Microfluidic *In Vivo* Laser Microsurgery Screen for Identification of Compounds Enhancing Neural Regeneration

by

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Abstract

Discovery of small molecules and novel mechanisms for enhancing neurite regeneration in animal models is significant for therapeutics of central nervous system injuries and neurodegenerative disorders. *C. elegans* is a widely studied model organisms 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 still remained unchanged. This thesis details the development of automated, high-throughput optical and microfluidic technologies for screening *C. elegans* and demonstrates the production of a reliable system for screening over ten thousand animals. Using the screening system, femtosecond laser microsurgery was performed on thousands of animals followed by incubation in compounds from a chemical library. The screens revealed several high-scoring drug candidates that enhance regeneration after laser microsurgery of *C. elegans* mechanosensory neurons.

Thesis Supervisor: Mehmet Fatih Yanik
Title: Assistant Professor of Electrical Engineering and Computer Science

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Chapter 1

Introduction

This chapter provides context for the research into the use of high throughput screening technologies[1,2] using whole animals models to study *in vivo* neural regeneration. The first section explains the motivation behind this research and introduces some of the relevant disease models. The second section gives an overview of the content of each chapter in this thesis.

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[3] as well as intrinsic neuronal factors[4-6]. 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 *in vitro* cell culture systems. However, these systems do not replicate conditions *in vivo*. In addition, off-target, toxic or lethal effects often manifest only *in vivo*. Thus, a thorough investigation of neuronal regeneration mechanisms requires *in vivo* neuronal injury models.

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 *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.

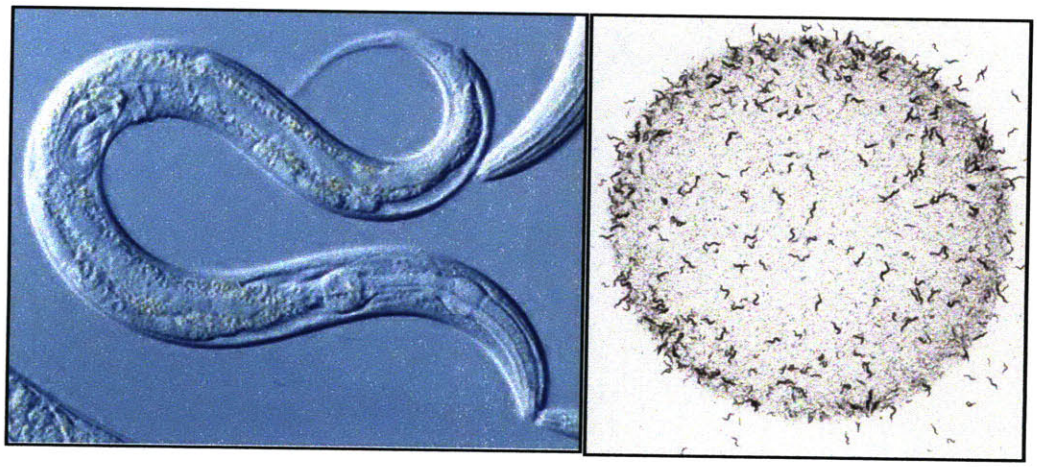


Figure 1. (L) Adult stage *C. elegans* with length of 1mm and diameter of 50μm (R) Cultured in bacteria on agar plate. Image credits: wormbook.org.

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*[7,8]. 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[9,10]. 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[11].

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 (NaN_3), levamisole and tricaine/tetrimisole, have significant and/or uncharacterized effects on nematode physiology, which may affect the regeneration process[12]. 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[13], cooling[14,15] and exposure to CO_2[16,17]. However the physiological consequence of such exposure to low temperatures and CO_2 are unknown.

1.2 Thesis Outline

The next chapter, Chapter 2, introduces in vivo neural regeneration screening of small molecules in combination with laser surgery. Chapter 3 details the fabrication of the microfluidic devices while chapter 4 continues with integration of the microfluidic system. Chapter 5 details the optical components involved in the system for laser surgery and chapter 6 discusses future work and applications of the system.

Chapter 2

In Vivo Laser Microsurgery Screening of Small Molecules

This chapter introduces the microfluidic screening system and discusses the application of the technology to perform large-scale *in vivo* neural regeneration assays. The rest of the thesis continues with the fabrication of the microfluidic devices and system integration.

2.1 Microfluidic System Overview

We developed microfluidic technologies for high-throughput small-animal studies at cellular resolution[1,2], which allow large-scale genetic and compound assays. These technologies rapidly isolate and orient single animals purely by mechanical means. Subsequently the animals are briefly and non-invasively immobilized for sub-cellular resolution imaging and femtosecond-laser microsurgery (**Fig. 2, 3**).

