



Quantifying and Modeling Dynamics of Heat Shock Detection and Response in the Intestine of *Caenorhabditis Elegans*

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Quantifying and modeling dynamics of
heat shock detection and response in the
intestine of *Caenorhabditis elegans*

A DISSERTATION PRESENTED
BY
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TO
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Quantifying and modeling dynamics of heat shock detection and response in the intestine of *Caenorhabditis elegans*

ABSTRACT

The heat shock response is the organized molecular response to stressors which disrupt proteostasis, potentially leading to protein misfolding and aggregation. While the regulation of the heat shock response is well-studied in single cells, its coordination at the cell, tissue, and systemic levels of a multicellular organism is more poorly understood. To probe the interplay between systemic and cell-autonomous responses, I studied the upregulation of HSP-16.2, a molecular chaperone induced throughout the intestine of *Caenorhabditis elegans* following a heat shock, by taking longitudinal measurements in a microfluidic environment. Based on the dynamics of HSP-16.2 accumulation, I showed that a combination of heat shock temperature and duration defines the intensity of stress inflicted on the worm and identified two regimes of low and high intensity. Modeling the underlying regulatory dynamics implicated the saturation of HSP mRNA production in defining these two regimes and emphasized the importance of time separation between transcription and translation in establishing these dynamics. By applying heat shock and measuring response in separate parts of the animals, I implicated thermosensory neurons in accelerating the response and transducing information within the animal. I discuss possible implications of the systemic and cell level aspects and how they coordinate to facilitate the organismal response.

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O

Introduction

ONE OVERARCHING QUESTION OF INTEREST IN BIOLOGY is how organisms respond to their environment. Organisms sense environmental cues of all sorts and must find some way to interpret and represent these cues internally. The physiological state of the organism, in addition to any memory it has of past cues, can affect this internal representation, and together, all of these contribute to the

organism's ultimate response to this environmental cue. Organismal responses may affect only the organism, but they can also feedback on the environment, changing the cues the organism receives. My thesis work focuses on the question of organismal response to environmental cues when the cues it receives are stressful, or *stress response*.

Stress response is a complex, dynamic process that occurs at the molecular, cellular, tissue, and systemic levels. My thesis work seeks to gain quantitative insight into one particular stress response, the heat shock response (HSR), in a multicellular organism. I used a microfluidics device, WormSpa, to administer precise changes in environmental cues and take high spatiotemporal resolution data of the HSR in *Caenorhabditis elegans*, a small roundworm. Combining this data with a minimal model of the cellular HSR, I uncovered essential determinants of the HSR dynamics in a multicellular organism. While the work I undertook was basic research, stress response in general is of major biomedical importance. Dysfunction of the HSR has consequences in health and disease, as well as implications in oncogenic processes. HSR specifically deals with maintaining proteostasis in cells that are under heat stress or other stressors, and failure to sustain proteostasis is a hallmark of several important human protein conformational diseases including Alzheimer's, Parkinson's, and some types of cancer. A better understanding of the hierarchy of regulation of the HSR at the level of basic research could lead to new applications and novel therapies for disease intervention.

In Chapter One, I discuss background and previous work on the HSR and what is known about its function in a variety of organisms, including my model organism of choice *Caenorhabditis elegans*. I also discuss why I chose to work with nematodes. Next, I detail the construction of the microfluidics devices I used, my experimental set-up and methods, and my data acquisition and

analysis pipeline in Chapter Two. Chapter Three addresses the basic characteristics of the HSR in *C. elegans*, including phenomenological characteristics and spatial features. Next, I introduce a simple, minimal model of the cellular level HSR in Chapter Four and use it to interpret my quantitative data. In Chapter Five, I consider systemic features of the *C. elegans* HSR and what interpretation my minimal model can provide. Finally, in Chapter Six, I discuss the implications of my findings and consider future research directions. Detailed protocols for techniques used, my heat shock experiments, microfluidics construction, and molecular biology methodologies can be found in Appendices A and B.

I always have stress.

