UNIVERSITY OF CALIFORNIA,
IRVINE

Real-time egg laying dynamics in *Caenorhabditis elegans*

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Philip Vijay Thomas

Dissertation Committee:
Professor Elliot Hui, Chair
Professor Olivier Cinquin
Professor Abraham Lee

2015

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGMENTS

This work was supported in part by multiple funding sources including: a NIH T32 Systems biology of development predoctoral training award under grant HD060555, the Howard Hughes Medical Institute and National Institute of Biomedical Imaging and Bioengineering (HHMI-NIBIB) Interfaces Initiative, and the Center for complex biological systems at the University of California, Irvine.

CURRICULUM VITAE

Philip Vijay Thomas

EDUCATION

Doctor of Philosophy in Biomedical Engineering — **2015**
University of California, Irvine — *Irvine, CA*

Master of Science in Biomedical Engineering — **2015**
University of California, Irvine — *Irvine, CA*

Bachelor of Science in Physics — **2008**
University of Texas at Austin — *Austin, TX*

RESEARCH EXPERIENCE

Graduate Research Assistant — **2009–2015**
University of California, Irvine — *Irvine, California*

R&D Intern — **2007–2008**
Schlumberger Technology Center — *Sugar Land, Texas*

TEACHING EXPERIENCE

Teaching Assistant — **2009–2014**
University of California, Irvine — *Irvine, California*

REFEREED CONFERENCE PROCEEDINGS AND PUBLICATIONS

S. Ahrar, T.V. Nguyen, Y. Shi, T. Ikrar, **P. Thomas**, X. Xu, and E.E. Hui, "Optical Stimulation and Imaging of Functional Brain Circuitry in a Segmented Laminar Flow Chamber", the 15th International Conference on Miniaturized systems for Chemistry and Life Sciences (μTAS), 2011, pp. 873-875.

P. Thomas, A. Paz, O. Cinquin, E.E. Hui, "Multiplexed media delivery to study nutrient adaptation in *C. elegans*", Annual meeting of Biomedical Engineering Society (BMES), Austin, Texas, 2010.

J.L. Kickhofel, A. Mohamide, J. Jalfin, J. Gibson, **P. Thomas**, G. Minerbo, H. Wang and D.M. Homan. 2010. "Inductive conductivity tensor measurement for flowline or material samples". Review of Scientific Instruments 81.

ABSTRACT OF THE DISSERTATION

Real-time egg laying dynamics in *Caenorhabditis elegans*

By

Philip Vijay Thomas

Doctor of Philosophy in Biomedical Engineering

University of California, Irvine, 2015

Professor Elliot Hui, Chair

Caenorhabditis elegans (*C. elegans*) is a powerful model organism for aging studies due to its short generation time and the ease of performing genetic screens. Assaying egg-laying window and output in the worm complements prior lifespan studies because the effects of aging on dividing and non-diving tissues can be studied in tandem. However worms are routinely sterilized in multiday studies because separating a large number of adults from their progeny quickly becomes impractical.

We demonstrate a liquid culture system to deliver and remove food and pharmacological agents from individually housed worms. Worm reproduction is a sensitive indicator of health, so we show worms lay a comparable amount of eggs in our liquid culture system as on agar. Our fast environmental control allows us to withhold food for a short enough time to avoid matricidal hatching, but a long enough time to extend reproductive span. To solve biofilm issues within the system, we identified a biofilm-deficient strain of *E. coli* that the worms thrive in. This system counts progeny in real-time, measuring embryo size as well as allowing for embryo recovery from individual mothers for further developmental assays. Loaded worms can be examined for their whole lifespan, and they can be recovered to do fixation for morphological analysis or other molecular biology.

It is our hope that this system will allow reexamination of many lifespan extending factors

to see how their timed dosing affects reproduction.

Chapter 1

Introduction and motivation

1.1 The impact of *C. elegans* in aging and lifespan studies along with current limitations

C. elegans are an important model in aging in part because they are comparatively cheap to culture and have a relatively short generation time and lifespan[2,3]. The *daf-2*[4,5] loss-of-function mutant worm was the first single gene mutant discovered to double lifespan in an organism (Fig 1.1D,F). This helped promote the hypothesis that lifespan has a strong genetic component, and since then researchers have been studying how genotype and environment interact in worms as they age. The *daf-2* gene corresponds to the insulin/IGF-1 like receptor[6], which senses high nutrient levels and signals to downregulate *daf-16*[7,8] (Fig 1.1C), a forkhead box transcription factor. When nutrient levels are low, the reduction in signaling leads to nuclear import of *daf-16* (Fig 1.1E), turning on a suite of genes as part of a stress response linked to longer lifespan[9,10]. Today we know polymorphisms in the analogous mammalian IIS pathway[11–13] are also associated with longer lifespans[14]. In addition to running forward and reverse genetic screens, pharmacological factors are routinely tested

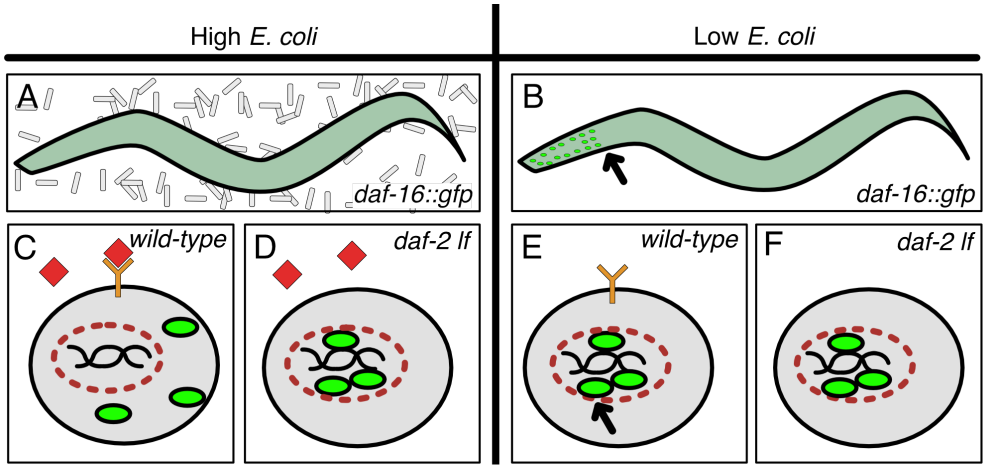

Figure 1.1: The effect of insulin-like signaling under different conditions is illustrated in *wild-type* worms and various mutant strains. (**A**) *daf-16::gfp* worm in nutrient rich media shows diffuse green fluorescence due to *daf-16::gfp* TF in the cytoplasm.(**B**) *daf-16::gfp* worm in *E. coli* free media shows nuclear localization of gfp. (**C**) In *wild-type* worms under high nutrient conditions, insulin-like peptides bind to the *daf-2* receptor. This initiates a signaling cascade that excludes *daf-16* TF from the nucleus. (**D**) *Daf-2* loss of function mutants have no receptor that binds insulin-like peptides, so *daf-16* is constitutively imported into the nucleus. (**E**) In *wild-type* worms under low nutrient conditions, *daf-16* is imported into the nucleus and promotes transcription of stress-response genes. (**F**) Low nutrient levels mean there is little insulin-like hormone present. Regardless, *daf-2 lf* mutants constitutively import *daf-16* TF, initiating a program that increases lifespan.

on worms to see their impact on lifespan, and they are often tested in combination with loss of function mutants and RNAi knockdowns to test for epistatic interactions[15–20]. The most common lifespan assay compares groups of worms assigned to different experimental conditions on agar plates. Worms are tracked daily, and their day of death is recorded, using that data to construct percent survival curves for each group. In these assays live adults are carefully removed and replated from their newly hatched larvae daily to avoid confusing them with their progeny and also to avoid exhausting their food supply. To avoid replating large numbers of worms, adults are usually sterilized with fluorodeoxyuridine (FUdR)[21–23], a DNA synthesis inhibitor. This prevents active division within the worm gonad and sterilizes the worms, allowing for uninterrupted study of lifespan, though it can itself extend lifespan in certain mutants[24–26]. Worm lifespan studies employing FUdR offer no opportunity to observe the effects aging has in self-renewing tissues. Humans experience age-related pathology

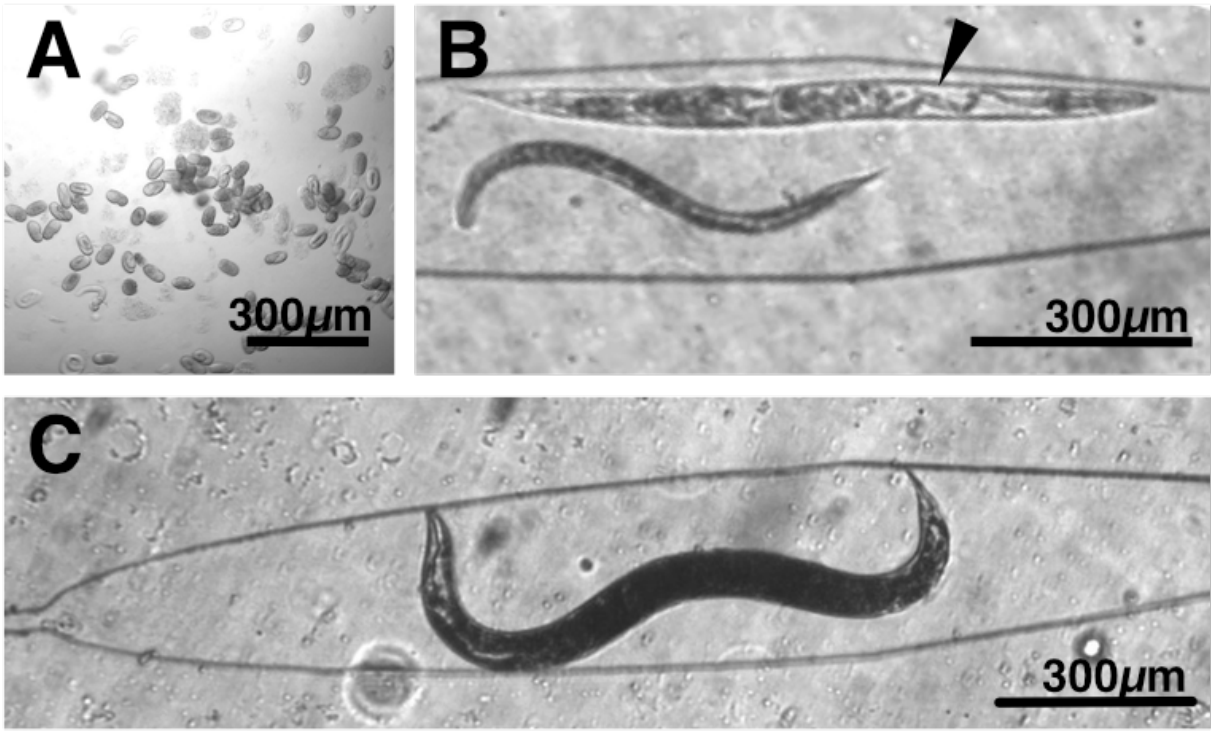

Figure 1.2: Individual nematodes are small enough that large numbers are easily cultured in petri dishes, yet they are big enough to be manipulated by a metal pick or within a pipet tip. (**A**) *C. elegans* eggs are comparable in size to the young adult body width. (**B**) Due to prolonged starvation, several larvae have hatched within the mother. (**C**) An adult hermaphrodite confined within a single microfluidic habitat is isolated from other worms.

within self-renewing tissues such as their liver, skin, and gastrointestinal lining. FUdR also prevents the researcher from studying the regulation of lifespan by the reproductive system. The regulation of lifespan by the insulin-like pathway through the reproductive system has been demonstrated in *C. elegans*[27,28]. Assaying worm reproduction is a good model for self-renewing organs and can complement lifespan analysis, though we have to consider how to read reproductive aging in the worm gonad. For example we know the brood size of *daf-2* and feeding impaired (*eat-2*) mutants is only 60% of wild-type worms[29–31], but these mutants have an extended egg-laying window[32]. In the previous experiments, there is a tradeoff between extending the egg-laying window and total brood size. This thesis will demonstrate that the specific timing of caloric restriction delays reproductive aging by increasing fertile

span without such a tradeoff. Current reproductive assays study many of the same genetic and pharmacological factors tested in lifespan assays except they focus on logging daily egg counts (progeny counting) as well as the cessation of egg-laying (reproductive lifespan). Most worms are hermaphroditic (genotype XX), so they produce around 300 sperm and self-fertilize as many oocytes, even though they have the capacity to produce many more[32,33]. Males also occur spontaneously from nondisjunction (genotype XO) and occur only at 0.1% frequency[34]. Actively mating males to hermaphrodites in our experiments is the best way to transfer an excess of sperm so that the mated hermaphrodites will lay fertilized eggs until their germ lines are exhausted. This allows us to see reproductive span extension that may only be apparent in mated worms[6,30,32,35].

1.2 Starvation and its effect on worms

Starvation is of particular interest in aging research, because caloric restriction is a healthspan and lifespan increasing intervention that is well-conserved across animals from worms to primates[36–41]. CALERIE is an example of a large two year study on dietary restriction in humans that concluded recently, finding a reduction in many risk factors associated with poor health such as inflammatory markers, cholesterol levels, and blood pressure[42,43]. Intermittent fasting (IF) is a subset of dietary restriction where extended fasting is interspersed with periods where food availability is not restricted—IF has been shown to delay aging even when the total caloric intake is similar to an unrestricted diet[44]. Recent IF experiments in worms and mice have corroborated these results and shown that IF can be even more efficacious and works in a distinct way from constant caloric restriction[44–46]. A long term view of these studies is not that a new kind of diet is the only answer for increased longevity, but rather that drug mimics of starvation and other related therapies should be tested[47–49]. Currently a big limitation in agar assays is the inability to change environmental factors

quickly. Starvation is difficult to study in fertile worms because starved worms often retain embryos until they hatch internally and kill the mother (Fig 1.2B). The standard practice is to censor[50,51] such worms or make the embryos nonviable[52,53]. FUdR sterilized worms were used in the recent *C. elegans* IF study because worms cannot tolerate being starved for two days without matricidal hatching. To get an idea of how short an interesting fasting period could be in worms, it was shown that *daf-16::gfp*, a fluorescent transcription factor fusion, enters and leaves the nucleus within five minutes and saturates within fifteen minutes in response to starvation and refeeding[54] (Fig 1.1A,B), so we wanted a system that could switch food concentrations every five minutes, which is much too fast for agar.