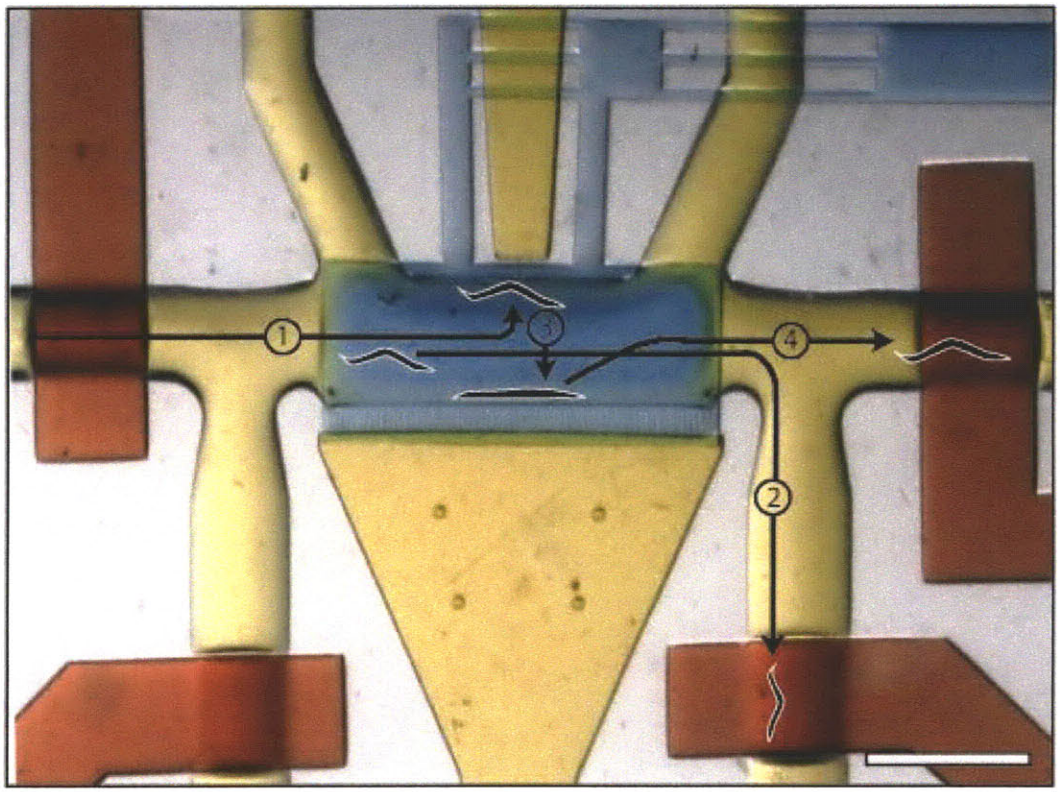


Figure 2. Laser microsurgery screen for small molecules affecting axonal regeneration *in C. elegans*. (a) Micrograph of chip with numbered arrows showing microfluidic *C. elegans* manipulation steps (see supplementary movies). 1: Loading of nematodes and capture of a single animal by one aspiration channel. 2: Washing of the channels to remove and recycle the rest of the nematodes. 3: Release of the captured animal from single aspiration port, and recapture and orientation of it by a linear array of aspiration ports. 4: Collection of the animal after surgery. Scale bar: 1 mm.

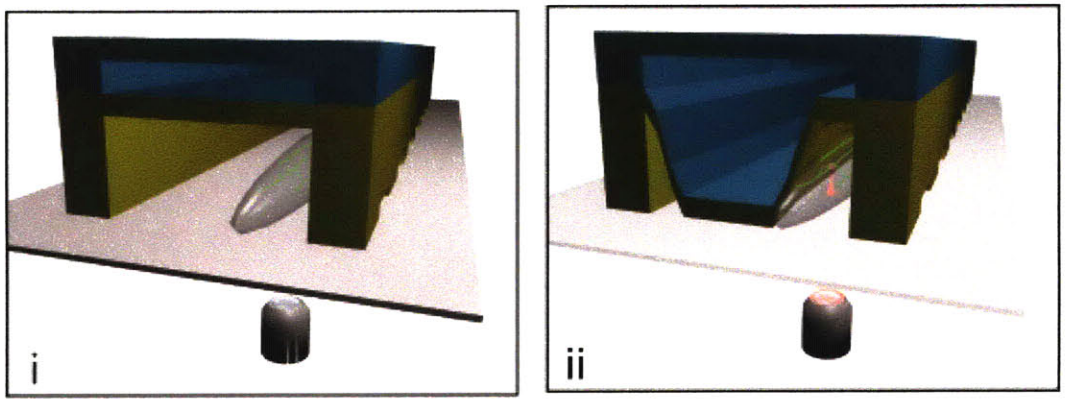


Figure3. Illustration of the final immobilization and laser axotomy: Once a single animal is captured and linearly oriented (i), a channel above it is pressurized pushing a thin membrane downwards (ii). This membrane wraps around the animal significantly increasing immobilization stability for imaging and surgery. Precise laser targeting of sub-cellular features is achieved using a femtosecond laser tightly focused inside the *C. elegans* by a high numerical aperture objective.

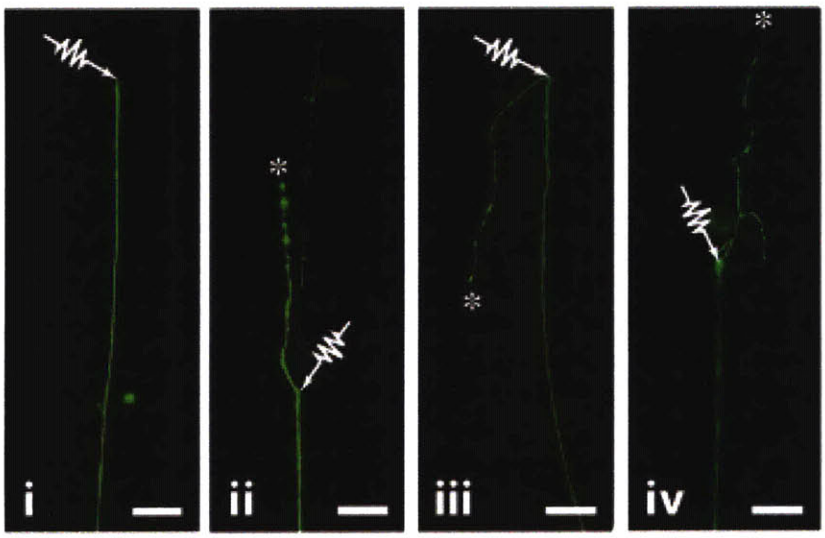


Figure 4. Common regeneration phenotypes observed 72 hours following axotomy and compound exposure: (i) No axon regrowth, (ii) forward regrowth, (iii) backward regrowth, and (iv) regrowth with branching. Arrows (⇝) and asterisks (*) indicate start and end points of regenerated axons respectively. For regrowth with branching, indicated start and end points are for the longest regrown branch. Scale bars: 20 μm.

