, could be made hydrophilic by exposure to ultraviolet (UV) light and ozone in a specially designed equipment called a UV/ozone cleaner [18][19]. Whereas oxygen plasma treatment hydrophilizes PDMS surface for only a few minutes, UV/ozone treatment lasted months. The procedure was simple and worked regardless of whether the PDMS had already been bonded to the rest of the compressor. In preparation, the areas on the PDMS that were to remain untreated—in this case, the area beyond the outer wall of the PDMS structures—were covered with transparent office tape. The PDMS was placed into the UVO Cleaner (Jelight Company Inc) and treated for 15 minutes. Durations longer than 20 minutes caused cracking of the hardened PDMS surface.

Experiments confirmed that liquid buffer flowed rapidly across the treated, hydrophilic area of the PDMS. Trapping was easily achieved; given that the platform had been properly made, the success rate of trapping approached 100%. Biologically, however, neuron death evidenced by beading remained a problem. After conducting extensive controlled tests, phototoxicity was ruled out as a possible reason for beading of the neurons. Worms

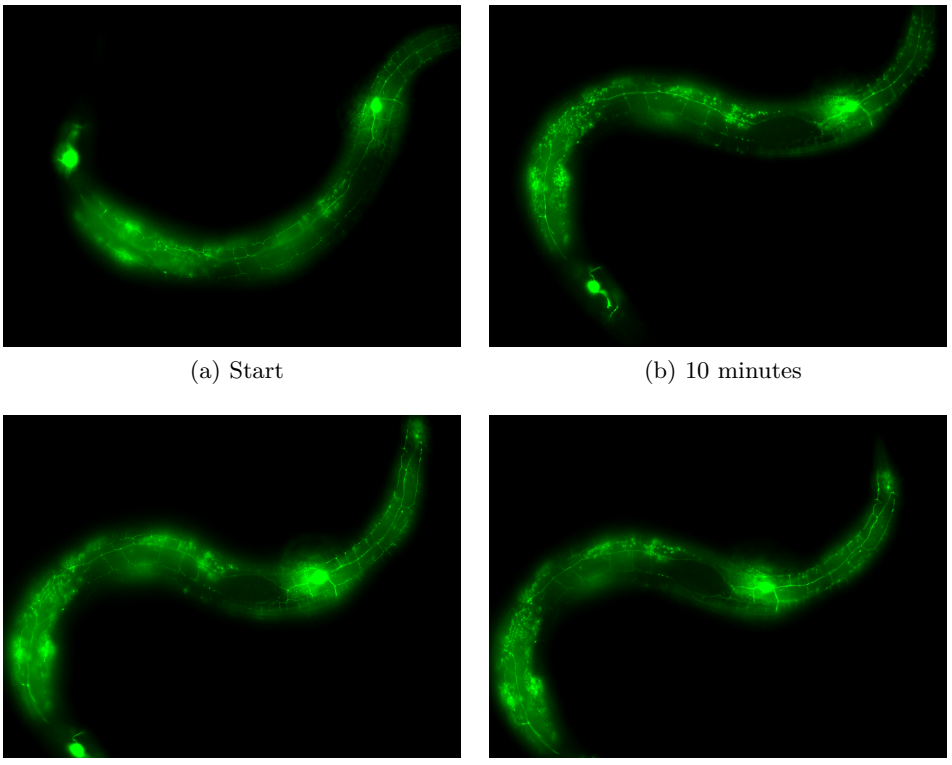


(a)



(b)

Figure 16: Design schematic of the PDMS platform containing an array of long rectangular slots. A series of posts with a $10\,\mu$m $\times$ $20\,\mu$m cross section formed the lining of each $830\,\mu$m $\times$ $30\,\mu$m slot. The post capping the end of each slot was of the same size. The distance separating adjacent posts was $5\,\mu$m. As with previous PDMS platform designs, holes for perfusion would be punched at either end of the device inside the outer wall, and a PDMS droplet was used to elevate the center prior to plasma bonding, as described in Section 2.5.3. **(a)** The entire design, including the pill shaped boundary wall. **(b)** Detail of a subset of the grid structure, designed to fit a *C. elegans* into any one of the long rectangular spaces lined with posts.