1.3 Microfabricated systems for *C. elegans* biology

The reason microfabricated systems are commonly used in worm biology is because they allow for high numbers of individual measurements on worms, complex worm handling, and fast switching of soluble factors. They may allow traditional worm experiments to be performed on a large scale, but they can also be used to interrogate worms in new ways. Devices have been made for individual or pooled culture in droplets[55,56], microwells[57,58], and chambers[59–63] to study lifespan, sleep, growth, and drug toxicity. They have been made to measure volume[64] and force generation[65]. A number of groups use them to assay learning[66,67] and behavior[68–72]. There are fast or high throughput systems for imaging[73–79], microinjection[80], and laser ablation[81–83]. Valve and compression based immobilization methods can be integrated into sorters that optically sort based on fluorescence or internal structure[82,84,85], but there are also passive sorters based on motor behavior[86] or physical size[87,88]. Microfluidic worm culture systems have been used to assay reproductive activity, though current systems either cannot house worms for their whole reproductive lifespan[54], or worms lay much smaller broods in them compared to agar[35]. Previous on-chip culture systems flow in

low food concentrations that are low relative to those used on agar, probably because high density *E. coli* in worm media has a tendency to form aggregates and grow biofilm. For running fast switching experiments in worms, we made a long term *C. elegans* culture device that can assay egg laying and reproductive span. After showing mated worm reproduction was not significantly lowered in our liquid culture system compared to solid agar culture, we tested dynamically changing food conditions to show that IF extends reproduction with no loss in embryo output.

Chapter 2

Real-time *C. elegans* embryo cytometry to study reproductive aging

---

The following work was performed in collaboration with a number of individuals. Adrian Paz conceived and tested the adult recovery as well as the transgenerational applications of the microfluidic reproductive device. He also devised protocols to improve mating efficiency over a short mating window so the entire mating reproductive window could be observed in a microfluidic system. Michael Chiang wrote the video acquisition and image processing software to identify *C. elegans* progeny. Alysha Chi and Matthew Cao helped with the habitat and bubble trap fabrication and testing.

We set out to create a liquid culture system that could adequately assay reproductive out-

put for the whole reproductive window. To do this, we would need to load the worms at the beginning of this window and house them for more than five days. We chose to keep them in separate chambers to get individual progeny counts and prevent pheromone based communication, so it was important to present the same flow rates and bubble-free food to each chamber. Lastly, we needed to clear embryos away from the parent for counting and to avoid confusing progeny with the parent.

2.1 High capacity low-weight passive bubble trap

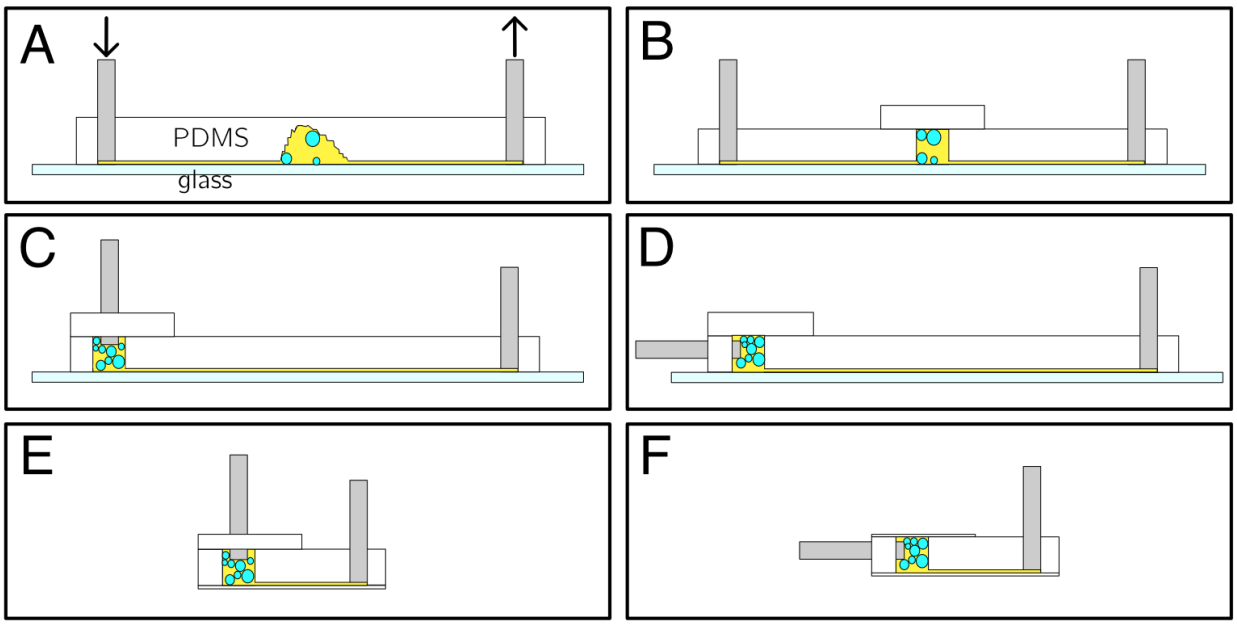

Figure 2.1: Different types of high-aspect ratio bubble traps were tested for their air capacity. (**A**) Carving the trap using a razor blade creates a jagged chamber that varies in quality. (**B**) Hole punching and bonding a PDMS piece on top standardized the design. We observed only 20% of the trap capacity was used and bubbles had a tendency to trace along the floor of the chamber to the exit. (**C,D**) Top and side inlet bubble trap trapped 100% of their chamber volume in tests. (**E,F**) Removing the glass slide and bonding these devices to PDMS membranes lightened these traps such that there is no chance they pull apart from the downstream worm chip.

As media travels from the syringe to the worm device (Fig 2.5), bubbles need to be removed. Without the addition of a bubble trap, nutrient control will be interrupted by bubbles,

ruining the experiment in progress . Significant nutrient interruptions from large bubbles will also starve the worms within a few hours and stall embryos that will permanently stick and occlude the habitat exit. Several microfluidic solutions have been developed, and we chose a high-aspect ratio chamber that traps bubbles that rise to the top of moving liquid. This style of trap can be prototyped quickly without photolithography and monolithically fabricated in PDMS[89].

At first we tried a single inlet-single outlet chip containing a central carved tall chamber to trap bubbles (Fig 2.1). The chambers were individually carved for each chip and worked inconsistently due to this variation, so we standardized the design by bonding a clear PDMS roof on top of a hole punched chamber, an extra fabrication step. The clear PDMS cover also had an advantage by making it easy to observe bubble accumulation. If a trap appears to be at capacity, it is convenient to replace it when media is changed. We observed that a very small volume of bubbles relative to the chamber size could be trapped before they would pass on to the worm chip, so we guessed that injected bubbles were traveling along the chamber floor to the exit instead of being trapped. This led us to test inlet ports elevated with respect to the chamber outlets[90]. Using several such designs, we were able to improve the bubble trap from using 20% of its trap volume to 100% of the trap volume, which we tested using an air-filled syringe. The very last change we made was to remove the glass slide and switch to an all PDMS design, where the top and bottom are membranes that make the trap light enough to be suspended in mid air while connected with tubing. This prevents the bubble trap and worm device from stretching apart and disconnecting during experiments. We also checked that suspending the trap does not negatively affect the trapping capacity.

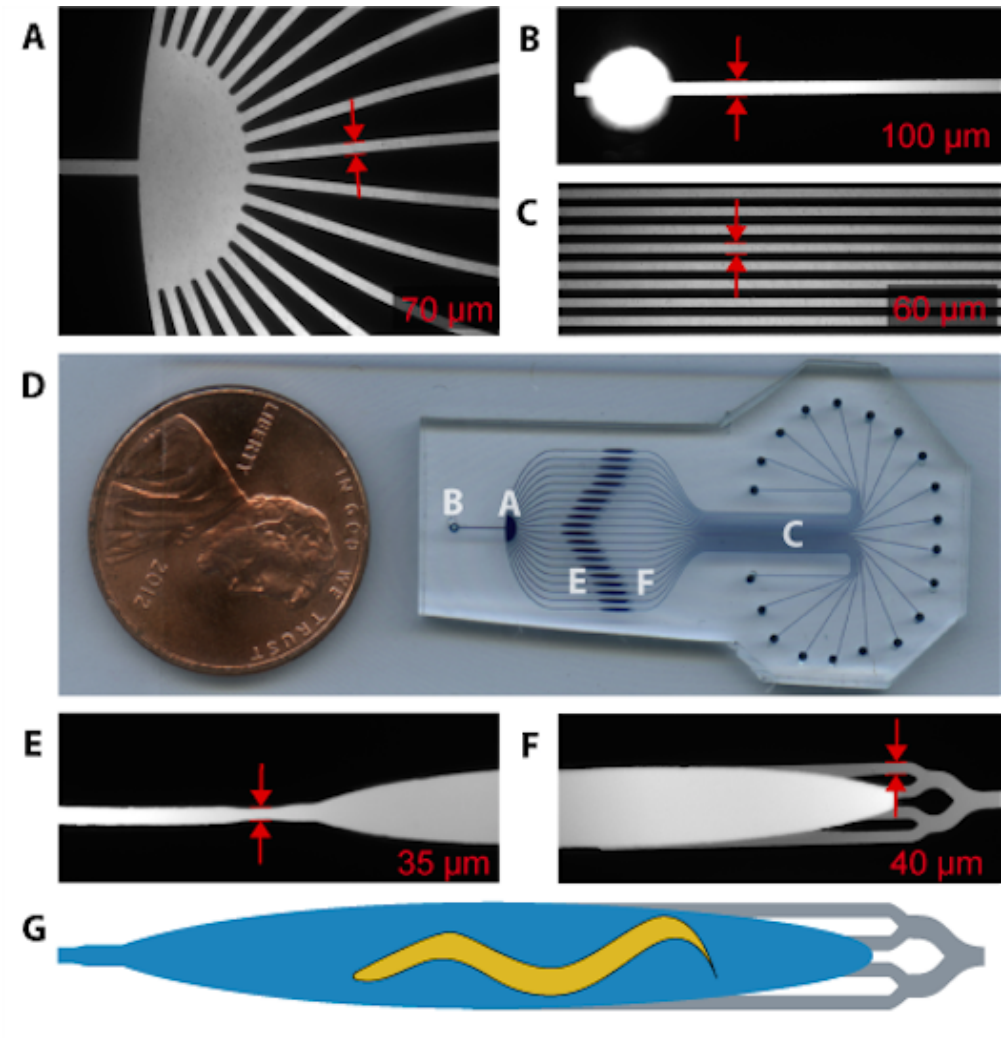

Figure 2.2: Overview of the device critical dimensions and their relevance. (**A**) Device fork where worms are guided individually to a downstream habitat. (**B**) Inlet channel is sized relatively large so adult worms easily go through and has 1 or 2 holes punched depending if the worms are being intermittently fasted. (**C**) Portion of the egg observation area where eggs and larvae are filmed indexed to each habitat. (**D**) Large-scale device view. (**E**) Prehabitat restriction prevents worms from flowing back out of the habitat when media is switched. (**F**) Multiple exits post-habitat help clear eggs and keep the worm from getting flushed out as it grows older and swims less (**G**) Device height drops by 50 µm post habitat to retain the larger worm (100 µm) while allowing eggs (45 µm) to exit.

2.2 Microfluidic device layout

The design of our worm culture system (Fig 2.2) feeds twenty worms housed individually in elliptical habitats, which is an adequate number of worms to characterize mated reproduction

based on a previous study[32]. Media enters through the source inlet and is equally divided towards each habitat so that each worm is presented the same food concentration and flow shear. For a lifespan assay, it may be preferable to crowd many worms per habitat[61] to simultaneously screen many lifespan extending conditions. However a reproductive span readout asks what fraction of worms are still egg-laying on a given day, so this was never an option. There are other advantages to using a single worm per habitat. It helps us avoid pheromone-based signaling between worms that communicates nutritional state and has been shown to affect embryos[91–93]. In addition, examining reproductive output from individual worms allows us to see if our population stratifies into subpopulations[94,95] due to the nutrient stresses being imposed. Each habitat is connected to an individual outlet which allows us the ability to profile progeny from an individual mother by collecting individual broods.

2.3 Tuning habitat exit sizes to flush out embryos while retaining worms

Our first design used a habitat exit width of 25 μm that would taper out to 50 μm similarly to a previous *C. elegans* culture device[59], but this failed to clear eggs reliably from the habitat so that they could be counted. The habitats would routinely clog and media could not be delivered, resulting in the experiment's failure. We chose to test habitat exit widths from 30-50 μm for their capability to clear embryos while still retaining the parent (Fig 2.3C). The best design started at 40 μm and would taper out to 45 μm, but there was a significant tradeoff between retaining adult worms and clearing eggs, so we could often lose up to 30% of young adults loaded in the first 12 h. Young adult worms (80 μm) are much more deformable than embryos, only growing to their maximum size after a few days within the device. For this reason we switched to a multi-height design. The area preceding the habitat

exit (including the habitat) was made 100 μm high, while the exiting channels would drop to 50 μm. In a single height design, the cross sectional area of the channel was around 90% of the loaded worm at its widest. Halving the height (Fig 2.3E) halved this area.

2.4 Equal flow resistance to make identical habitats

In going from a single-inlet single-outlet design to a single-inlet multiple-outlet design, we had to make each path to an outlet equal length, but this took up too much mask space, reducing the number of available devices. To avoid relying on symmetry or equal lengths to balance the habitats and also to improve the capability of the device to separate embryos from adults, we employed channels with reduced height (and thus increased resistance) in the sections downstream of the worm habitats. In order to calculate the total hydraulic resistance of each rectangular microchannel, we used an equation[96] based on cross-sectional area (A), length (L), perimeter (P), viscosity (η), and a shape-dependent parameter (α)

$$R = \alpha \eta L / A^2 \tag{2.1}$$

$$\alpha = \frac{22}{7}\frac{P^2}{A} - \frac{65}{3} \tag{2.2}$$

After choosing a total path length, we could solve for the pre-habitat (100 μm) and post-habitat (50 μm) lengths such that the total length and total resistance was the same. According to the formulas, 5-10% changes in channel height which might arise due to fabrication error do not significantly affect the balancing.