Using these high-throughput on-chip technologies we screened several thousands of nematodes for regenerative effects upon exposure to members of a chemical library. Fluorescently labeled ALM mechanosensory neurons were axotomized in larval stage 4 (L4) nematodes approximately 200 μm away from the cell body. Mechanosensory neurons grow long axonal processes devoid of any lateral branches. This facilitates highly precise microsurgery and subsequent imaging and characterization of outgrowing processes, which allows investigation of specific modulators of regeneration. Following microsurgery animals were incubated in the presence of small molecules. Axon regeneration was assessed post axotomy by measuring the length of the regrown processes (**Fig 4**).

Chapter 3

Microfluidic Device Fabrication

This section describes the process of fabricating the microfluidic device for screening of C. elegans including photolithography to create template for PDMS layer molding, thermal bonding of PDMS layers, and plasma bonding to cover glass for imaging and surgery.

3.1 Photolithography

Photolithography is a widely used technique to create well-defined structures on the surface silcon wafers. This section will describe the process of fabricating the control mold. Please reference the Appendix A2 for immobilization and flow layer protocol.

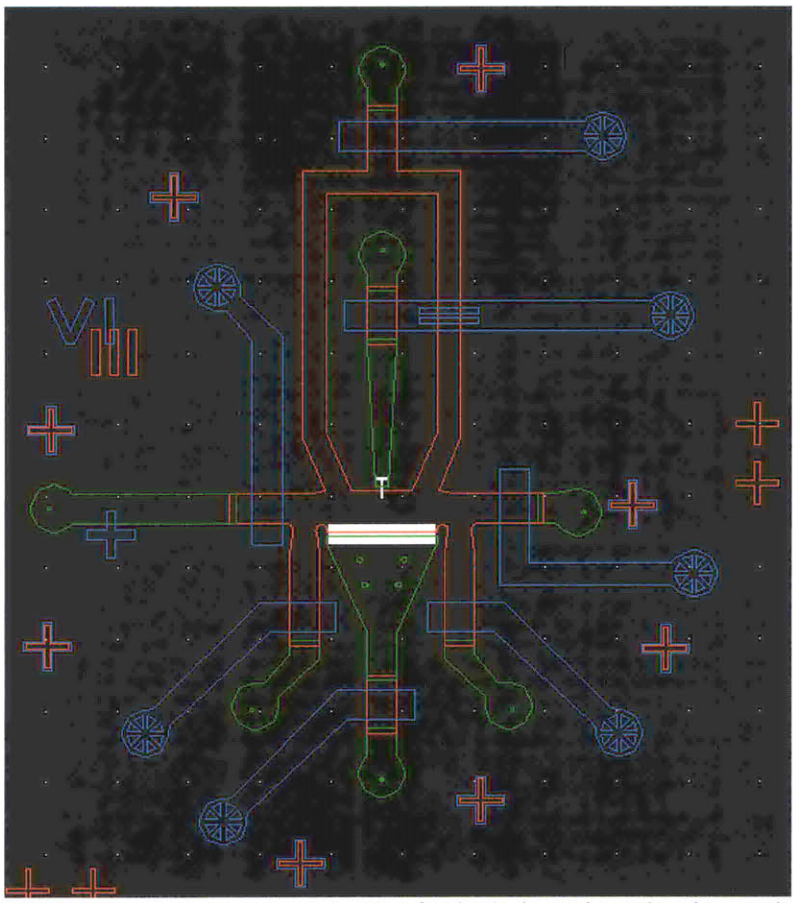


Figure 5. AutoCad design schematic of chip design. The red and green layer indicate flow layer where the animals are transported. The blue layer indicates the control valve layers. The white structures represent the multi-suction and single-suction ports. The crosses are used for aligning multiple layers.

The process begins with pre-treating the standard silicon wafer by dehydrating the wafer on a hotplate for 10 minutes at 135C followed by spincoating HMDS for 10 seconds at 1000rpms to promote adhesion of photoresist. A 70μm thick layer of SU-8 2075 photoresist is then spincoated by ramping the spin speed from 0 to 500rpm over 5 seconds, 500 to 3500rpm for 5 seconds, followed by 3500rpm for 30 seconds. The wafer is then soft baked at 65C for 3 minutes followed by 95C for 8 minutes to make the photoresist layer more rigid. Edge bead removal is necessary if sections of photoresist are raised at the edges to allow flat surface for proper mask contact and alignment. Once mask is aligned the UV lamp is exposed through the mask pattern on the mask to

crosslink the photoresist. The wafer is then post-baked at 65C for 90 seconds followed by 95C for 6.5 minutes. The photoresist is then developed with Polymond Acetate while the wafer is spun at 500rpm to removed the unexposed photoresist leaving the desired structure on the wafer surface. The wafer is then rinsed with IPA at 1000rpm and dried with nitrogen.

3.2 PDMS fabrication

PDMS is a widely used material for fabricating microfluidic devices. The top layer provides structural support for interfacing with the devices. The top immobilization layer is poured onto the immobilization layer mold and degassed to remove air bubbles. The flow layer is then spin coated and both layers are soft baked to provide rigidity before being thermally bonded. The control layer is then spin coated, soft baked and then thermally bonded to the flow and immobilization layers. The necessary holes are punched to access each layer between thermal bonding steps. Please reference the appendix A.3 for fabrication protocol.