(a) Start (b) 10 minutes

(c) 20 minutes (d) 30 minutes

Figure 17: *C. elegans* trapped in a compressor employing the PDMS platform with array of slots. The slots were not utilized; the worm was simply pinned down. The worm was loosely held and wiggled during image acquisition and between time points. For each time point a stack of images was taken; depicted here is the slice in best focus from the stack.

were exposed to continuous excitation light for 10 minutes, followed by compression and immediate observation. No beading of the neurons was observed for each worm tested. It was assumed that compression would not *immediately* cause beading, which was most likely true given past experimental observations. The viability of worms in M9 buffer alone without compression was also confirmed by overnight incubation. More recent experiments demonstrated that *C. elegans* viability seemed to improve if the worm was loosely held in the compressor (Figure 17), indicating that too much compression was likely to harm the organism.

3 Discussion and conclusions

3.1 Compression and *C. elegans* viability

Regardless of the type of PDMS platform used, *C. elegans* immobilization experiments hint at the trend that the tighter the compression, the shorter the specimen viability. The relationship is intuitive and not unexpected; it is reasonable to hypothesize that greater compression leads to greater stress and malcontent in the worm, and thus reduced viability. However, what remains puzzling is that it has been demonstrated that *C. elegans* has been physically immobilized for extended periods of time in microfluidic devices [13][20][21], apparently without ill effects, although the true success rates of these devices are typically not stated. What features were different about the compressor (plus various PDMS platforms) that caused it produce less viable results? It is possible that certain vital organs in *C. elegans* were over-stressed by the compressor, although one would expect that the same level of physical stress would be also be imposed by microfluidics devices if the same level of immobilization were to be achieved. It is more likely that trapped worm were suffocating inside the compressor: the top coverslip was not air permeable like PDMS, and the amount of fresh oxygen supplied by the perfusion was not enough to keep the trapped worm healthy. Another reason for the shortened viability might be that worms do not survive for long under *continuous* compression; published works that employed microfluidic devices generally seem to avoid continuously applying stress on the worm. Further research is needed to determine the cause for short worm viability inside the compressor and to make design choices that would avoid these shortcomings. Given that there exist many advantages such as precision, ease of loading, and optical superiority to the use of an open-faced device like the compressor over the use of microfluidic devices, the effort to employ the compressor to immobilize *C. elegans* remains a worthy pursuit.

3.2 Other applications

Although the present work concerned the study of only two specimens, *S. cerevisiae* and *C. elegans*, the Commodore Compressor may be easily adapted for other specimen types and experimental scenarios. For instance, some fellow laboratory members are using the device to compress the social amoeba *Dictyostelium discoideum* for total internal reflection

fluorescence (TIRF) microscopy. Other laboratory members have begun investigations using the model organisms *Tetrahymena thermophilia* and *Euplotes vannus*. With a working perfusion system to allow long-duration trapping, plus infinite possibilities in the design of the optional patterned PDMS insert, the COMMODORE COMPRESSOR is a truly versatile device.

4 Acknowledgments

The present work would not have been possible without extensive use of VIIBRE's facilities and the helpful advice of Dr. John Wikswo, Phil Samson, Ron Reiserer, and other VIIBRE members. In particular, I would like to thank Gideon Searle and Dr. Kevin Seale for the SyBBURE program, which introduced me to the exciting world of microfluidics.

Many others have been influential during this long but rewarding journey. Instrumental to the construction of the COMMODORE COMPRESSOR were John Fellenstein and Bob Patchin, masters of their art at the physics machine shop. I would like to also thank Vanderbilt's Beckman Scholars Program, headed by Dr. Jeffrey Johnston, for its generous support. In the laboratory, I am indebted to my fellow researchers as well as my collaborators for their encouragement and support. Above all, I would like to give my sincere thanks to my adviser, Dr. Chris Janetopoulos, whose unwavering support and passion fueled the train of scientific progress.

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