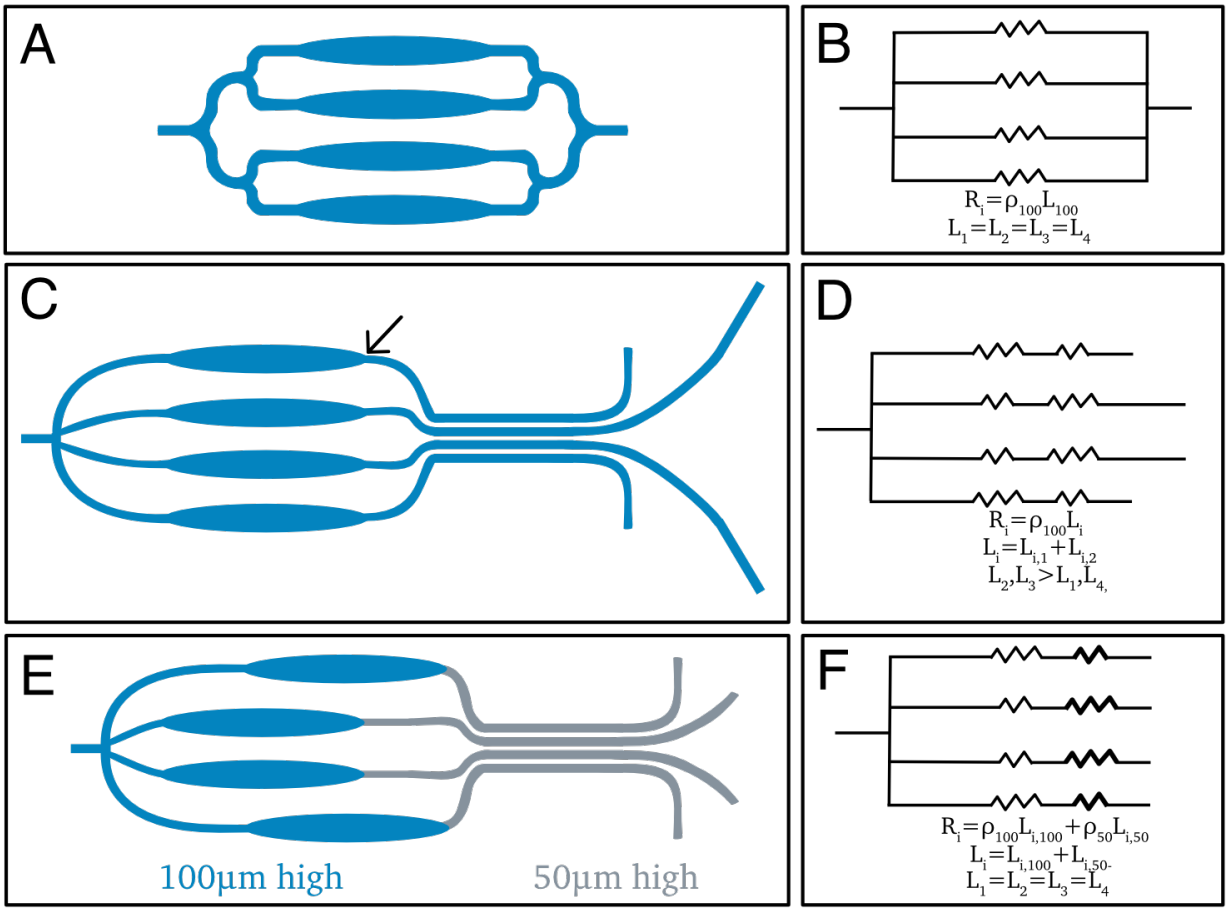

Figure 2.3: Design progression for balanced habitats. (**A**) Following a common microfluidic motif, channel bifurcations from inlet and towards the exit naturally create equal habitats but worm progeny cannot be individually collected. (**C**) The center channels have a shorter path to the habitat, so the length to their outlets are longer to balance the resistances. This design required adjusting flow rates for the first 36 h to prevent worms from exiting the habitat. (**E**) The final version was designed to keep constant the total length and resistance to each path; this reduces the device area and makes it easier to fabricate. Dropping the height at the habitat exit helped to retain worms much better. (**B,D,F**) Each path resistance is dependent on the resistance per unit length and the segment length. When the channel height drops by half, the resistance per unit length increases by a factor of five.

2.5 Video enumeration of eggs

After the eggs are cleared from the habitat, they flow into a section of the device where all the channels are closely packed and parallel to each other (Fig 2.2C). This egg observation area fits cleanly in the field of view of a videocamera attached to a dissection scope. This

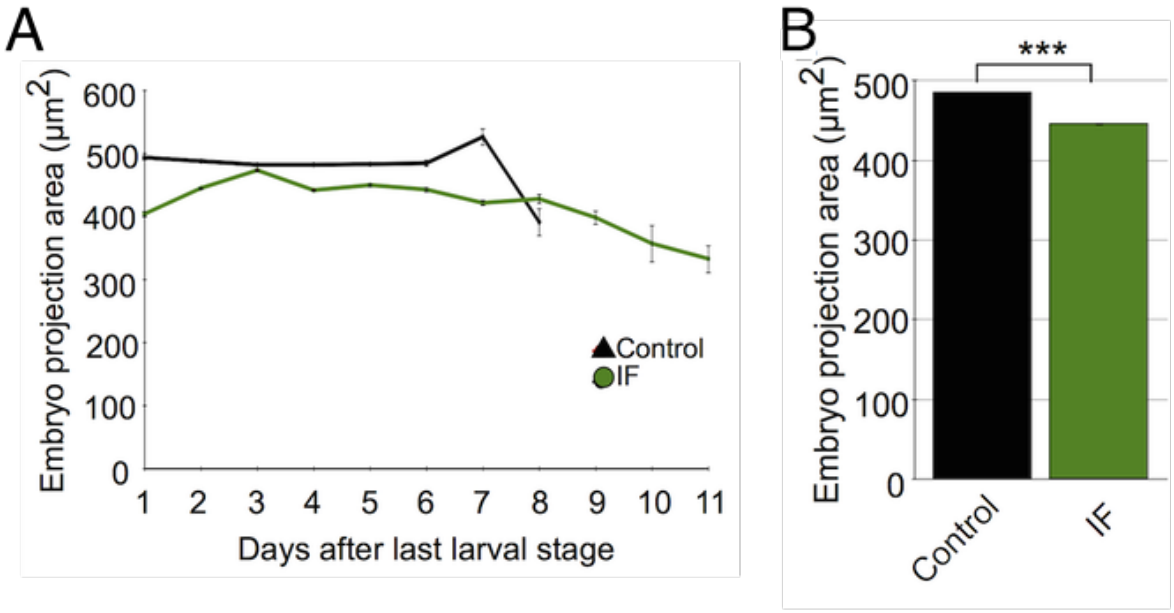

Figure 2.4: Embryo size can be profiled in the video to track size changes over time and nutritional state[1]. Here it reduces embryo size in IF worms. (**A**) Embryo comparison for worms at 8.6×10^9 cells/mL (control) and worms switched between 6 h at 1×10^{10} cells/mL and 1 h with no food (IF).

microscope is used for its large field of view, long working distance, and a tunable magnification. Microscope images of the egg observation channels are captured at around 5 frames per second. Initially, all frames were saved to disk and eggs were counted manually, but software was written to save those that are most likely to save eggs. If there are no eggs flowing past the screen, the difference images between two successive frames will look completely dark, but if an embryo flows past the change in intensity will appear as a bright ellipse. We save to hard disk the images where the difference image intensity sum over all the pixels crosses a preset threshold. Due to noise varying from frame-to-frame, there are a lot of randomly dispersed bright pixels throughout the difference image that may trigger saving a frame, so later on the images are processed further using a blob detection algorithm which searches the difference images for bright blobs matching the size of an egg. Identified egg blobs are tagged so a viewer can see all the detection events quickly and make any corrections if necessary. We also measure embryo size (Fig 2.4) to assess changes over the reproductive lifespan. We hope to correlate these with embryo health through further lifespan and dauer formation

assays.

2.6 Switching between discrete and continuously varying media concentrations

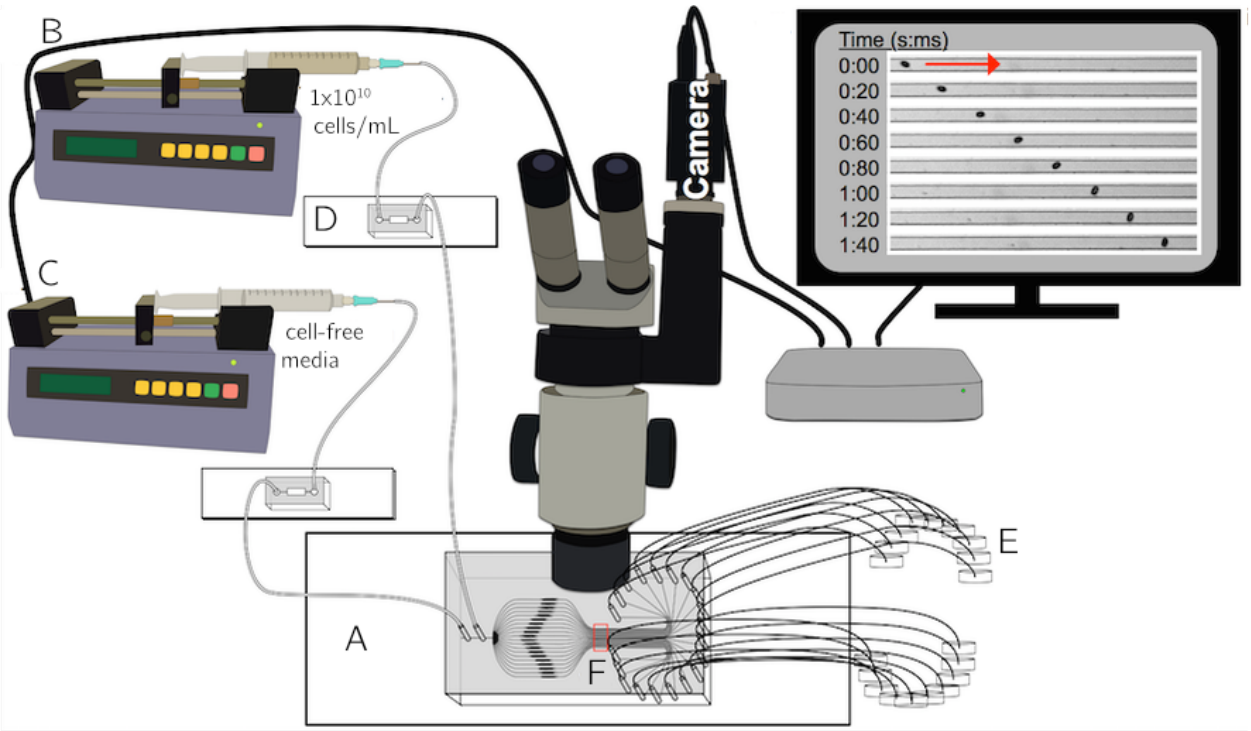

Figure 2.5: Food regimen control and embryo laying detection setup[1]. Up to 20 mated hermaphrodites are loaded into each habitat on the (**A**) microfluidic chip. Food delivery to the chip is controlled using two syringe pumps. (**B**) The first pump delivers the food source, 1×10^{10} cells/mL in S-medium, and the (**C**) second pump delivers bacteria-free S-medium. Syringe pumps are programmed to alternate between on and off states at desired time intervals; 5 min is the minimum time it takes for media to switch. Silastic tubing connects each syringe to its own (**D**) bubble trap which then goes to the (**A**) worm chip. Media flows in continuously, flushing embryos out which can be (**E**) collected and plated for each channel. A camera attached to a dissection scope takes images of the (**F**) egg observation area at a rate of 5 frames per second.

In our culture system (Fig 2.5), media is delivered to the microfluidic device from two computer controlled syringe pumps. We make sure the media does not settle between media changes (24 h) by placing stirbars in the syringe and placing a running stirplate close to

the pumps. When controlling the pumps over a long period of time, the most robust pump operation strategy is to communicate the desired flowrate every 5 min. Using this method it is unlikely that the pumps are incorrect for more than 5 min if a switch happens. According to previous results with *C. briggsae*, a close relative of *C. elegans*, nutrient intake is directly proportional to food concentration in liquid culture[97], so we expect that injected food concentrations match worm nutrient intake.

Chapter 3

Optimizing worm health in *C. elegans* microfluidics

The following work was performed in collaboration with a number of individuals. Adrian Paz and Jafar Al Souz performed the majority of the biofilm tests with worms and found the use of Tween-80 best minimizes egg clumping within the device.

Long-term culture in previous *C. elegans* liquid culture systems results in poor embryo counts relative to agar. On agar plates, worms are crawling on a lawn of bacteria concentrated by evaporation, so food densities approach 1×10^{13} cells/mL. It was previously found that egg-laying in liquid culture reaches a maximum[98] at 1×10^{9} cells/mL. The first on-chip liquid culture system[59] also used this concentration, explaining that lifespan also dropped when bacterial concentration was increased. Lowered eggcounts in liquid are often explained as a result of poor oxygen transfer within liquid; in a closed system it is a resource that live

bacteria and the worm would be competing for. We focused on increasing oxygen transfer to support higher nutrient density in the culture medium, but also needed to eliminate biofilm formation by the denser *E. coli* food source.

3.1 *E. coli* densities of 10^{10} cells/mL maintain egg-laying in liquid worm culture

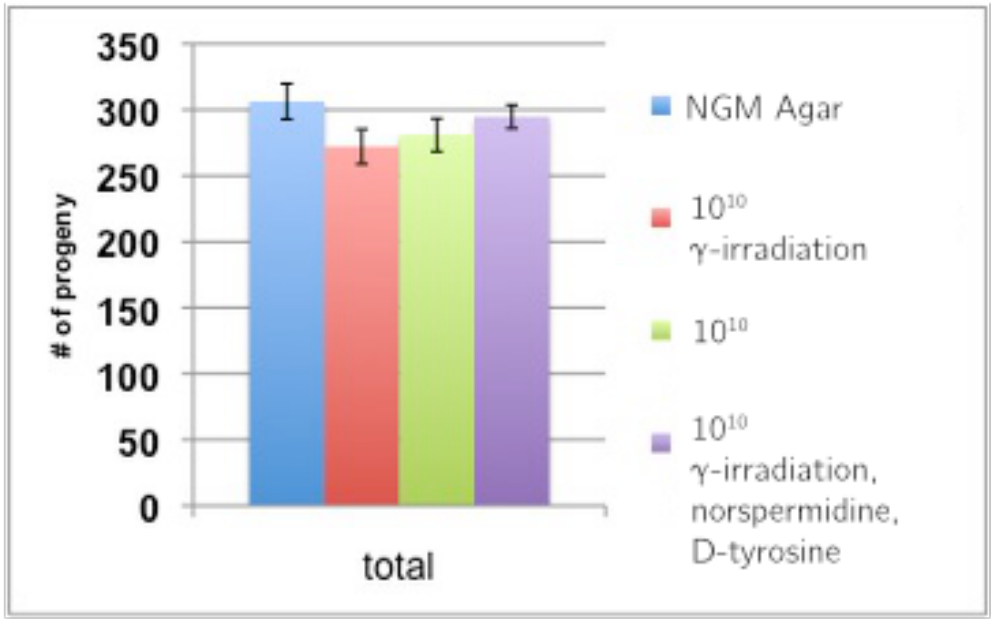

Figure 3.1: In wellplate liquid culture, we were able to show that 1×10^{10} cells/mL is sufficient to recapitulate normal egg laying as seen on NGM agar[1]. Media was prepared from saturated *E. coli* grown in LB a number of different ways: (**A**) seeded on nematode growth agar; (**B**) γ-irradiated for 24 h, centrifuged and frozen as pellets, then resuspended in S-medium; (**C**) prepared as in (B) but without γ-irradiation; (**D**) prepared as in (B) with 10 µM norspermidine and 2.5 µM D-tyrosine added to S-medium.

We hoped to demonstrate that egg-laying should increase as bacterial concentrations are increased past 10^9 cells/mL because that is what is seen on agar. Employing liquid culture in standard 24-well plates, we compared the reproduction of unmated hermaphrodites in liquid with 10^{10} cells/mL with worms plated on NGM agar, showing that we did not need to

increase concentration further to match agar (Fig 3.1). It was crucial not to deprive liquid cultures of oxygen, so we only filled each 24-well with 250 µL of *HB101 E. coli* and subjected the wellplates to gentle agitation on a laboratory nutator. In these tests we also examined two biofilm-inhibiting treatments for their effects on worm reproduction. We limited ourself to treatments that would not lyse the bacteria because worms do not reproduce well feeding on *E. coli* lysate. Here we tested γ-irradiation because it has shown to be effective for wastewater treatment[99–102] and is better at killing bacteria in turbid media (saturated bacterial solutions) than UV[101]. Norspermidine and D-tyrosine were also added together in one condition to worm media because they are amino acids that had been previously shown to inhibit biofilm formation[103].