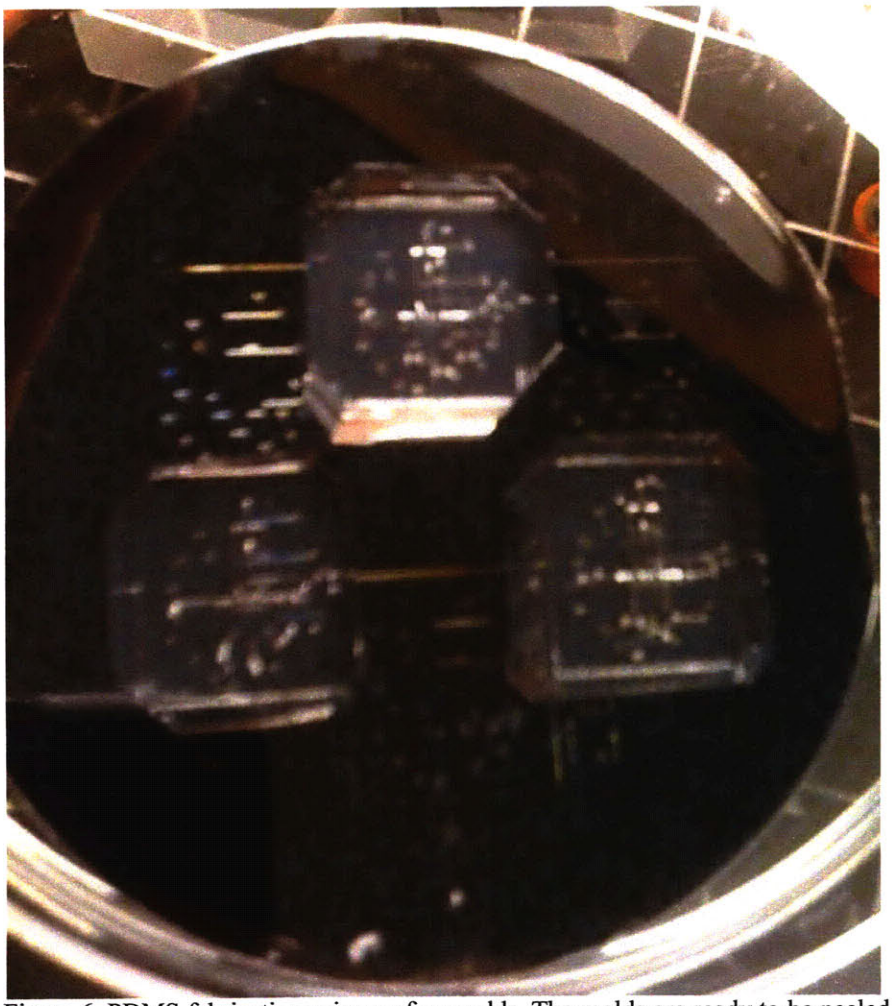


Figure 6. PDMS fabrication using wafers molds. The molds are ready to be peeled off and bonded to cover glass. (Scale: wafer is 4in in diameter)

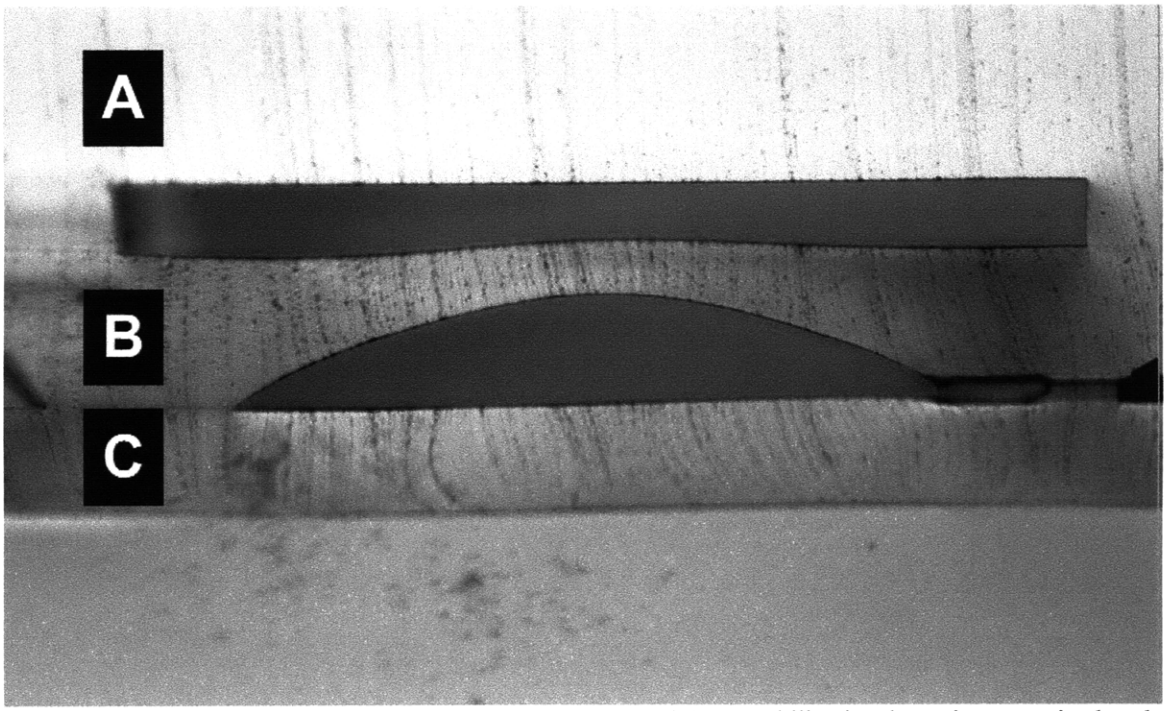


Figure 7. Thermally bonded PDMS layers in cross-section. (A) Immobilization layer is pressurized and pressed down onto layer B. (B) Flow channel where animals are transported. (C) Control valve layer where thin layers are pressurized to close off layer B (valve section not pictured). (Scale : Layer A width is 500μm)

3.3 Plasma Bonding

The PDMS layer are then plasma bonded to the cover glass to form a permanent seal. The cover glass and PDMS device are placed with bonding sides facing up into the plasma bonder then placed under vacuum. The samples are then exposed to RF energy while a small amount of oxygen is allowed to enter the chamber to enhance the plasma reaction.