Takashi Murakami

1

Background

OVER THE YEARS, the HSR has received much attention from the systems and quantitative biology and biophysics communities. Many properties make this system particularly attractive for studying and modeling. First, its function is of major biomedical importance, with implications to the survival, fitness, and evolution of organisms from all kingdoms of life. Second, the heat shock re-

sponse is an ancient and universal mechanism that is required for the marginal stability of much of the proteome and the exposure of cells to a broad range of temperatures and other proteotoxic environmental and physiological conditions. Third, despite differences in the identity and origins of some of the proteins involved, the core design of the regulatory network that controls the heat shock response, which is based on titration of a master regulator by heat shock proteins, is conserved from bacteria to humans. Finally, this regulatory network is well-defined, separated from other regulatory functions in the cell, and composed of a relatively small number of protein families, making it amenable for modeling.

1.1 PROTEOSTASIS AND THE HSR

Proteostasis is a key regulatory process in organisms that maintains the balance between protein synthesis, folding and assembly, and degradation^{13,194}. It is an especially important process, as mammalian cells express upwards of 10,000 different protein species, each of which plays a crucial role in the functioning of a cell⁷⁰. A protein begins its life as a sequence of base pairs in an organism's DNA. From the DNA, it is transcribed into an mRNA, then decoded by a ribosome into an amino acid chain, or polypeptide. Each protein's amino acid chain then has a three-dimensional, native protein configuration it wants to be folded into that minimizes the free energy of the system¹³². To reach this final state, molecular chaperones help each protein fold properly, including taking it through a series of folding intermediary stages. Molecular chaperones are any proteins that interact with another protein to help it attain its final state but are not present in this final state. Proteins in

their native configuration or in a folding intermediate can then unfold or misfold into an unwanted protein configuration, which can then lead to aggregation of misfolded proteins. Molecular chaperones also help these misfolded proteins refold into their native configuration or help tag them for degradation if they cannot be rescued^{40,70,182}.

This process is further complicated by the fact that proteins must retain their structure and function under small thermal fluctuations. In addition, in order to function, many proteins require some conformational flexibility, and indeed around 20 - 30% of proteins in eukaryotic cells adopt their final three-dimensional structure only after interaction with their binding partners⁴⁴. To maintain proteostasis, cells have several options and tactics. Asymmetrically dividing cells can segregate damaged proteins into one daughter cell, allowing the other daughter cell to be produced damage-free³⁵. In cells and organisms that cannot do this, damaged proteins are often compartmentalized into nuclear or cytoplasmic deposits to sequester the damage¹¹⁷. To refold these, or tag them for degradation, there are several pathways cells use. The unfolded protein response, or UPR, helps maintain proteostasis in the endoplasmic reticulum (UPR^{ER})^{51,187,26} and mitochondria (UPR^{mt})¹⁸¹. In eukaryotic cells, there exists a different stress response pathway termed the integrated stress response (ISR)¹³³.

One pathway of special note is the heat shock response (HSR), a highly conserved molecular response whose main goal is to prevent protein misfolding and aggregation that can lead to interference with cellular function^{7,49,122}. The HSR has been studied in innumerable organisms including (but not limited to): camels⁸, mice⁴¹, sponges⁶⁹, sea turtles¹⁷⁹, various plants^{203,131}, flies^{7,65}, yeast (including *Candida albicans*^{100,99} and *Saccharomyces cerevisiae*^{28,110,16,84}), bacteria (especially *Es-*

Escherichia coli^{62,67,149,170}), and human cell lines^{152,50,55}. It is tasked with maintaining the stability and integrity of the proteome under normal and stress conditions. After a stressor or disruption, it is activated to restore proteostasis^{131,59}. It also has a housekeeping functionality under normal conditions, helping to maintain protein folding under small thermal fluctuations^{12,165}. While there are other negative effects on cellular processes, such as DNA damage⁸³, following a stressor, the main focus of research has been on the restoration of proteostasis.

The HSR is so named because it was discovered as a response to heat shock specifically, and the corresponding molecular chaperones were named heat shock proteins, or “hsp’s” in the 1970s⁷. While the name of the HSR implies that is a response to heat stressors only, the HSR is actually triggered in response to a variety of other stressful environmental and internal cues that also disrupt proteostasis such as heavy metal stress^{92,130}, metabolic stress^{57,143}, pathogen stress^{119,139}, and aging^{58,123}. Other stressors of interest to which the HSR apply include human protein conformational diseases such as Alzheimer’s, Parkinson’s, and some types of cancer^{136,116,121}. In addition, a number of more strange substances induce the HSR as well, such as caffeine^{71,2,24}. Heat shock, however, remains a popular stress mechanism by which to study the HSR, as precisely controlled heat shocks are easy to apply in a laboratory setting.