3.2 *E. coli* biofilms in devices

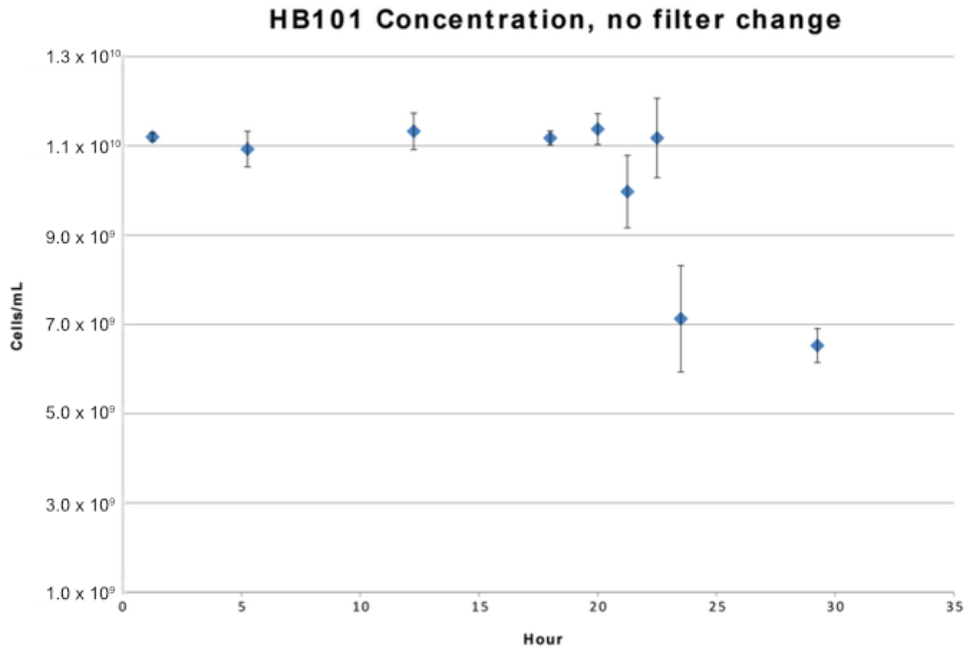

Figure 3.2: *HB101* clumps after 24 h even with continuous stirring. *HB101* at 1.1×10^{10} cells/mL is injected through a 2 µm syringe filter and the concentration is checked using a hemocytometer. Over time the concentration through the syringe filter drops.

Once the reproductive output was optimized, the largest barrier to continuous device culture over the lifespan of the worm have been *E. coli HB101* biofilms and their capability to interrupt the device flow. Distinct from the planktonic phase seen in log-phase growth, bacterial biofilms in *E. coli* and other bacteria are bacterial aggregates along with their surrounding protective extracellular polysaccharide matrix which form in response to stressful conditions such as low nutrients, low temperatures, flow-based shear, low salt, or the presence of antibiotics[104]. Worms in liquid culture are fed a suspension of *E. coli* in buffer solution. Concentrating live *E. coli* in nutrient-free media fulfills the conditions to initiate this stress response. The resulting biofilms often caused bacteria to aggregate and block flow within the device. They would also overload the syringe filter preceding the device and cause the experiment to fail before biofilms were seen because the effective concentration seen by the worm was much lower than we expected (Fig 3.2).

3.3 Amino acid addition to S-media, γ irradiation of bacteria, and elevated syringe temperatures are ineffective in reducing biofilms in devices

We treated bacteria in numerous ways to minimize biofilms, but most of these failed because worms did not take well to a modified food source, or the treatment caused no significant reduction in biofilm formation. Norspermidine and D-tyrosine are two amino acids that have mixed evidence of inhibiting biofilms[103,105] and did not negatively affect worm reproduction (Fig 3.1), but the biofilm reduction we saw was insufficient and inconsistent for our experiments (Fig 3.3).

UV[15,44], and β[106] irradiation have been previously used to arrest *E. coli* growth in *C. elegans* agar culture and is a proven food source, so we hoped radiation would prevent biofilm

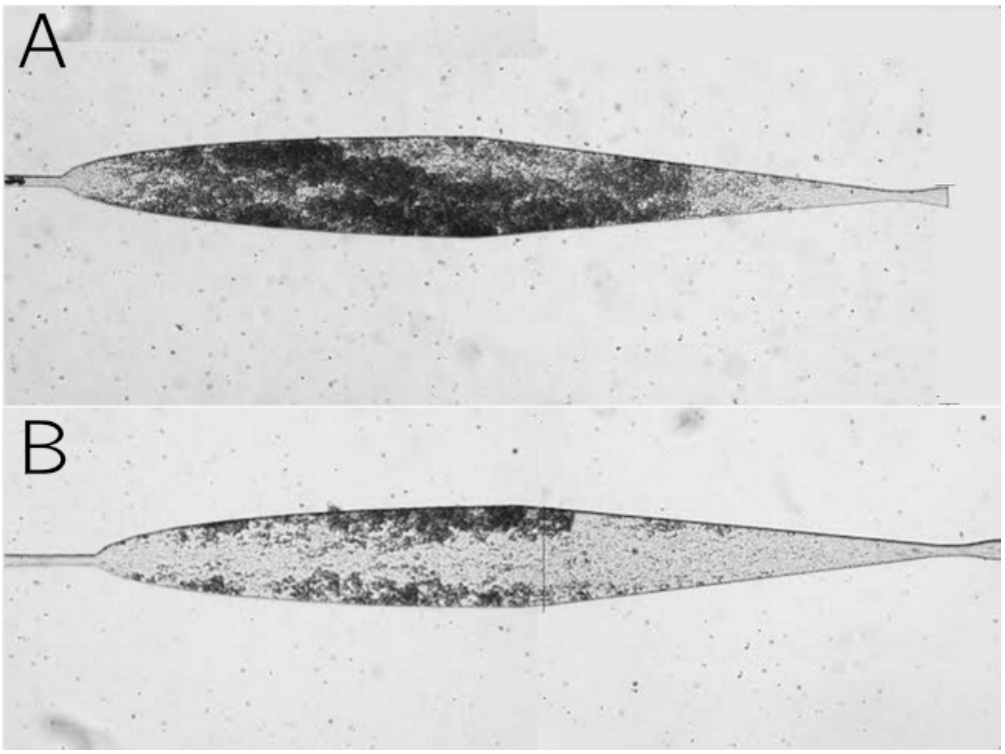

Figure 3.3: The effect of norspermidine and D-tyrosine added to media after 36 h flow in device. Biofilm is reduced but not sufficient for weeklong on-chip culture of worms. (**A**) Biofilm buildup using 10^{10} *HB101* cells/mL in S-media with norspermidine at 10 µM and D-tyrosine at 2.5 µM. (**B**) Biofilm buildup using 10^{10} *HB101* cells/mL in S-media.

formation in liquid culture while keeping bacteria intact. UV irradiation has trouble killing particle-associated microorganisms or sterilizing turbid media[101], and our liquid culture experiments required us to process hundreds of milliliters of saturated *E. coli* solution, so we decided to try gamma irradiation, which has been investigated for wastewater treatment[99–102]. 24 h treatment for us was equivalent to 400 kGy. This should correspond to a 3-log fold decrease in CFUs, which was not enough to prevent plated colonies from appearing on its own but prevented colony growth when combined with freeze-thawing. When tested in devices, γ-irradiation was again insufficient and inconsistent for our experiments (Fig 3.4).

Low temperatures, especially below 23 °C are known to promote biofilm formation in *E. coli*[107]. Our media syringes that feed the device are stored in the 20 °C incubator which is the preferred temperature for worms, so we tried elevating the syringe temperature to 37 °C.

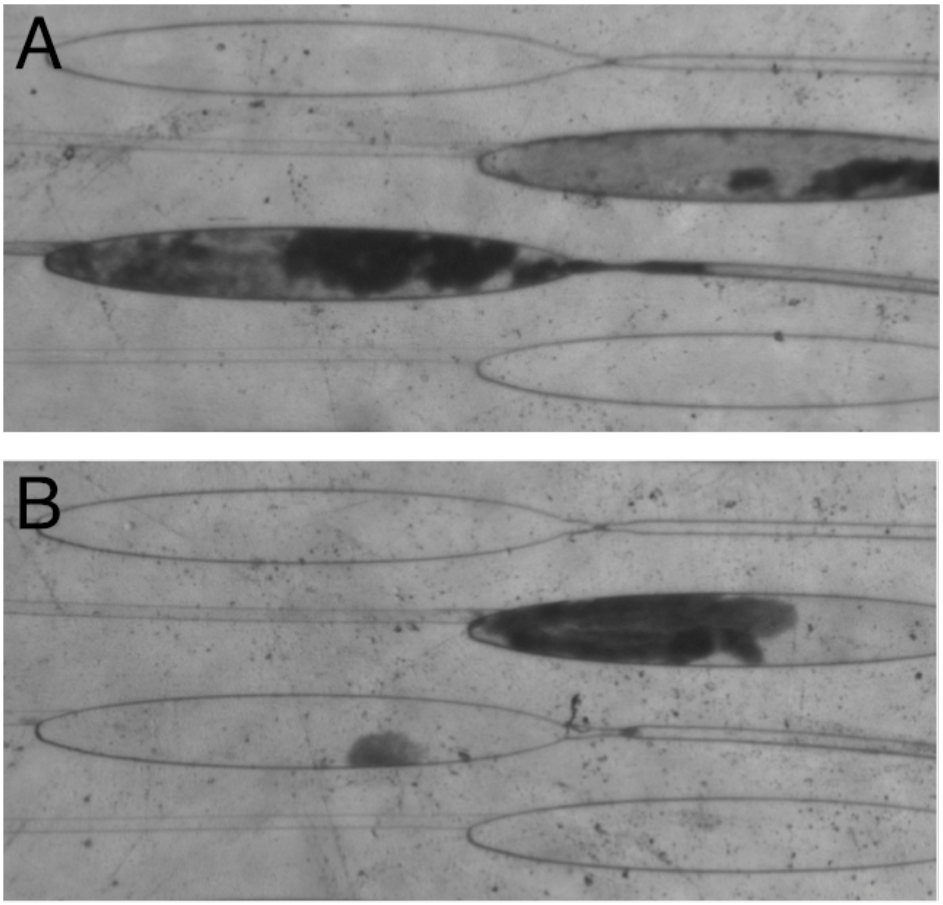

Figure 3.4: The effect of pre-treated γ-irradiated bacteria after 39 hour flow in device. Biofilm is reduced but not sufficient for weeklong on-chip culture of worms. (**A**) Biofilm buildup using 10^{10} *HB101* cells/mL in S-media. Bacteria were irradiated for 24 hour, subjecting them to approximately 400 kGy. (**B**) Biofilm buildup using 10^{10} *HB101* cells/mL in S-media.

The media cools to the incubator temperature by the time it reaches the worms, so the worms experience the same temperature regardless of the syringe condition. In our hands, the syringe kept at 37 °C did not reduce biofilm development enough to be of use in worm experiments.

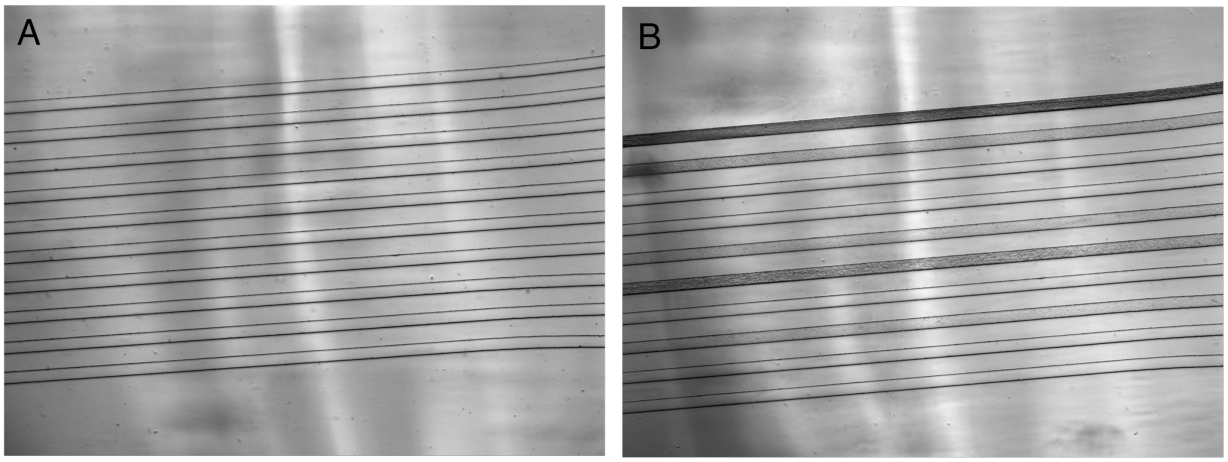

Figure 3.5: 36 hour flow in device with media syringe kept at 37 °C. Biofilm is not significantly reduced. (**A**) Biofilm buildup with media syringe kept at 20 °C. (**B**) Biofilm buildup with media syringe kept at 37 °C.

3.4 Use of a *curli* major subunit deletion strain significantly reduces biofilm in S-media

The choice of *E. coli* strain in worms has a large effect on lifespan and reproductive output because worms see bacteria as a food source, though they are also colonized by them. This relationship becomes pathogenic towards worms at the end of their lifespan, and bacteria leak into the body, so worms show a preference for slow-growing, less pathogenic strains[108]. Two strains in particular, *HB101* and *OP50*, have been previously used in *C. elegans* culture. In *E. coli*, multiple biofilm associated factors are under operon control. The operons generally contain structural genes for one or more components, genes facilitating synthesis such as chaperones, and genes facilitating export since these factors are displayed extracellularly. The functions of these genes were originally identified because knockouts for critical factors inhibit biofilm formation. We attempted to test several knockout for biofilm machinery chosen from the Keio collection, a library of all the nonessential single gene knockouts in a K-12 *E. coli* strain (*BW25113*)[109,110]. The genes we chose were within operons controlling *curli*, *fimbriae*, and colanic acid, because single knockouts within the three pathways severely

abrogate biofilm formation[111].

Curli consists of secreted amyloid (protein) fibers made up of repeating units of the *curli* major subunit (*csgA*)[112] which nucleate from the *curli* minor subunit (*csgB2012*) attached to the bacterial exterior surface[113]. Both are necessary to form the protein component of biofilm[112,114] important in adhesion, so we stocked their knockouts *JW1025-1* and *JW1024-1*.

Fimbriae, or type I pili, are partially responsible for *E. coli* attachment to surfaces. *FimA* makes up the bulk of the pilius and *fimH* forms the tip while *fimD* is responsible for extracellular export of the structural monomers[115,116]. Knockouts in one or both of these genes has been shown to reduce surface attachment. For this reason we chose the knockouts *JW4277-1*, *JW4283-1*, and *JW5780-1*.