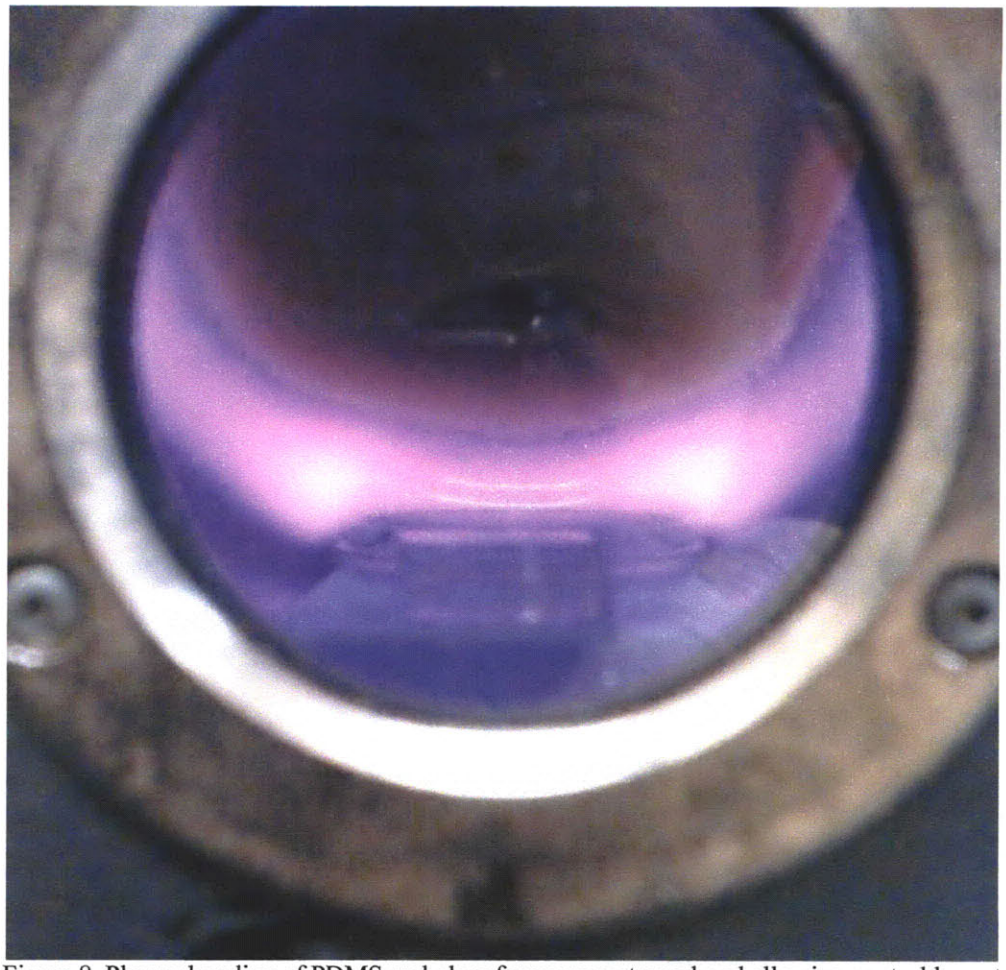


Figure 8. Plasma bonding of PDMS and glass forms very strong bond allowing control layer to be pressurized.

Chapter 4

Microfluidic System Integration

This chapter details the complete microfluidic system that enables automated screening. The system begins with software controlling the national instruments digital card activating the pneumatic valves that operate the quake valves on the microfluidic chip. The pressurized supply bottles are controlled by the on-chip quake valves and pinch valves. The microscope is controlled by the custom MATLAB software to change objectives, filter cubes, and location of the stage.

Figure 9. External components to microfluidic chip. (A) pressure gauges/regulators (B) pneumatic valves (C) Pinch valves (D) vacuum flask (E) Pressurized M9 media supply bottles