1.2 SINGLE CELL HSR

Dynamical systems properties of the HSR have been well-studied in single cell organisms. In a single cell, the accepted paradigm posits that the HSR involves three main sets of players – at least one heat

shock transcription factor (HSF), a class of molecular chaperones called heat shock proteins (HSPs), and the diverse group of misfolded proteins themselves. Under normal conditions, HSF is located predominantly in the cytoplasm of a cell in a repressor complex with at least one HSP. Some evidence suggests two highly conserved heat shock proteins, HSP-70^{163,204} and HSP-90^{205,17}, or both, are often a part of this repressor complex. The influx of misfolded proteins caused by a stressor is thought to be the major cue that starts the HSR by titrating the HSPs away from HSF^{78,100}. HSF then undergoes a series of activation steps, including trimerization and relocation to the nucleus^{153,80}. There, it binds to a heat shock element on DNA⁸⁶ and induces the transcription of several HSPs. Post-translational modifications also exist that promote nuclear retention of HSF and DNA binding¹⁵³. Some of the transcribed HSPs act as molecular chaperones, restoring proteostasis by clearing misfolded proteins and helping others refold¹⁸². Other HSPs that are transcribed are part of the repressor complex and enact a negative feedback loop that represses the activity of HSF¹⁶³. As the misfolded proteins are cleared and refolded, more HSPs are left free to bind back to HSF and repress further transcription⁶⁸. Other post-translational modifications to HSF promote DNA dissociation and inactivation as well, including acetylation^{86,131,191}. In addition, as the HSR ends, genes unrelated to the HSR, which are silenced by miRNAs (microRNAs, or small, non-coding RNA molecules) during the heat shock, return to their normal activity⁵⁵.

There are slight differences in the HSR in each of the single cell organisms. Eukaryotic HSR variations all depend on some version of HSF. For example, the HSR in mammalian and human cell lines is moderated by the transcription factors Hsf1 and Hsf2^{29,50,172}. Yeast (*Saccharomyces cerevisiae*) only use Hsf1; however two additional, partially redundant, zinc-finger transcription factors, Msn2

and Msn4, also play in role in the HSR and help mediate the response to other stressors as well²⁸. Bacteria are slightly different. While prokaryotes have transcription factors similar to eukaryotes, arguably the most fundamental molecule for orchestrating broad changes in gene expression states is the sigma factor, a bacterial transcription initiation factor. Sigma factors are a component of the holoenzyme, an RNA polymerase that initiates gene regulation, and they both recruit the holoenzyme to the promoter as well as being responsible for promoter recognition¹⁵⁹. The sigma factor responsible for the majority of the upregulation of the HSR in *Escherichia coli* is σ^{32} ⁶². Given the great similarity between single celled organisms, the HSR has not only been studied experimentally, but also has been modeled in bacteria⁸², yeast^{28,204}, and HeLA cells¹⁴⁴.

1.3 MULTICELLULAR HSR AND *CAENORHABDITIS ELEGANS*

The same single cell players - HSF, HSPs and misfolded proteins - remain at play in the cells of multicellular organisms. In addition, the same main heat shock proteins - HSP-70 and HSP-90 - also still have important roles in the multicellular HSR, and an influx of misfolded proteins can also trigger the HSR in cells within multicellular organisms. For example, the introduction of polyglutamine aggregates in *Caenorhabditis elegans* was shown to induce the HSR without an increase in temperature or the application of any other external stressor¹⁵⁵. While the HSR pathway was originally thought to be cell autonomous in multicellular organisms, more recent work provides evidence for additional layers of regulation and the involvement of multiple signaling pathways^{66,176,184}, and questions remain as to how the HSR is regulated at the systemic and tissue levels. A better under-

standing of the hierarchy of regulation of the HSR could lead to novel therapies for disease intervention^{20,12,192,138}. In order to study this, the systems biology community has turned to a number of multicellular model organisms.