Lastly, very large biofilms have a significant polysaccharide component that in *E. coli* is made up of colanic acid[117]. This operon has numerous genes associated with colanic acid synthesis from four different sugars and extracellular export. *wcaJ*[118] and *wcaF*[119] are both linked to lower biofilm formation and correspond to *JW2032-1* and *JW2039-1*.

We expected the $\Delta csgA$ (*curli* major subunit deletion) strain or *JW1025-1* to reduce biofilm the most since it is the major protein component of biofilm, so we tested the strain with worms and noticed that it consistently reduced biofilm with respect to *HB101* (Fig 3.6) in a device while maintaining worm progeny production. (Fig 3.7). We acquired multiple single knockout strains with the intention of forming multiple knockout strains if necessary. Since biofilm components are expressed on the cell surface or secreted into culture, we wanted multiple targets in case any of them are important for worm health. The *curli* major subunit deletion leads to less biofilm formation within microfluidic worm culture. Mated worm reproduction is also higher for those worms (Fig 3.7B), so it would be interesting to see if this is due to the background strain *JW1025-1* was derived from or directly resulting from the lack of *curli* major subunit.

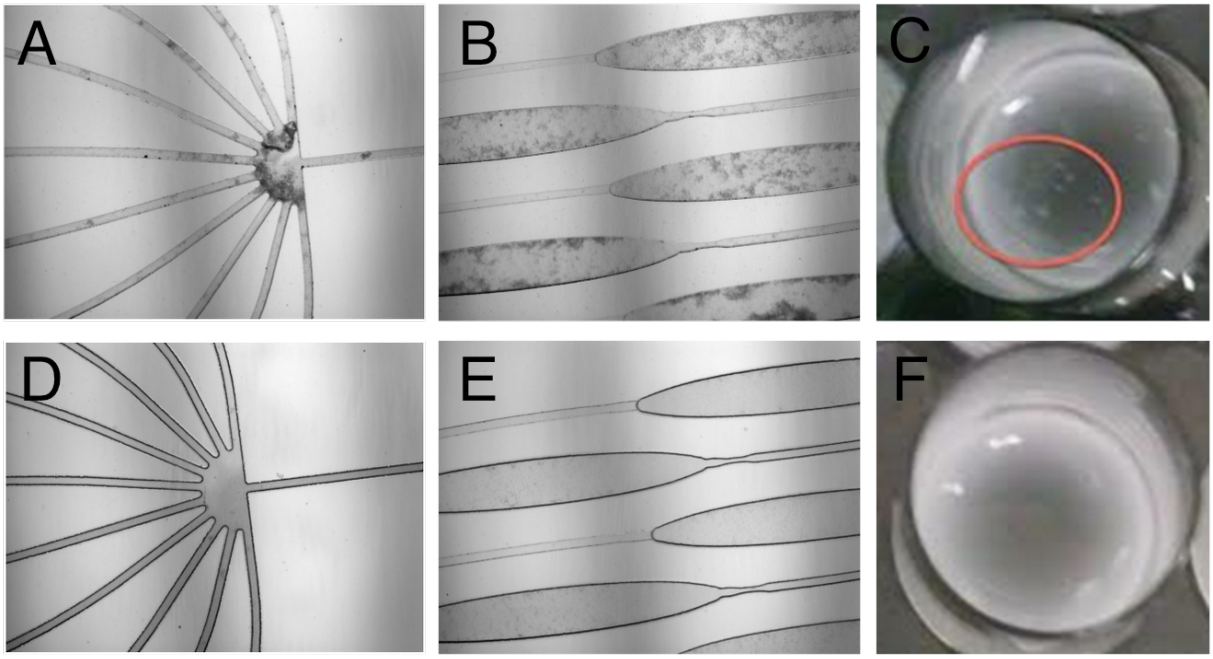

Figure 3.6: In device and wellplate comparisons of *HB101* and *JW1025-1*[1]. (**A-C**) *HB101* has less biofilm buildup after 36 hour. (**D-F**) *JW1025-1* shows no biofilm formation after 36 hour. (**A-B**) *HB101* biofilm formation within before and in the worm habitats. (**C**) *HB101* shows clumping even in a wellplate placed on a nutator. (**D-E**) *JW1025-1* shows no biofilm formation before and in the worm habitats.(**F**) *JW1025-1* remains well dispersed in wellplate based tests.

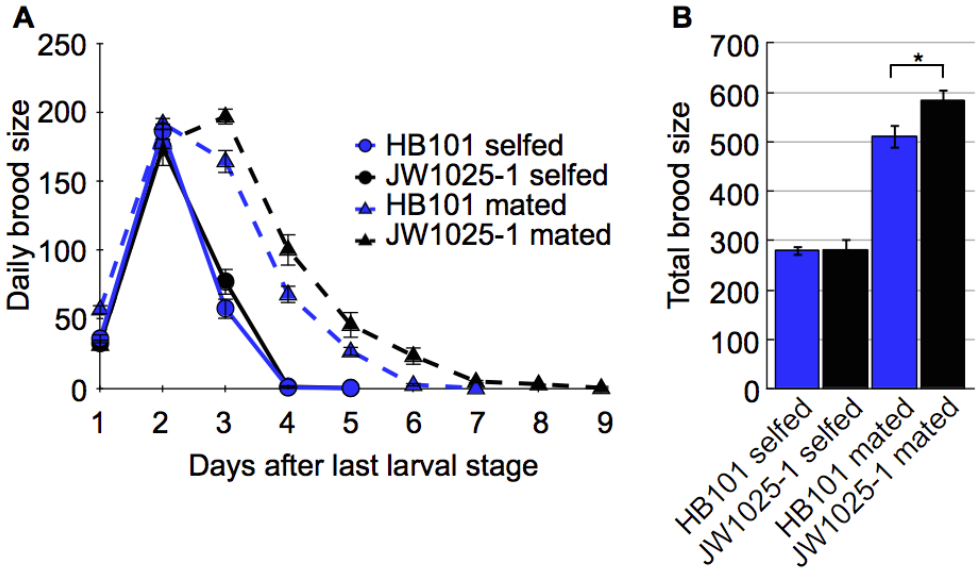

Figure 3.7: *JW1025-1* is as good as *HB101* in maintaining progeny production in mated and nonmated worms[1]. (**A**) Daily progeny counts. (**B**) Total progeny over the reproductive period.

Chapter 4

Conclusions and future directions

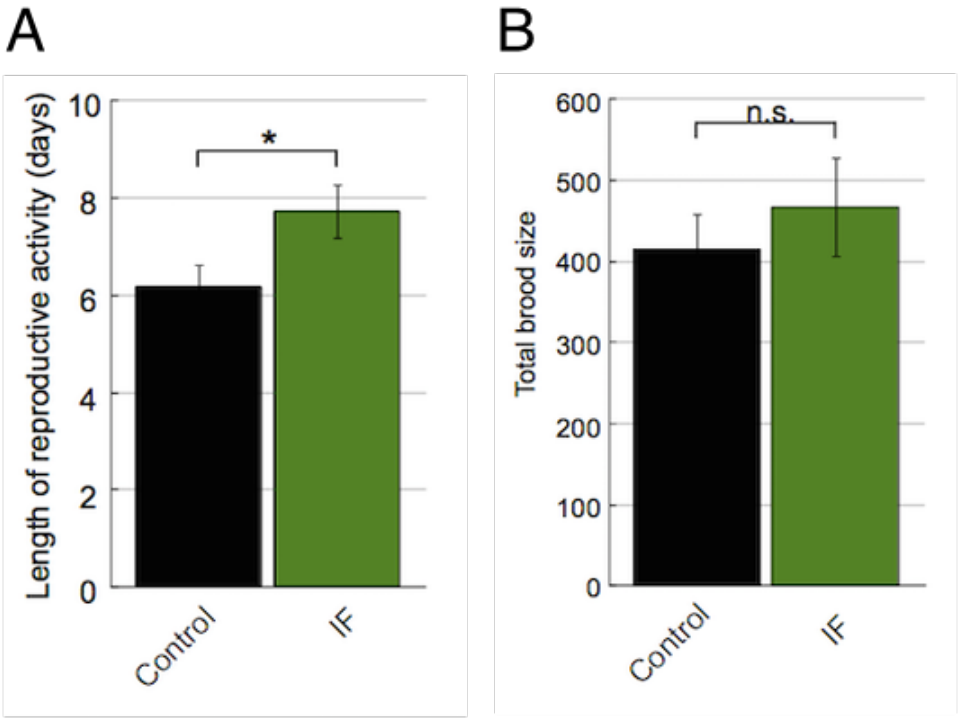

Figure 4.1: IF increases reproductive lifespan without decreasing brood size. The IF condition was 6 hour at 1×10^{10} cells/mL and 1 hour with no food. The control condition was 8.6×10^{9} cells/mL, such that the average food concentration over time is the same in both conditions. (**A**) Reproductive window is increase by 25%. (**B**) Brood size is maintained under IF.

Microfabricated systems that complemented agar culture have demonstrated novel behavioral interrogation of worms that cannot be done on plate assays. Past *C. elegans* microfluidic culture systems have shown great promise in short term worm sorting, screening, and observation, but give results that cannot be directly compared to agar based assays. Because of this they cannot be expected to replace agar culture because of the large volume of prior studies and results derived from agar. The most comparable results were seen in worms cultured on chip for 24 hour and transferred to plates for followup comparison. We spent a great deal of care optimizing the conditions for worms to thrive long term in microfluidic liquid culture taking into account food concentrations, oxygen availability in liquid, and low biofilm accumulation.

If we consider the two recent liquid culture systems that assay reproduction, the one by Kopito et al[54] is very different because a tight habitat size constrains natural worm growth but allows for subcellular imaging. Membrane based immobilization[60] and taper based immobilization[59] are two alternatives we could integrate into our device because they have been demonstrated in amply sized microfluidic habitats like ours. Capturing the eggs and photographing them in their system is much simpler than real-time video based cytometry, so it is an alternate method of egg counting that would be interesting for us to incorporate. The system by Li et al[35] is more comparable in term of their design and goals, though differences in survival and egg-laying can be attributed to minor unpublished details. They did not aim to demonstrate the same amount of egg-laying as on agar plates, but if they used higher food concentrations like us, they may in fact observe similar brood sizes with other details being equal. Another difference is that our device measures egg laying in real-time, while theirs looks at live progeny hatching in real-time. Our measurement can be interpreted as a real-time measurement of the mother, while theirs is a direct measurement of progeny health but does not give real-time resolution of the mother's state. Eggs take several hours to hatch, so progeny hatching data tells something about the mother within the past six hours. All the above mentioned systems use a fixed viewing area, but a panning stage can

increase the available viewing area with the tradeoff of a more complicated experimental setup.

With our ability to switch soluble factors on fast timescales we are able to show for the first time that a particular type of intermittent fasting can increase reproductive span in an organism at the same time while maintaining progeny output (Fig 4.1). There is no compromise between reproductive lifespan and progeny output. There are a variety of follow up directions that can be taken with regard to these results.

It would be interesting to extend the IF results further to show there is no tradeoff between fertility and lifespan. Given that starved worms have extended reproductive lifespan and smaller brood size, it is likely that IF will extend lifespan, even if the increase is more modest than harsher diets. In worms, a highly asymmetric IF regimen (6 h food, 1 h no food) increases reproductive span, but a 40% lifespan increase was seen in sterilized worms with symmetric fasting. Whether these are mediated by *daf-2*, *daf-16*, and *rheb-1* can be shown with loss of function mutants and RNAi knockdown, as these are standard techniques in this field. Worms can be observed in our chip for the duration of their lifespan or it may be more convenient to extract and observe them on plates.

To screen through more compounds in the lifespan literature to see their effect on reproduction, the system can be improved to screen more conditions simultaneously. The maximum number of worms that can be assayed with a similar design is fifty, but the multiple outlets complicate the waste collection so they should be pooled to make running experiments more simple. The mating efficiency in our protocol is such that we can assume all worms were mated, or we can mate to worms with fluorescently labeled sperm.

One that is done, inlet channel sizes could be iterated to allow for simultaneous loading as is done in the various clamp designs, and two sets of 25 worms could be run simultaneously, one control and one experimental condition. Since the *JW1025-1* strain eliminates biofilm,

it is possible to run the system without an inlet syringe filter and switch to a pressure-driven setup. This would be a fluidic setup that can scale up switching experiments for nutrients or other soluble factors. This could lead to high throughput reproductive assays in worms.

Bibliography

[1] Adrian Paz. *Environmental effects on reproductive longevity and cell cycle progression of the Caenorhabditis elegans hermaphrodite germline*. PhD thesis, University of California, Irvine, December 2015.

[2] Lorenzo Galluzzi, Ilio Vitale, Oliver Kepp, and Guido Kroemer, editors. *Cell Senescence*, volume 965 of *Methods in Molecular Biology*. Humana Press, Totowa, NJ, 2013.

[3] Heidi A Tissenbaum. Using C. elegans for aging research. *Invertebrate Reproduction & Development*, December 2014.

[4] Koutarou D Kimura, Heidi A Tissenbaum, Yanxia Liu, and Gary Ruvkun. daf-2, an Insulin Receptor-Like Gene That Regulates Longevity and Diapause in Caenorhabditis elegans. *Science (New York, N.Y.)*, 277(5328):942–946, August 1997.

[5] C Kenyon, J Chang, E Gensch, A Rudner, and R Tabtiang. A C. elegans mutant that lives twice as long as wild type. *Nature*, 366(6454):461–464, December 1993.

[6] Cheng Huang, Chengjie Xiong, and Kerry Kornfeld. Measurements of age-related changes of physiological processes that predict lifespan of Caenorhabditis elegans. *Proceedings of the National Academy of Sciences*, 101(21):8084–8089, May 2004.

[7] Siu Sylvia Lee, Scott Kennedy, Andrew C Tolonen, and Gary Ruvkun. DAF-16 target genes that control C. elegans life-span and metabolism. *Science*, 300(5619):644–647, April 2003.

[8] S Ogg, S Paradis, S Gottlieb, G I Patterson, L Lee, H A Tissenbaum, and G Ruvkun. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature*, 389(6654):994–999, October 1997.

[9] Coleen T Murphy, Steven A McCarroll, Cornelia I Bargmann, Andrew Fraser, Ravi S Kamath, Julie Ahringer, Hao Li, and Cynthia Kenyon. Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature*, 424(6946):277–283, July 2003.

[10] Nataliya Libina, Jennifer R Berman, and Cynthia Kenyon. Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. *Cell*, 115(4):489–502, November 2003.

[11] Karen T Coschigano, David Clemmons, Linda L Bellush, and John J Kopchick. Assessment of Growth Parameters and Life Span of GHR/BP Gene-Disrupted Mice1. *Endocrinology*, July 2013.

[12] Andrzej Bartke, J Chris Wright, Julie A Mattison, Donald K Ingram, Richard A Miller, and George S Roth. Longevity: Extending the lifespan of long-lived mice. *Nature*, 414(6862):412–412, November 2001.