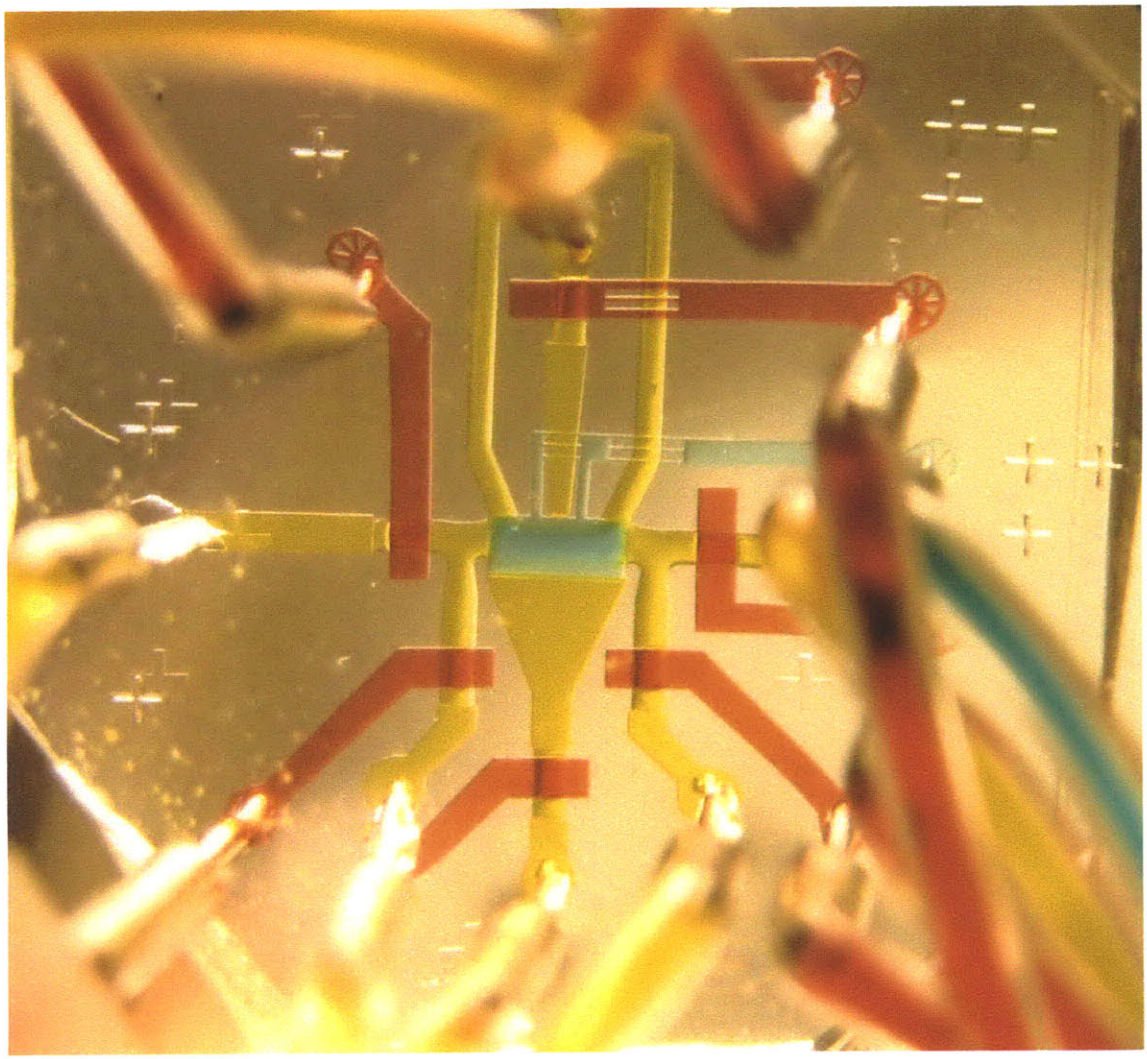
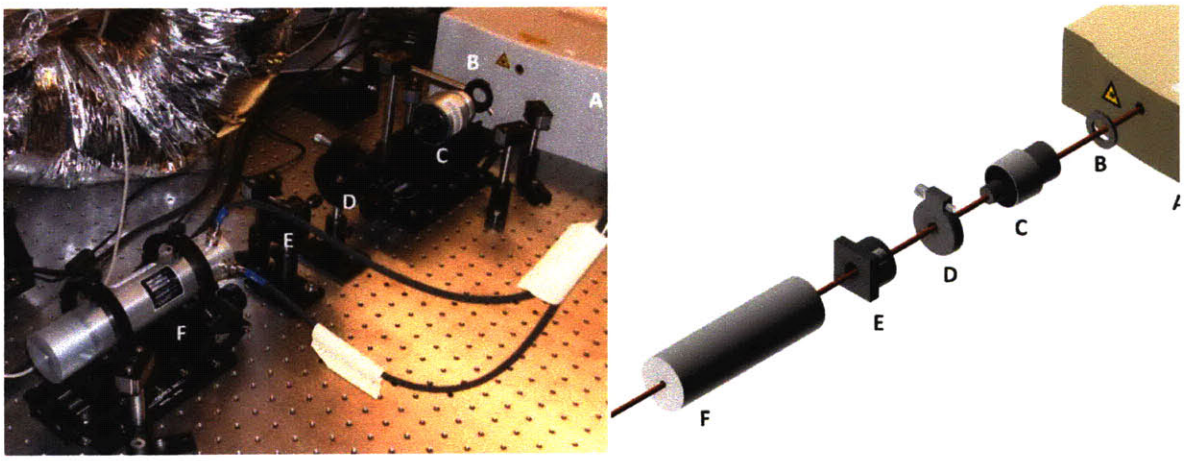


Figure 10. Microfluidic Device viewed from top side with dye-filled channels and pins interfacing with pressurized supply and control lines.

Chapter5

Optical System Integration

The optical system is composed of a femtosecond laser, electro-optical modulator (EOM) and amplifier, half wave plate, beam polarizer, telescoping lenses, and mirrors for alignment, dichroic and objectives.



(a) (b)

Figure 11. Components of the Optical Path as they appear in the setup (a) and with supporting hardware removed for clarity (b). A: The short-pulse laser. B: Aperture for (re)alignment purposes. C: Faraday Isolator. D: Adjustable λ/2 Plate. E: 45° Linear Polarizer. F: Electro-optical Modulator (EOM). Beam path is shown in red in (b).

5.1. Laser Source: Spectra Physics Mai Tai 3042, TiSapphire Laser

The system employs a femtosecond laser (Spectra Physics Mai Tai HP TiSapphire) with minimum recommended pulse energy exceeding ~20 nJ (i.e. average power 1.6 Watts for a laser with 80 MHz pulse repetition rate) taking into account losses in optical components

5.2. Optical Isolator:

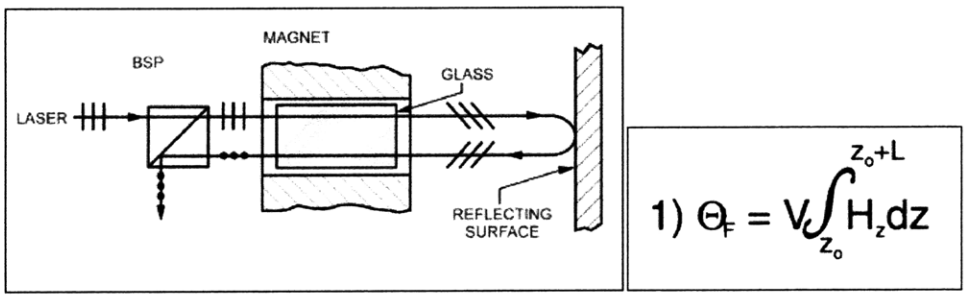


Figure 12. Optical Isolator diagram shows the effect on beam polarization with governing equation for the phase imparted. Image credit: www.ConOptics.com