In *Drosophila melanogaster*, there exists a transcription factor, HSF, and a class of heat shock proteins that are transcribed after a heat stress. (In fact, *Drosophila melanogaster* is the model organism in which HSPs were discovered and thusly named^{7!}) However, the HSR in flies is also characterized by the abundant transcription of the hsr-omega gene, which is encoded by a “heat shock puff”, which refers to the specific morphology and structural changes of the fly’s polytene chromosomes^{134,7}. While there are many conserved aspects to the HSR in *Drosophila*, there are also the former distinctions, which make it harder to draw parallels to human health and disease pathways. Instead, I chose to use *Caenorhabditis elegans* as my model organism. *Caenorhabditis elegans*, or *C. elegans*, is a nematode, or round worm. Adult worms are around 1 mm in length and exist primarily as self-fertilizing hermaphrodites. They have a well-defined cell lineage and tissue structure as well as an invariant number of somatic cells. In addition, they also have a rapid life cycle for easy cultivation in a laboratory setting - at 25°C, it takes them three days to grow from an egg to an egg-laying adult³⁷.

C. elegans is a great model system for a variety of reasons. Firstly, *C. elegans* is transparent for ease of imaging, including both phase or differential interference contrast (DIC) imaging of individual cells and subcellular details at high magnification and visualization of developmental processes or protein responses and interactions using fluorescent protein tags³⁷. In addition, its nervous system is small - only 302 neurons - and the circuitry is entirely mapped out¹⁹³. In fact, it is the only organism

to have a fully constructed connectome. While this is an extraordinarily small number of neurons, even compared to other common model organisms such as the fruit fly and larval zebrafish (250,000 and 100,000 neurons respectively), it still retains enough complexity for active research into the nervous system. There is also a wide range of genetic tools²³ available to probe its biological processes, including the more recently developed tools of MosSCI⁵³ and miniMos⁵⁴. Lastly, *C. elegans* retains many highly conserved molecules, processes, and pathways, including the stress response pathway. The *C. elegans* genome contains orthologs, or genes in different species that evolved from a common ancestral gene and retain the same functionality, of 60 - 80% of human genes, including 40% of genes known to be associated with human diseases^{81,39}.

1.4 THE *C. ELEGANS* HSR

Behavioral responses to a range of temperatures in *C. elegans* have been thoroughly studied previously. *C. elegans* live happily within a range of temperatures from around 15°C to 25°C but have a preference for the temperature they were cultivated at within this range. They exhibit thermotaxis toward their cultivation temperature when exposed to a gradient of temperatures^{61,72}. In fact, wild strains of *C. elegans* have been shown to have genotypic correlations with the environmental parameters of the niche they are from, including temperature, and wild isolates from climates with different climates have different preferred growth temperatures⁴⁸. When exposed to an extreme temperature (i.e. a temperature outside of the 15°C - 25°C range), they will move to a preferred temperature if possible (i.e. noxious heat or cold avoidance^{157,103}). However if they cannot escape, they exhibit a

stress response. While response to cold shock is poorly studied¹⁴⁶, several behavioral responses to heat shock have been experimentally observed. These behaviors include a reduction in thrashing movement⁵⁶ and induction of a sleep-like state known as quiescence^{73,127,125}.

In addition to behavioral responses to heat shock, the HSR has also been studied at the molecular, tissue, and systemic levels in *C. elegans*. At the molecular level, the response remains similar to that of a single cell, with a transcription factor, HSF-1, inducing the transcription of a variety of heat shock proteins. Activation of downstream HSR genes has also been shown to be tissue specific in *C. elegans*^{109,184,66}. Since the proteome can vary greatly between different tissues and cells, the regulatory network of a multicellular organism must be able to allow for different modes of activation within its individual cells and tissues¹⁴⁵. Cell non-autonomous levels of regulation are shown in relation to the HSR as well as the regulation of other stress responses including both the endoplasmic reticulum and mitochondrial unfolded protein responses^{178,45,162}, hypoxic and oxidative stress responses^{102,112}, pathogen immunity²⁷, and aging³.

Considering the systemic response, temperature sensing in *C. elegans* is mediated by cyclic guanosine monophosphate (cGMP) signaling in thermosensory neurons, most notably the AFD sensory neuron pair, which is considered to be the main thermosensory neuron^{5,142}. The sensory endings of the AFD extend into the environment at the end of a worm's head, and they appear to sense temperature directly, as they can respond to temperature changes even after being separated from the cell body³⁴. The AFD's temperature sensation is thought to be regulated by a combination of multiple elements. Two transient receptor potential ion channels (TRPA and TRPV) help mediate thermosensory transductions and thermotaxis¹⁸⁰. In addition, the AFD's temperature sensation is also

regulated by three receptor-type guanylyl cyclases (GCs), which may be the actual thermosensors¹⁷⁵, as well as phosphodiesterase (PDE) and the cyclic-nucleotide-gated channel (CNG)⁵.