[13] Martin Holzenberger, Joëlle Dupont, Bertrand Ducos, Patricia Leneuve, Alain Géloën, Patrick C Even, Pascale Cervera, and Yves Le Bouc. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature*, 421(6919):182–187, January 2003.

[14] Luigi Fontana, Linda Partridge, and Valter D Longo. Extending Healthy Life Span—From Yeast to Humans. *Science (New York, N.Y.)*, 328(5976):321–326, April 2010.

[15] Filipe Cabreiro, Catherine Au, Kit-Yi Leung, Nuria Vergara-Irigaray, Helena M Cochemé, Tahereh Noori, David Weinkove, Eugene Schuster, Nicholas D E Greene, and David Gems. Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism. *Cell*, 153(1):228–239, March 2013.

[16] Mohan Viswanathan, Stuart K Kim, Ala Berdichevsky, and Leonard Guarente. A Role for SIR-2.1 Regulation of ER Stress Response Genes in Determining C. elegans Life Span. *Developmental cell*, 9(5):605–615, November 2005.

[17] Piper R Hunt, Tae Gen Son, Mark A Wilson, Quian-Sheng Yu, William H Wood, Yongqing Zhang, Kevin G Becker, Nigel H Greig, Mark P Mattson, Simonetta Camandola, and Catherine A Wolkow. Extension of Lifespan in C. elegans by Naphthoquinones That Act through Stress Hormesis Mechanisms. *PloS one*, 6(7):e21922, July 2011.

[18] Yoko Honda, Masashi Tanaka, and Shuji Honda. Trehalose extends longevity in the nematode Caenorhabditis elegans. *Aging cell*, 9(4):558–569, August 2010.

[19] Tanja Heidler, Kai Hartwig, Hannelore Daniel, and Uwe Wenzel. Caenorhabditis elegans lifespan extension caused by treatment with an orally active ROS-generator is dependent on DAF-16 and SIR-2.1. *Biogerontology*, 11(2):183–195, 2010.

[20] Silvestre Alavez, Maithili C Vantipalli, David J S Zucker, Ida M Klang, and Gordon J Lithgow. Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. *Nature*, 472(7342):226–229, April 2011.

[21] Schiva Gandhi, John Santelli, David H Mitchell, J Wesley Stiles, and D Rao Sanadi. A simple method for maintaining large, aging populations of Caenorhabditis elegans. *Mechanisms of ageing and development*, 12(2):137–150, February 1980.

[22] George L Sutphin and Matt Kaeberlein. Measuring Caenorhabditis elegans life span on solid media. *Journal of visualized experiments : JoVE*, (27):e1152–e1152, 2009.

[23] Gregory M Solis and Michael Petrascheck. Measuring Caenorhabditis elegans life span in 96 well microtiter plates. *Journal of visualized experiments : JoVE*, (49):e2496–e2496, 2011.

[24] Layla Aitlhadj and Stephen R Stürzenbaum. The use of FUdR can cause prolonged longevity in mutant nematodes. *Mechanisms of ageing and development*, 131(5):364–365, May 2010.

[25] Jeremy Michael Van Raamsdonk and Siegfried Hekimi. FUdR causes a twofold increase in the lifespan of the mitochondrial mutant gas-1. *Mechanisms of ageing and development*, 132(10):519–521, October 2011.

[26] Sarah K Davies, Armand M Leroi, and Jacob G Bundy. Fluorodeoxyuridine affects the identification of metabolic responses to daf-2 status in Caenorhabditis elegans. *Mechanisms of ageing and development*, 133(1):46–49, January 2012.

[27] Honor Hsin and Cynthia Kenyon. Signals from the reproductive system regulate the lifespan of C. elegans. *Nature*, 399(6734):362–366, May 1999.

[28] Kui Lin, Honor Hsin, Natasha Libina, and Cynthia Kenyon. Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nature genetics*, 28(2):139–145, June 2001.

[29] P L Larsen, P S Albert, and D L Riddle. Genes that regulate both development and longevity in Caenorhabditis elegans. *Genetics*, 139(4):1567–1583, April 1995.

[30] David Gems, Amy J Sutton, Mark L Sundermeyer, Patrice S Albert, Kevin V King, Mark L Edgley, Pamela L Larsen, and Donald L Riddle. Two Pleiotropic Classes of daf-2 Mutation Affect Larval Arrest, Adult Behavior, Reproduction and Longevity in Caenorhabditis elegans. *Genetics*, 150(1):129–155, September 1998.

[31] Heidi A Tissenbaum and Gary Ruvkun. An Insulin-like Signaling Pathway Affects Both Longevity and Reproduction in Caenorhabditis elegans. *Genetics*, 148(2):703–717, February 1998.

[32] Stacie E Hughes, Kimberley Evason, Chengjie Xiong, and Kerry Kornfeld. Genetic and Pharmacological Factors That Influence Reproductive Aging in Nematodes. *PLOS Genet*, 3(2):e25, February 2007.

[33] Christopher L Pickett, Nicholas Dietrich, Junfang Chen, Chengjie Xiong, and Kerry Kornfeld. Mated progeny production is a biomarker of aging in Caenorhabditis elegans. *G3 (Bethesda, Md.)*, 3(12):2219–2232, December 2013.

[34] Lise Frézal and Marie-Anne Félix. C. elegans outside the Petri dish. *eLife*, 4:e05849, 2015.

[35] Siran Li, Howard A. Stone, and Coleen T. Murphy. A microfluidic device and automatic counting system for the study of c. elegans reproductive aging. *Lab Chip*, 15:524–531, 2015.

[36] Ruiqian Wan, Simonetta Camandola, and Mark P Mattson. Intermittent Food Deprivation Improves Cardiovascular and Neuroendocrine Responses to Stress in Rats. *J Nutr*, 133(6):1921–1929, June 2003.

[37] R M Anson, Z Guo, R de Cabo, and T Iyun. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. In *Proceedings of the National Academy of Sciences of the United States of America*, 2003.

[38] Fenni Rusli, Mark V Boekschoten, Arantza Aguirre Zubia, Carolien Lute, Michael Müller, and Wilma T Steegenga. A weekly alternating diet between caloric restriction and medium fat protects the liver from fatty liver development in middle-aged C57BL/6J mice. *Molecular Nutrition & Food Research*, 59(3):533–543, March 2015.

[39] Reyhan Westbrook, Michael S Bonkowski, Oge Arum, April D Strader, and Andrzej Bartke. Metabolic Alterations Due to Caloric Restriction and Every Other Day Feeding in Normal and Growth Hormone Receptor Knockout Mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 69(1):25–33, January 2014.

[40] L K Heilbronn, A E Civitarese, and I Bogacka. Glucose Tolerance and Skeletal Muscle Gene Expression in Response to Alternate Day Fasting. *Obesity Research*, 2005.

[41] C L Goodrick, D K Ingram, M A Reynolds, J R Freeman, and N Cider. Effects of intermittent feeding upon body weight and lifespan in inbred mice: interaction of genotype and age. *Mechanisms of ageing and development*, 55(1):69–87, July 1990.

[42] James Rochon, Connie W Bales, Eric Ravussin, Leanne M Redman, John O Holloszy, Susan B Racette, Susan B Roberts, Sai Krupa Das, Sergei Romashkan, Katherine M Galan, Evan C Hadley, William E Kraus, and CALERIE Study Group. Design and conduct of the CALERIE study: comprehensive assessment of the long-term effects of reducing intake of energy. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 66(1):97–108, January 2011.

[43] Eric Ravussin, Leanne M Redman, James Rochon, Sai Krupa Das, Luigi Fontana, William E Kraus, Sergei Romashkan, Donald A Williamson, Simin N Meydani, Dennis T Villareal, Steven R Smith, Richard I Stein, Tammy M Scott, Tiffany M Stewart, Edward Saltzman, Samuel Klein, Manju Bhapkar, Corby K Martin, Cheryl H Gilhooly, John O Holloszy, Evan C Hadley, Susan B Roberts, and CALERIE Study Group. A 2-Year Randomized Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 70(9):1097–1104, September 2015.

[44] Sakiko Honjoh, Takuya Yamamoto, Masaharu Uno, and Eisuke Nishida. Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. *Nature*, 457(7230):726–730, February 2009.

[45] Pilar Caro, José Gómez, Mónica López-Torres, Inés Sánchez, Alba Naudi, Manuel Portero-Otín, Reinald Pamplona, and Gustavo Barja. Effect of Every Other Day

Feeding on Mitochondrial Free Radical Production and Oxidative Stress in Mouse Liver. *Rejuvenation Research*, 11(3):621–9, July 2008.

[46] M Mattson, Mark P and Ruiqian Wan. Beneficial effects of intermittent fasting and caloric restriction on the cardiovascular and cerebrovascular systems. *The Journal of nutritional biochemistry*, 16(3):129–137, March 2005.

[47] Colin Selman. Dietary restriction and the pursuit of effective mimetics. *Proceedings of the Nutrition Society*, 73(02):260–270, May 2014.

[48] S Rohrbach, M Aslam, and B Niemann. Impact of caloric restriction on myocardial ischaemia/reperfusion injury and new therapeutic options to mimic its effects. *British journal of Pharmacology*, 2014.

[49] Minaxi S Gami and Catherine A Wolkow. Studies of Caenorhabditis elegans DAF-2/insulin signaling reveal targets for pharmacological manipulation of lifespan. *Aging cell*, 5(1):31–37, February 2006.

[50] Nicholas Stroustrup, Bryne E Ulmschneider, Zachary M Nash, Isaac F López-Moyado, Javier Apfeld, and Walter Fontana. The Caenorhabditis elegans Lifespan Machine. *Nature methods*, 10(7):665–670, May 2013.

[51] Francis Raj Gandhi Amrit, Ramesh Ratnappan, Scott Alexander Keith, and Arjumand Ghazi. The C. elegans lifespan assay toolkit. *Methods (San Diego, Calif.)*, 68(3):465–475, August 2014.

[52] Hannah S Seidel and Judith Kimble. The oogenic germline starvation response in C. elegans. *PloS one*, 6(12):e28074, 2011.

[53] Eugeni V Entchev, Dhaval S Patel, Mei Zhan, Andrew J Steele, Hang Lu, QueeLim Ch'ng, and Oliver Hobert. A gene-expression-based neural code for food abundance that modulates lifespan. *eLife*, 4:e06259, May 2015.

[54] Ronen B. Kopito and Erel Levine. Durable spatiotemporal surveillance of caenorhabditis elegans response to environmental cues. *Lab Chip*, 14:764–770, 2014.

[55] Weiwei Shi, Jianhua Qin, Nannan Ye, and Bingcheng Lin. Droplet-based microfluidic system for individual Caenorhabditis elegans assay. *Lab on a chip*, 8(9):1432–1435, September 2008.

[56] Guillaume Aubry, Mei Zhan, and Hang Lu. Hydrogel-droplet microfluidic platform for high-resolution imaging and sorting of early larval Caenorhabditis elegans. *Lab on a chip*, 15(6):1424–1431, 2015.

[57] Henrik Bringmann. Agarose hydrogel microcompartments for imaging sleep- and wake-like behavior and nervous system development in Caenorhabditis elegans larvae. *Journal of neuroscience methods*, 201(1):78–88, September 2011.

[58] S J Belfer, H S Chuang, B L Freedman, J Yuan, and M Norton. Caenorhabditis-in-drop array for monitoring C. elegans quiescent behavior. *Sleep*, 2013.

[59] S. Elizabeth Hulme, Sergey S. Shevkoplyas, Alison P. McGuigan, Javier Apfeld, Walter Fontana, and George M. Whitesides. Lifespan-on-a-chip: microfluidic chambers for performing lifelong observation of c. elegans. *Lab Chip*, 10:589–597, 2010.

[60] Hui Wen, Weiwei Shi, and Jianhua Qin. Multiparameter evaluation of the longevity in C. elegans under stress using an integrated microfluidic device. *Biomedical microdevices*, 14(4):721–728, August 2012.

[61] Bo Xian, Jie Shen, Weiyang Chen, Na Sun, Nan Qiao, Dongqing Jiang, Tao Yu, Yongfan Men, Zhijun Han, Yuhong Pang, Matt Kaeberlein, Yanyi Huang, and Jing Dong J Han. WormFarm: a quantitative control and measurement device toward automated Caenorhabditis elegans aging analysis. *Aging cell*, 12(3):398–409, June 2013.

[62] Jan Krajniak and Hang Lu. Long-term high-resolution imaging and culture of C . elegans in chip-gel hybrid microfluidic device for developmental studies. *Lab on a chip*, 10(14):1862–1868, 2010.

[63] Kwanghun Chung, Mei Zhan, Jagan Srinivasan, Paul W Sternberg, Emily Gong, Frank C Schroeder, and Hang Lu. Microfluidic chamber arrays for whole-organism behavior-based chemical screening. *Lab on a chip*, 11(21):3689–3697, 2011.

[64] Jaehoon Jung, Masahiro Nakajima, Masaru Kojima, Katsutoshi Ooe, and Toshio Fukuda. Microchip device for measurement of body volume of C. elegans as bioindicator application. *Journal of Micro-Nano Mechatronics*, 7(1-3):3–11, 2012.

[65] Shazlina Johari, Volker Nock, Maan M Alkaisi, and Wenhui Wang. On-chip analysis of C. elegans muscular forces and locomotion patterns in microstructured environments. *Lab on a chip*, 13(9):1699–1707, May 2013.

[66] Jianhua Qin and Aaron R Wheeler. Maze exploration and learning in C. elegans. *Lab on a chip*, 7(2):186–192, 2007.

[67] Yun Zhang, Hang Lu, and Cornelia I Bargmann. Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. *Nature*, 438(7065):179–184, November 2005.

[68] Dirk R Albrecht and Cornelia I Bargmann. High-content behavioral analysis of Caenorhabditis elegans in precise spatiotemporal chemical environments. *Nature methods*, 8(7):599–605, July 2011.

[69] Jesse M Gray, David S Karow, Hang Lu, Andy J Chang, Jennifer S Chang, Ronald E Ellis, Michael A Marletta, and Cornelia I Bargmann. Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue. *Nature*, 430(6997):317–322, July 2004.

[70] Sungsu Park, Hyejin Hwang, Seong-Won Nam, Fernando Martinez, Robert H Austin, and William S Ryu. Enhanced Caenorhabditis elegans Locomotion in a Structured Microfluidic Environment. *PloS one*, 3(6):e2550, June 2008.

[71] S R Lockery, K J Lawton, J C Doll, S Faumont, S M Coulthard, T R Thiele, N Chronis, K E McCormick, M B Goodman, and B L Pruitt. Artificial Dirt: Microfluidic Substrates for Nematode Neurobiology and Behavior. *Journal of Neurophysiology*, 99(6):3136–3143, June 2008.

[72] Nikos Chronis, Manuel Zimmer, and Cornelia I Bargmann. Microfluidics for in vivo imaging of neuronal and behavioral activity in Caenorhabditis elegans. *Nature methods*, 4(9):727–731, September 2007.