The optical isolator protects the laser source from harmful back reflections. The change in polarization is induced by the magnetic field of the surrounding magnet, Hz, the birefringent glass Verdet constant, V, and the length of the crystal. The first element is a beam splitter that will pass vertically polarized beams and reject horizontally polarized beams. As the vertically polarized beam passes through the crystal is will rotate the beam by 45 degrees. Any reflected beams will pass backward through the crystal and undergo

an additional 45 degree rotation to horizontally polarized beam which is rejected by the beam splitter thus protecting the source. This optical isolator is paired with a 45 degree polarizer to ensure that any beam reflected backward through the second 45 degree polarizer will gain additional 45 degree to be horizontally polarized rejected before damaging the laser source.

5.3. Half Wave Plate:

The half wave plate consists of slow and fast axes oriented orthogonally to one another. The half wave plate axes are tuned to rotate the polarization of the beam between 0 degrees and 90 degrees depending upon the tuning angle. The beam is then projected on the 45 degree polarizer thus tuning the intensity of the beam between 0% and 100% of the input beam; however, losses occur due to dispersion in a simple wave plate.

6.4 45 Degree Linear Polarizing Beam Splitter

In conjunction with the half wave plate the polarizer will only allow 45 degree polarized light into the Electro-Optical Modulator. This allows the EOM to control the output of the laser with an electrical signal.

5.4 Electro-Optical Modulator

The electro optical modulator operates by applying an electric field inside of a crystal thus changing the refractive index along the extraordinary axis allowing the electric field to induce a change in phase. Two linear polarizers are aligned at 45 degrees in the x direction at both ends. The half wave voltage is applied to act as a half wave plate by rotating the beam polarization by 90 degrees to prevent the beam from passing through the second linear polarizer. When the EOM is switched off the bias voltage is removed allowing the phase of the beam to be unchanged and pass through the second polarizer. In our case we send an electrical signal to the EOM controller which removes the bias voltage for 2.2 seconds to let the 80MHz pulse of 120fs pulses pass through to perform laser surgery.

Chapter6

Future Work

The screening system is currently being adapted to study *zebrafish* larvae to study axonal regeneration in a vertebrate model. The current system is still in full production mode performing drug/genetics screens to identify compounds and genes that affect axonal regeneration. Ongoing improvements to system reliability, automation and throughput are in progress as well with the possibility of commercialization.

Appendix

Appendix A

A.1 Diagram of device interface ports and operation logic table

Chip Logic	V1	V2	V3	V4	V5	V6	V7	Press down	A/B/G	E/D	C/F
Capture worm(ca)	0	1	0	1	1	1	1	0	7~9 psi	6~8 psi	0.5~1.5 psi
Wash single suction port(was)	1	1	0	1	0	1	0	0	7~9 psi	6~8 psi	10 psi
Wash Multi suction port(wam)	1	1	1	1	0	0	0	0	7~9 psi	6~8 psi	10 psi
Clear flow channel (cl)	1	1	1	1	0	1	0	0	7~9 psi	6~8 psi	0.5~1.5 psi
Relocate worm to multi suction (ms)	1	0	1	1	1	0	1	0	7~9 psi	6~8 psi	0.5~1.5 psi
Immobilize worm by Press down (pr)	1	0	1	1	1	0	1	1	7~9 psi	6~8 psi	0.5~1.5 psi
Release worm (allup)	1	1	1	1	1	1	1	0	7~9 psi	6~8 psi	0.5~1.5 psi
Collect worm (co)	1	1	1	0	1	1	0	0	7~9 psi	6~8 psi	0.5~1.5 psi
all valves off (off)	0	0	0	0	0	0	0	0	0 psi	0 psi	0 psi

A.2 Photolithography of Wafer Molds

Control mold, SU-8 2075, thickness ~ 70micron

1. wafer pre-treat
   - dehydrate at 135C for 10min;
   - HMDS, 1000rpm for tens of seconds.

2. coating
   - start from 500rpm for 5sec;
   - 500 -> 3500rpm in 5sec;
   - 3500rpm for 30sec;

3. soft-bake
   - 65C for 3min;
   - 95C for 8min.

4. edge bead removal if necessary

5. exposure
   - without filter, 40sec

6. post-bake
   - 65C for 1.5min;
   - 95C for 6.5min.

7. develop
   - Polymond Acetate, at 500rpm
   - Rinse with IPA, at 1000rpm
   - Dry with nitrogen

Thin feature on flow layer, SU-8 2025, thickness ~ 25micron

1. wafer pre-treat
   - dehydrate at 135C for 10-15min;
   - HMDS, 1000rpm for tens of seconds.

2. coating
   - start from 500rpm for 5sec;
   - 500 -> 4000rpm in 5sec;
   - 4000rpm for 30sec;

3. soft-bake
   - 65C for 1min;
   - 95C for 5min.

4. edge bead removal
    - with Polymond Acetate.

5. exposure
    - without filter, 40sec

6. post-bake
    - 65C for 1min;
    - 95C for 5min.

7. develop
    - Polymond Acetate, at 500rpm
    - Rinse with IPA, at 1000rpm
    - Dry with nitrogen

8. hard-bake
    - 140C for at least 10min, before coating positive photo-resist.