The AIY interneuron is the AFD neuron's main postsynaptic partner. At the systemic level, previous research implicates the thermosensory AFD neuron and AIY interneuron in the regulation of HSF-1 dependent HSR. The AFD and AIY neurons are necessary for both thermotaxis and thermosensation in *C. elegans*^{120,108}, and the AFD neuron is necessary for magnetosensation as well¹¹. They also both play a role in regulating the longevity of *C. elegans* at different growth temperatures¹⁰¹. Mutant worms with genetic ablations of either neuron were shown to have a heat shock response that produced 5 - 10 times less HSPs than wild type worms following a heat stress¹³⁷. The HSR could also be triggered in the absence of heat with the optogenetic stimulation of the AFD neuron, which enhances serotonin release, or the direct optogenetic stimulation of serotonergic neurons¹⁷⁷.

1.5 IMPLICATIONS IN HUMAN HEALTH AND DISEASE

Proteostasis and stress responses like the HSR are inextricably linked to human health and aging. Studies indicate that aging is linked to a general decline in proteostasis capabilities and an increase in protein aggregation^{121,43,96}. Indeed, literature shows that restriction of cytoplasmic protein synthesis prevents induction of cellular senescence, or biological aging, in both normal cell lines and tumor-derived human cell lines. This was true in *C. elegans* as well; when protein synthesis was restricted, average lifespan was significantly extended¹⁷⁴. In addition, HSF1 DNA binding activity deteriorates

with age as well, contributing to the decline in proteostasis capabilities as well²⁰². In contrast, upregulation of the HSR pathway through HSF-1 can lead to an increased lifespan¹²³. Exposure to small doses of stress, such as a short, low temperature heat shock, can also help extend lifespan and provide higher tolerance for a variety of stressors^{139,93}.

In addition to its connection to aging, a basic understanding of the HSR also has direct connection to therapeutic strategies in human disease. For example, human protein conformational diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases are all characterized by reduced proteostasis capabilities, and a hallmark of all of these diseases is an increase in deleterious protein aggregations^{136,116,121}. Therapeutic treatments, therefore, often aim to restore proteostasis capabilities by leveraging stress response pathways like the HSR^{12,20,192}. While HSR activity is impaired in many neurodegenerative diseases, research has shown cancer cells can actually co-opt HSF1 to upregulate a different transcriptional network than is upregulated in the HSR and support their own growth instead^{116,60}. In fact, HSPs are also often highly expressed in cancer cells and can thus be used as a diagnostic tool for detecting cancer or targeted for cancer therapy treatments^{151,195}. Contrastingly, upregulation of HSPs has also been used to induce regulatory T cells that can then help fight chronic inflammatory diseases¹⁴.

Lastly, while connections between the HSR, proteostasis, and human health usually center around protein misfolding diseases and aging, humans also have exposure to actual heat shock pulses during weather events such as heat waves. While our circulatory system allows us to maintain our core temperature without major fluctuations, even during a heat wave, our cells still undergo a transcriptomic response to exposure to extreme heat. In one study, humans were exposed to extreme

heat (around 75.7°C, or 168°F) via a sauna and their gene expression patterns were measured. Gene expression of proteins associated with stress pathways, including proteostasis, was found to be still amplifying at least one hour after the end of exposure²¹. In this regard, studying the HSR in *C. elegans* after application of a heat shock pulse may have very clear parallels to human exposure to extreme heat as well.

Some like it hot, so let's turn up the heat 'til we fry.