[73] Tina Schrödel, Robert Prevedel, Karin Aumayr, Manuel Zimmer, and Alipasha Vaziri. Brain-wide 3D imaging of neuronal activity in Caenorhabditis elegans with sculpted light. *Nature methods*, 10(10):1013–1020, October 2013.

[74] Jingjing Wang, Xiaojun Feng, Wei Du, and Bi-Feng Liu. Microfluidic worm-chip for in vivo analysis of neuronal activity upon dynamic chemical stimulations. *Analytica Chimica Acta*, 701(1):23–28, September 2011.

[75] S Elizabeth Hulme, Sergey S Shevkoplyas, Javier Apfeld, Walter Fontana, and George M Whitesides. A microfabricated array of clamps for immobilizing and imaging C. elegans. *Lab on a chip*, 7(11):1515–1523, 2007.

[76] Jan Krajniak, Yan Hao, Ho Yi Mak, and Hang Lu. C.L.I.P.–continuous live imaging platform for direct observation of C. elegans physiological processes. *Lab on a chip*, 13(15):2963–2971, 2013.

[77] Hyewon Lee, Shin Ae Kim, Sean Coakley, Paula Mugno, Marc Hammarlund, Massimo A Hilliard, and Hang Lu. A multi-channel device for high-density target-selective stimulation and long-term monitoring of cells and subcellular features in C. elegans. *Lab on a chip*, 14(23):4513–4522, December 2014.

[78] Brandon G Wong, Adrian Paz, Michael A Corrado, Brian R Ramos, Amanda Cinquin, Olivier Cinquin, and Elliot E Hui. Live imaging reveals active infiltration of mitotic zone by its stem cell niche. *Integrative biology : quantitative biosciences from nano to macro*, 5(7):976–982, July 2013.

[79] Trushal Vijaykumar Chokshi, Adela Ben-Yakar, and Nikos Chronis. CO 2 and compressive immobilization of C . elegans on-chip. *Lab on a chip*, 9(1):151–157, 2009.

[80] Xingfu Zhao, Fei Xu, Lichun Tang, Wei Du, Xiaojun Feng, and Bi-Feng Liu. Microfluidic chip-based C. elegans microinjection system for investigating cell–cell communication in vivo. *Biosensors and Bioelectronics*, 50:28–34, December 2013.

[81] Fei Zeng, Christopher B Rohde, and Mehmet Fatih Yanik. Sub-cellular precision on-chip small-animal immobilization, multi- photon imaging and femtosecond-laser manipulation. *Lab on a chip*, 8(5):653–656, 2008.

[82] Christopher B Rohde, Fei Zeng, Ricardo Gonzalez-Rubio, Matthew Angel, and Mehmet Fatih Yanik. Microfluidic system for on-chip high-throughput whole-animal sorting and screening at subcellular resolution. *Proceedings of the National Academy of Sciences*, 104(35):13891–13895, August 2007.

[83] Sertan Kutal Gokce, Samuel X Guo, Navid Ghorashian, W Neil Everett, Travis Jarrell, Aubri Kottek, Alan C Bovik, and Adela Ben-Yakar. A fully automated microfluidic femtosecond laser axotomy platform for nerve regeneration studies in C. elegans. *PloS one*, 9(12):e113917, 2014.

[84] Matthew M Crane, Kwanghun Chung, and Hang Lu. Computer-enhanced high-throughput genetic screens of C. elegans in a microfluidic system. *Lab on a chip*, 9(1):38–40, January 2009.

[85] Yuanjun Yan, Li Fang Ng, Li Theng Ng, Kwan Bum Choi, Jan Gruber, Andrew A Bettiol, and Nitish V Thakor. A continuous-flow C. elegans sorting system with integrated optical fiber detection and laminar flow switching. *Lab on a chip*, 14(20):4000–4006, 2014.

[86] Pouya Rezai, Sangeena Salam, Ponnambalam Ravi Selvaganapathy, and Bhagwati P Gupta. Electrical sorting of Caenorhabditis elegans. *Lab on a chip*, 12(10):1831–1840, 2012.

[87] Xiaoni Ai, Weipeng Zhuo, Qionglin Liang, Patrick T McGrath, and Hang Lu. A high-throughput device for size based separation of C. elegans developmental stages. *Lab on a chip*, 14(10):1746–1752, 2014.

[88] Xavier Casadevall i Solvas, Florian M Geier, Armand M Leroi, Jake G Bundy, Joshua B Edel, and Andrew J deMello. High-throughput age synchronisation of Caenorhabditis elegans. *Chemical Communications*, 47(35):9801–9803, 2011.

[89] David T. Eddington. Chips and tips: In-line microfluidic bubble trap.

[90] Wenfu Zheng, Zhuo Wang, Wei Zhang, and Xingyu Jiang. A simple PDMS-based microfluidic channel design that removes bubbles for long-term on-chip culture of mammalian cells. *Lab on a chip*, 10(21):2906–2910, 2010.

[91] Tsuyoshi Kawano, Naoya Kataoka, Sunao Abe, Mari Ohtani, Yoko Honda, Shuji Honda, and Yasuo Kimura. Lifespan Extending Activity of Substances Secreted by the Nematode Caenorhabditis elegans That Include the Dauer-Inducing Pheromone. *Bioscience, Biotechnology, and Biochemistry*, May 2014.

[92] Pan-Young Jeong, Mankil Jung, Yong-Hyeon Yim, Heekyeong Kim, Moonsoo Park, Eunmi Hong, Weontae Lee, Young Hwan Kim, Kun Kim, and Young-Ki Paik. Chemical structure and biological activity of the Caenorhabditis elegans dauer-inducing pheromone. *Nature*, 433(7025):541–545, February 2005.

[93] Marco Gallo and Donald L Riddle. Effects of a Caenorhabditis elegans Dauer Pheromone Ascaroside on Physiology and Signal Transduction Pathways. *Journal of Chemical Ecology*, 35(2):272–279, 2009.

[94] D Wu, S Rea, A Yashin, and T Johnson. Visualizing hidden heterogeneity in isogenic populations of C. elegans. *Experimental Gerontology*, 41(3):261–270, March 2006.

[95] Patrick D. McMullen, Erin Z. Aprison, Peter B. Winter, Luis A. N. Amaral, Richard I. Morimoto, and Ilya Ruvinsky. Macro-level modeling of the response of ¡italic¿c. elegans¡/italic¿ reproduction to chronic heat stress. *PLoS Comput Biol*, 8(1):e1002338, 01 2012.

[96] Niels Asger Mortensen, Fridolin Okkels, and Henrik Bruus. Reexamination of Hagen-Poiseuille flow: shape dependence of the hydraulic resistance in microchannels. *Physical review. E, Statistical, nonlinear, and soft matter physics*, 71(5 Pt 2):057301, May 2005.

[97] S. Viswanathan W.L. Nicholas, A. Grassia. The efficiency with which caenorhabditis briggsae (rhabditidae) feeds on the bacterium, escherichia coli. *Nematologica*, 19(4):411–420, 1973.

[98] Michael R. Klass. Aging in the nematode caenorhabditis elegans: major biological and environmental factors influencing life span. *Mechanisms of Ageing and Development*, 6(6):413–429, Nov-Dec 1977.

[99] Andrej Trampuz, Kerryl E Piper, James M Steckelberg, and Robin Patel. Effect of gamma irradiation on viability and DNA of Staphylococcus epidermidis and Escherichia coli. *Journal of Medical Microbiology*, 55(9):1271–1275, September 2006.

[100] John E Thompson and Ernest R Blatchley III. Gamma Irradiation for Inactivation of C. parvum, E. coli, and Coliphage MS-2. *Journal of Environmental Engineering*, August 2000.

[101] Fariborz Taghipour. Ultraviolet and ionizing radiation for microorganism inactivation. *Water Research*, 38(18):3940–3948, November 2004.

[102] R Sommer. Inactivation of bacteriophages in water by means of non-ionizing (uv-253.7nm) and ionizing (gamma) radiation: a comparative approach. *Water Research*, 35(13):3109–3116, September 2001.

[103] Live L Nesse, Kristin Berg, and Lene K Vestby. Effects of Norspermidine and Spermidine on Biofilm Formation by Potentially Pathogenic Escherichia coli and Salmonella enterica Wild-Type Strains. *Applied and Environmental Microbiology*, 81(6):2226–2232, March 2015.

[104] J W Costerton and Z Lewandowski. Microbial biofilms. *Annual Reviews in Microbiology*, 49:711–745, 1995.

[105] Laura Hobley, Sok Ho Kim, Yukari Maezato, Susan Wyllie, Alan H Fairlamb, Nicola R Stanley-Wall, and Anthony J Michael. Norspermidine Is Not a Self-Produced Trigger for Biofilm Disassembly. *Cell*, 156(4):844–854, February 2014.

[106] Isabelle Lenaerts, Glenda A Walker, Luc Van Hoorebeke, David Gems, and Jacques R Vanfleteren. Dietary Restriction of Caenorhabditis elegans by Axenic Culture Reflects Nutritional Requirement for Constituents Provided by Metabolically Active Microbes. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 63(3):242–252, March 2008.

[107] Christine A White-Ziegler, Suzin Um, Natalie M Pérez, Abby L Berns, Amy J Malhowski, and Sarah Young. Low temperature increases expression of biofilm-, cold-shock- and RpoS-dependent genes in Escherichia coli K-12. *Microbiology*, 154(1):148–166, January 2008.

[108] Joshua D Meisel and Dennis H Kim. Behavioral avoidance of pathogenic bacteria by Caenorhabditis elegans. *Trends in immunology*, 35(10):465–470, October 2014.

[109] T Baba, T Ara, M Hasegawa, Y Takai, Y Okumura, M Baba, KA Datsenko, M Tomita, BL Wanner, and H Mori. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*, 2(2006.0008), February 2006.

[110] Natsuko Yamamoto, Kenji Nakahigashi, Tomoko Nakamichi, Mihoko Yoshino, Yuki Takai, Yae Touda, Akemi Furubayashi, Satoko Kinjyo, Hitomi Dose, Miki Hasegawa, Kirill A Datsenko, Toru Nakayashiki, Masaru Tomita, Barry L Wanner, and Hirotada Mori. Update on the Keio collection of Escherichia coli single-gene deletion mutants. *Molecular Systems Biology*, 5(1):335, January 2009.

[111] Bong Hyun Sung, Choong Hoon Lee, Byung Jo Yu, Jun Hyoung Lee, Ju Young Lee, Mi Sun Kim, Frederick R Blattner, and Sun Chang Kim. Development of a Biofilm Production-Deficient Escherichia coli Strain as a Host for Biotechnological Applications. *Applied and Environmental Microbiology*, 72(5):3336–3342, May 2006.

[112] Vijay K Sharma and Bradley L Bearson. Hha Controls Escherichia coli O157:H7 Biofilm Formation by Differential Regulation of Global Transcriptional Regulators FlhDC and CsgD. *Applied and Environmental Microbiology*, 79(7):2384–2396, April 2013.

[113] Luz P Blanco, Margery L Evans, Daniel R Smith, Matthew P Badtke, and Matthew R Chapman. Diversity, biogenesis and function of microbial amyloids. *Trends in microbiology*, 20(2):66–73, February 2012.

[114] Neal D Hammer, Bryan A McGuffie, Yizhou Zhou, Matthew P Badtke, Ashley A Reinke, Kristoffer Brännström, Jason E Gestwicki, Anders Olofsson, Fredrik Almqvist, and Matthew R Chapman. The C-Terminal Repeating Units of CsgB Direct Bacterial Functional Amyloid Nucleation. *Journal of molecular biology*, 422(3):376–389, September 2012.

[115] Garry Laverty, Sean Gorman, and Brendan Gilmore. Biomolecular Mechanisms of Pseudomonas aeruginosa and Escherichia coli Biofilm Formation. *Pathogens*, 3(3):596–632, July 2014.

[116] James Lillington and Gabriel Waksman. Ordered and ushered; the assembly and translocation of the adhesive type I and p pili. *Biology*, 2(3):841–860, 2013.

[117] P Lembre, C Lorentz, and P Di Martino. Exopolysaccharides of the biofilm matrix: A complex biophysical world, 2012.

[118] Kinnari B Patel, Evelyn Toh, Ximena B Fernandez, Anna Hanuszkiewicz, Gail G Hardy, Yves V Brun, Mark A Bernards, and Miguel A Valvano. Functional Characterization of UDP-Glucose:Undecaprenyl-Phosphate Glucose-1-Phosphate Transferases of Escherichia coli and Caulobacter crescentus. *Journal of Bacteriology*, 194(10):2646–2657, May 2012.

[119] Paul N Danese, Leslie A Pratt, and Roberto Kolter. Exopolysaccharide Production Is Required for Development of Escherichia coli K-12 Biofilm Architecture. *Journal of Bacteriology*, 182(12):3593–3596, June 2000.

[120] Alvaro Mata, Aaron J Fleischman, and Shuvo Roy. Fabrication of multi-layer SU-8 microstructures. *Journal of Micromechanics and Microengineering*, 16(2):276, February 2006.

Appendix A

Appendix Title

A.1 Methods

A.1.1 Device fabrication

Culture device designs were drawn up for 100 mm diameter wafers using AutoCAD and printed at 20000 dpi on transparency photomasks by CAD/Art (Bandon, OR). Test grade silicon wafers (UniversityWafers) were cleaned with Cyantek Nanostrip to improve adhesion. The first 50 μm layer was made by spinning SU-8 3035 (MicroChem) at 1900 RPM on a Laurell spin coater. The 50 μm features were exposed on a Karl Suss MA6 mask aligner and then post-exposure baked to reveal the features. To increase the height to 100 μm a second layer of SU-8 3035 was spun at 2100 RPM, aligned, and exposed for double the single layer time. A higher speed was necessary for the same thickness because of the increased SU-8-to-SU-8 adhesion. The pattern heights were checked using a stylus profilometer to make sure they were within 10% error.

The mold wafer was then hard baked at 150 °C and placed overnight under vacuum with

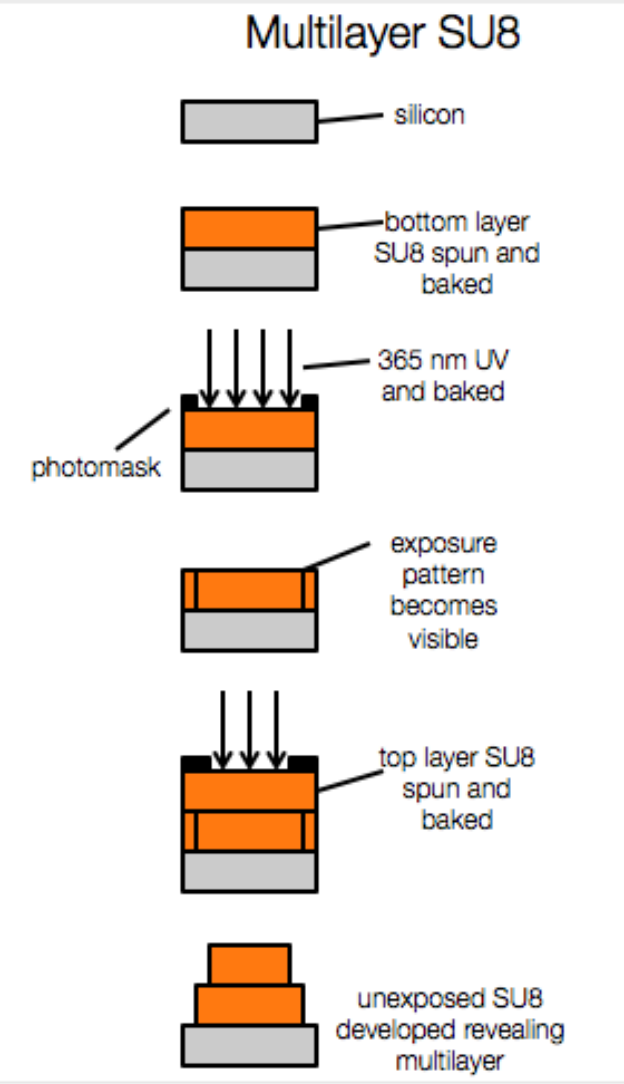

Figure A.1: Multilayer SU8 photolithography process[120].