Thick feature on flow layer, SIPR-7123, thickness ~ 100-110micron

1. wafer pre-treat
    - hard-bake if it is not done during the thin feature fabrication

2. 1st coating
    - 600rpm for 100sec;
    - Bake, 40C->100C at 360C/hr, so it will take 10min;
    - 140C for 10min.

3. edge bead removal
    - with IPA

4. 2nd coating
    - 600rpm for 100sec;
    - Bake, 40C->100C at 360C/hr, so it will take 10min;
    - 140C for 10min.

5. edge bead removal
    - with IPA

6. exposure
    - without filter, 4.5min

7. develop after the wafer is cooled down
   - with AZ 440, in a beak, for about 30min

8. reflow
   - 10C/hr ->150C, usually overnight.
   - Turn off the hot plate, let the wafer sit on until it cools down

A.3 PDMS Chip fabrication

Project Name	**Worm Immobilization**		
Material	RTV-615		
Valve action	Push-up & push-down		
# of layers	3		
Layer A	top-control (pour)	a few mm thick	
layer B	flow (spin)	710rpm/60sec, gives a PDMS coating thickness ~120um, which corresponds a membrane ~ 15-20um	590-600rpm/60sec, gives a PDMS coating thickness ~145um, which corresponds to a membrane ~ 45-50um. The chip still works.
Layer c	bottom-control (spin)	870rpm, give a PDMS coating thickness~90um, corresponding a membrane ~ 17um	
	Instruction	***Parameter***	***Comments/Recipe Changes***
CHIP FA-BRICATION			
1)	**Clean molds if necessary**		
	Prepare 20g of 10:1 Sylgard for each mold	Part A: 20g Part B: 2g	Sylgard is cheaper than RTV615

	Pour 20g of 10:1 on each mold and degas for 15 min.			
	Bake molds for 30min at 80C until PDMS cures			
	Peel off PDMS from mold and begin fabrication			
2)	**Prepare Layer A (thick/top-control)**			
	Expose Flow mold to TMCS in hood in closed container for 2min minimum (first time using the mold do TMCS 20min)			
	Store Flow mold in closed petri dish (covered by aluminum foil) until use			
	Combine 60g 5:1 RTV615 for the mold in labelled mixing cup	Part A: 50g Part B: 10g		
	Mix PDMS in hybrid mixer	Mix time: 2 min Degas time: 3 min		
	Pour PDMS mixture onto mold in aluminum foil			
	Place mold+foil in dessicator to degas until all bubbles disappear. Let air into chamber if bubbles overflow.	Degas time: 20-30min		
3)	**Prepare Layer B (thin/flow) - (Do While Degassing Layer A)**			

	Expose Flow mold to TMCS in hood in closed container for 2min minimum			
	Store Flow mold in closed petri dish (covered by aluminum foil) until use			
	Combine 31.5g 20:1 RTV615 for the mold in labelled mixing cup	Part A: 30g Part B: 1.5g		
	Mix PDMS in hybrid mixer	Mix time: 2 min Degas time: 3 min		
	Place Flow mold on PDMS spinner and pour about half PDMS onto mold (fill center with 2inch circle)	Spin: 710rpm Spin time: 60s	For thin samples, you have to spin. Be careful not to spill over from the sides of the mold until the spinner is on. If you ever need to calibrate the thickness of PDMS, you cannot use profiler, instead you spin/bake multiple layers and look under the microscope sideways to measure their thickness.	
4)	**Cure Layer A and Cure Layer B**			
	When Layer A is done degassing, place both layer A and layer B into oven with foil covers on	Time: 30min Temp: 80C		
5)	**Cut & Punch Layer A (top-Control)**			
6)	**Clean Layer A**			
	Clean channel side of Layer A using tape and nitrogen			

7)	**Align & Bond Layer A to Layer B**			
	Under stereo microscope align Layer B to Layer A with channel side facing down			
	Place petri dish with chips into oven for bonding	Time: 1.5 hr Temp: 80C		
8)	**prepare layer C (bottom-control)**			
	Expose Flow mold to TMCS in hood in closed container for 2min minimum			
	Store Flow mold in closed petri dish (covered by aluminum foil) until use			
	Place bottom-control mold on PDMS spinner and pour the other half 20:1 PDMS onto mold (fill center with 2 inch circle)	Spin: 870rpm Spin time: 60s	For thin samples, you have to spin. Be careful not to spill over from the sides of the mold until the spinner is on. If you ever need to calibrate the thickness of PDMS, you cannot use profiler, instead you spin/bake multiple layers and look under the microscope sideways to measure their thickness.	
9)	**Cure Layer C**			
	Place layer C into oven with foil covers on	Time: 30min Temp: 80C	This can be done when 30 min left for the curing Layer A+B	

10)	**Cut & Punch Layer A+B (top-control+flow)**			
	Remove both Layer A+B and layer C from oven			
	Using scalpel cut out Layer A+B chips and peel off from wafer			
	Punch holes in chip with channel side (Layer B) facing up			
10)	**Align & Bond Layer A+B to Layer C**			
	Under stereo microscope align Layer A+B to Layer C with channel side facing down			
	Place petri dish with chips into oven for bonding	Time: at least overnight Temp: 80C		
11)	**Cut & Punch Layer C (bottom-control)**			
	Remove petri with Layer A+B+C from oven			
	Using scalpel cut out Layer A+B+C chips and peel off from wafer			
	Punch holes in chip with channel side (Layer C) facing up			
12)	**Plasma bonding PDMS and glass**			

	Clean glass slide using DI water and Micro 90 detergent cleaner, dry with nitrogen gun.			
	Clean channel side of PDMS using tape and nitrogen			
	plasma bonding PDMS and glass slide	follow the instruction attached on the plasma machine in the common room		
	put chips (PDMS+glass) into oven to increase the bonding, and get rid the toxicity of PDMS	Time: 36 hours Temp: 80C		

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