10 µL of (tridecafluoro-1,1,2,2-tetrahydrooctly)triethoxysilane (Gelest) to passivate it. 25 g of 10:1 Sylgard 184 was poured on the mold, degassed for 45 min, and then baked at 65 °C for 4 h. The PDMS devices were then diced and trimmed and punched with a 23 gauge hole punch (CR0350255N20R4, Syneo). The holes were cleaned with water, and the devices were tape cleaned with methanol, water, and and dried with an airgun. The devices along with Nanostrip cleaned slides were treated with 25 s of air plasma, bonded together, and baked at 65 °C for 20 min. The devices were filled with water and stored until the night before loading with worms. The device was prepared for worm loading by rinsing with 10% bleach

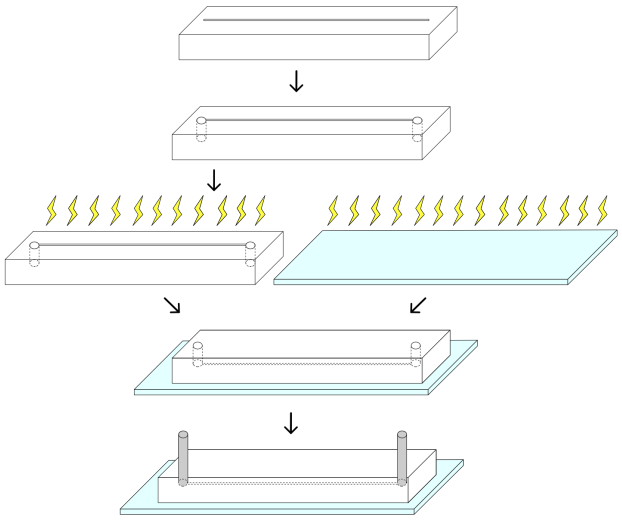

Figure A.2: PDMS fabrication and irreversible bonding.

and flushing generously with water, then gamma irradiating it overnight.

Bubble traps were casted and processed from 20 mm long 50 × 100 µm long channels made similarly to culture devices. A 3 mm diameter circle was punched at the expected inlet hole. A side inlet was punched into this formed chamber. 250 µm and PDMS membranes were bonded to the top and bottom of the device to close it.

A.1.2 Bacterial and worm preparation

JW1025-1 E. coli was grown to saturation overnight; the bacteria were centrifuged and stored as frozen pellets at −20 °C until ready for use. At that point they were diluted to 1×10^{10}cells/mL in S-media, filtered through a 5 µm filter (Millipore) and delivered to worms through a 2 µm filter (IDEX).

Mated *C. elegans N2 wild-type* strain was cultured on *JW1025-1 E. coli* seeded on nematode growth medium. At 20 °C, it takes approximately 48 h for worms of either sex to reach the L4 stage. L4 males were isolated from laid embryos and allowed to develop further for *20*h. At this point, L4 hermaphrodites were isolated from a separate culture and allowed

to develop for 4 h. Both isolated populations were added together on a 60 mm at a 5:1 male:hermaphrodite ratio and allowed to mate for 6 h.

Mated hermaphrodites were gently washed by pipet in filtered M9 then transferred into A 10 µL pipet placed at the device inlet. The worms were individually drawn into each habitat using a vacuum source at the corresponding outlet.

A.1.3 Long term worm culture

The worm microfluidic chip was perfused at a flowrate of 800 µL which works out to 40 µL through each habitat. Worms were imaged using a fiber optic red source on the dissection scope in a 20 °C incubator because they are sensitive to temperature and blue light[?] . Effluent from each habitat outlet was collected and analyzed for male offspring to verify that individual hermaphrodites were mated successfully.

A.2 Syringe pump bash scripts

A.2.1 HA11v6

```
#!/bin/bash

. "$( dirname "$0" )"/send2pumpv2

#v1. first version given to Adrian
#v2. added parameter ldelay to wait between sending each letter
#added pdelay to wait between sending each command
#v3. include files are now defined based on same folder as script and don't need explici
```

```
#fixed timing so it's accurate to the second by switching according to system time, not
#v4. added a lower limit rate so stop command is sent instead of being set OUT OF RANGE

#VARIABLE DEFINITIONS
#starting pump point (default=0). enter output of last program run
pumpcount=1
#rate tables
ftable="rampup24h.csv"
#pump command interval (in s)--for now independent of program clock, depends on the Matl
pumptime=900



#syringe diameter (mm 0.002 mL/hr is min rate for 14.5mm syringe)
syringe1d=14.5
syringe2d=14.5
#flow units (MLH-milliL/hr, ULH-uicroL/hr)
flowunits="mlh"
#lower limit rate, below which the pump becomes OUTOFRANGE, same units as flowunits
lowlimit=0.002
#pump addresses, pump1-max concentration, pump2-min concentration
pump1="01"
pump2="03"
#baudrate
brate=9600

#nonblocking input check interval (in s)--acts as program clock
keypresstime=1
```

```
#USB device name
USBdevice="/dev/cu.usbserial-FTK5KD41"
#delay between sending each letter of a command
ldelay=0.3
#delay between one command to the next
pdelay=0.3
screenlogdelay=1
logcheckdelay=10
#CONVERT PUMP COMMAND INTERVAL TO PROGRAM CLOCK INTERVAL
let pumptime_in_progclock=$pumptime/$keypresstime

#READ 2 COL CSV INTO ARRAY
count=0
while IFS=',' read -r R1 R2; do
  p1[count]=$R1
  p2[count]=$R2
  let count+=1
  #echo "$R1 $R2"
done <"$ftable"
#CHECKING FOR ENDING NEWLINE OR NOT
#if [[ $R1 != "" ]] ; then
#    echo "no ending newline"
#    #echo "$R1 $R2"
#fi
  p1[count]=$R1
  p2[count]=$R2
let trigpoints=$count+1
```

```
#NUMBER OF DATAPOINTS
#echo $trigpoints

#CSV TO ARRAY DEBUG
#echo p1
#echo ${p1[@]}
#echo p2
#echo ${p2[@]}

#SET SERIAL COMMUNICATION PARAMETERS
#8 data bits, 2 stop bits, no parity
#stty -f /dev/cu.$USBdevice $brate cs8 -parenb cstopb cooked clocal -crtscts cread
#OPEN CONNECTION, KILLING ANY OLD CONNECTION
Tinit="$(date +%s)"
flog="screen""$Tinit""_i.txt"
screen -S HAscreen -X quit
screen -d -m -S HAscreen $USBdevice $brate
screen -ls
screen -S HAscreen -p 0 -X logfile $flog
screen -S HAscreen -p 0 -X logfile flush $screenlogdelay
screen -S HAscreen -p 0 -X log on
#SET DIAMETERS AND CLEAR VOLUME
send2pump "HAscreen" "$pump1 mmd $syringe1d" $ldelay $pdelay $flog
sleep $pdelay
send2pump "HAscreen" "$pump2 mmd $syringe2d" $ldelay $pdelay $flog
sleep $pdelay
send2pump "HAscreen" "$pump1 clv" $ldelay $pdelay $flog
```

```
sleep $pdelay
send2pump "HAscreen" "$pump2 clv" $ldelay $pdelay $flog
sleep $pdelay

#START PUMP RIGHT AWAY AT PREVIOUS POINT, SET LOG TIMER
let pumpcountseed=$pumpcount
let pumpmod=$pumpcountseed%$trigpoints
let pumpcountf=pumpcountseed
let pumpcounti=pumpcountseed
#rm "$( dirname "$0" )"/log.txt
Tinit="$(date +%s)"
Tcurr=0
if [ $(echo "${p1[pumpmod]} < $lowlimit" | bc) -eq 1 ]; then
   send2pump "HAscreen" "$pump1 stp" $ldelay $pdelay $flog
   sleep $pdelay
   let p1stop=1
   echo "$pump1 stopped"
else
   let p1stop=0
   send2pump "HAscreen" "$pump1 $flowunits ${p1[pumpmod]}" $ldelay $pdelay $flog
   sleep $pdelay
   send2pump "HAscreen" "$pump1 run" $ldelay $pdelay $flog
   sleep $pdelay
   echo $pump1" "$flowunits" "${p1[pumpmod]}
fi
if [ $(echo "${p2[pumpmod]} < $lowlimit" | bc) -eq 1 ]; then
   send2pump "HAscreen" "$pump2 stp" $ldelay $pdelay $flog
```

```
   sleep $pdelay
   let p2stop=1
   echo "$pump2 stopped"
else
   send2pump "HAscreen" "$pump2 $flowunits ${p2[pumpmod]}" $ldelay $pdelay $flog
   sleep $pdelay
   send2pump "HAscreen" "$pump2 run" $ldelay $pdelay $flog
   sleep $pdelay
   let p2stop=0
   echo $pump2" "$flowunits" "${p2[pumpmod]}
fi
sleep $logcheckdelay
screen -S HAscreen -X quit

#echo "$Tcurr" >>"$( dirname "$0" )"/log.txt

#SET TO NON-BLOCKING INPUT
if [ -t 0 ]; then stty -echo -icanon time 0 min 0; fi

#LOOP UNTIL 'Q' IS PRESSED
echo press 'q' to quit
count=0
keypress=''
while [ "$keypress" != "q" ]; do
  #PROGRAM CLOCK
  let count+=1
  #echo $count'\r'
```

```
  T="$(date +%s)"
  let Tcurr=T-Tinit
  let pumpcounti=pumpcountf
  let pumpcountf=pumpcountseed+Tcurr/pumptime
  #echo "$pumpcounti" " $pumpcountf"
  #PUMP COMMAND INTERVAL REACHED, MODULAR DIVISION TO FIND PUMP DATAPOINT
  if [ "$pumpcounti" -ne "$pumpcountf" ]; then
  echo "switch at $Tcurr" |tee -a $flog
screen -d -m -S HAscreen $USBdevice $brate
screen -ls
screen -S HAscreen -p 0 -X logfile $flog
screen -S HAscreen -p 0 -X logfile flush $screenlogdelay
screen -S HAscreen -p 0 -X log on

   let pumpmod=$pumpcountf%$trigpoints
echo "$Tcurr s, line $pumpmod in table"
    #echo "$Tcurr" >>"$( dirname "$0" )"/log.txt
testcase1="${p1[pumpmod]}"
#echo "testcase1 $testcase1"
    #if [ $(echo "${p1[pumpmod]} < $lowlimit" | bc) = 1 ]; then
#FOR FIRST PUMP, IF ENTRY IS LOWER THAN THE PUMP CAN HANDLE, JUST STOP PUMP
if [[ $(bc <<< "$testcase1 < $lowlimit") == 1 ]]; then
   send2pump "HAscreen" "$pump1 stp" $ldelay $pdelay $flog
   sleep $pdelay
       let p1stop=1
   echo "$pump1 stopped"
    else
```

```
        if [ "$p1stop" -eq 1 ]; then
           let p1stop=0
           send2pump "HAscreen" "$pump1 run" $ldelay $pdelay $flog
  echo $pump1" run"
        fi
        send2pump "HAscreen" "$pump1 $flowunits ${p1[pumpmod]}" $ldelay $pdelay $flog
        sleep $pdelay
        echo $pump1" "$flowunits" "${p1[pumpmod]}
    fi
    testcase2="${p2[pumpmod]}"
#echo "testcase2 $testcase2"
#FOR SECOND PUMP, IF ENTRY IS LOWER THAN THE PUMP CAN HANDLE, JUST STOP PUMP
if [[ $(bc <<< "$testcase2 < $lowlimit") == 1 ]]; then
    #if [ $(echo "${p2[pumpmod]} < $lowlimit" | bc) = 1 ]; then
   send2pump "HAscreen" "$pump2 stp" $ldelay $pdelay $flog
   sleep $pdelay
        let p2stop=1
   echo "$pump2 stopped"
    else
        if [ "$p2stop" -eq 1 ]; then
           let p2stop=0
           send2pump "HAscreen" "$pump2 run" $ldelay $pdelay $flog
  echo $pump2" run"
        fi
        send2pump "HAscreen" "$pump2 $flowunits ${p2[pumpmod]}" $ldelay $pdelay $flog
        sleep $pdelay
        echo $pump2" "$flowunits" "${p2[pumpmod]}
```

```
    fi
#QUERY BOTH RATES FOR LOG
send2pump "HAscreen" "$pump1 rat" $ldelay $pdelay $flog
send2pump "HAscreen" "$pump2 rat" $ldelay $pdelay $flog
sleep $logcheckdelay
sleep $logcheckdelay

screen -S HAscreen -X quit

  fi

  read keypress
  sleep $keypresstime
done

#RETURN INPUT TO STANDARD
if [ -t 0 ]; then stty sane; fi

#STOP AND RETURN PUMPS TO KEYBOARD CONTROL
send2pump "HAscreen" "$pump1 stp" $ldelay $pdelay $flog
sleep $pdelay
send2pump "HAscreen" "$pump2 stp" $ldelay $pdelay $flog
sleep $pdelay
send2pump "HAscreen" "$pump1 key" $ldelay $pdelay $flog
sleep $pdelay
send2pump "HAscreen" "$pump2 key" $ldelay $pdelay $flog
sleep $pdelay
```

```
#KILL SCREEN SESSION
screen -S HAscreen -X quit
echo "You pressed '$keypress' on $pumpmod step"
exit 0
```

A.2.2 send2pumpv2

```
#!/bin/bash
# ############################
# send2pump
# ############################
# Description:
#
# This function splits string commands into individual characters to send #them to the #
#1 - screen window name
#2 - pump command (address command (value?))
#3 - sleep time between each letter

function send2pump(){
x=$2
ltime=$3
ptime=$4
fileout=$5
# ${#x} length of x
i=0
```

```
#echo "" |tee -a $fileout
echo "$x" |tee -a $fileout
while [ $i -lt ${#x} ]; do
#y[$i]=${x:$i:1};  i=$((i+1))
       screen -S $1 -p 0 -X stuff ${x:$i:1}
   #echo ${x:$i:1}
sleep $ltime
i=$((i+1))
done
screen -S $1 -p 0 -X stuff $'\r'
sleep $ptime
}
```