Nat King Cole

2

Methods

IN THIS DISSERTATION, I ask how activation and regulation of the HSR is coordinated within a tissue of a multicellular organism, *C. elegans*. To do this, I sought to measure and characterize the induction dynamics of a downstream heat shock protein in individual worms under well-defined stress conditions. More specifically, I looked at the the activation dynamics of HSP-16.2, a small

molecular chaperone expressed throughout the intestine of a worm and highly upregulated following a heat stress. HSR gene expression dynamics in *C. elegans* are conventionally studied by exposing a population of worms crawling on solid media to a warm environment using either a water bath or an incubator. At each time point, a sub-population of animals is taken off the plates and the level of the gene or genes of interests is assayed, using either biochemical techniques such as qPCR or imaging of anesthetized animals on a microscope slide. These are population-level approaches, precluding the possibility of following the dynamics of activation in individual animals^{105,137,115,66,114}. Approaches that do allow following individual animals are often behavioral assays that provide data that are more qualitative than quantitative^{118,180}. To facilitate long-term, high-time-resolution, longitudinal imaging, namely quantitative measurements that preserve the identity of each worm throughout an experiment, I turned to a microfluidics device.

2.1 MICROFLUIDICS AND SOFT LITHOGRAPHY

The microfluidics devices I used were extended two-layer versions of the previously published WormSpa, as seen in Figure 2.1. These devices allow the tracking of individual worms over a period of hours to days with high frequency sampling and immobilization of the animals that does not in itself cause stress⁸⁸. The bottom layer is a series of 32 chambers, each of which is roughly the size of an adult worm. Each chamber contains two rows of pillars and other structures that confine the worms for imaging while still allowing gentle movement and egg laying. A hierarchy of channels leads to these chambers to facilitate easy loading of a single worm into each chamber. The top layer is a water

channel that is located directly above the worm chambers, separated from them by a thin layer of PDMS, allowing for the delivery of precise, temperature-controlled heat shock pulses.

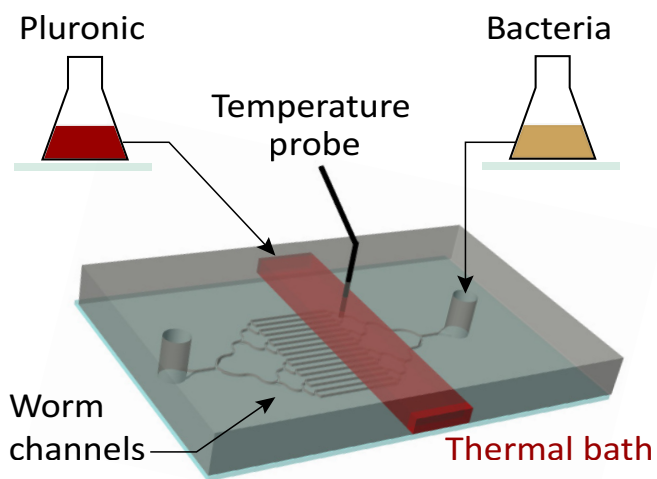


Figure 2.1: Two-layer microfluidics device. Worms are loaded into bottom channel in bacterial suspension. Heat shock pulse is applied via thermal bath layer located above worms. Temperature is controlled with in-line heater and measured with thermistor probe.

While there have been a multitude of applications of microfluidics in the study of worm biology⁷⁷, this device and approach is a novel one. Previous applications, mostly unrelated to our approach, but inspirational nonetheless, have included immobilizing worms for imaging, microsurgery, or microinjection^{6,76,166}, holding worms for quantification of undulatory dynamics^{140,31,190}, animal sorting^{10,198,148}, and animal screening^{36,38}. There have also been a number of studies that used microfluidics devices to control changes in the chemical environment in order to study the chemosensory system^{189,75,32}, and a previous study measuring thermotaxis in a microfluidics device¹⁹⁹. This thermotaxis study, however, only measures behavior; the temperature gradient produced does not allow for confinement of the worms and the high-resolution imaging that is needed

for quantitative gene expression assays. This is the most novel aspect of our device. While microfluidics devices that allow for long-term imaging of *C. elegans* have been developed previously, they rely on either immobilization, which triggers stress responses^{89,47,90}, or free movement, which precludes high-spatial-resolution imaging^{200,197,33}. Our device uses the strategy of confinement, allowing worms some freedom of movement while also keeping them still enough to take high-resolution images. Under these conditions, worms show a minimal decrease in size, but otherwise exhibit expected physiological cues including movement, egg laying, and pumping over the course of 24 hours and longer⁸⁸ (Figure 2.2B). They are also unstressed by this confinement, as extended stays in the microfluidics device produce no increase in the small heat shock protein HSP-16.2, which is up-regulated under stress conditions, and DAF-16 (another transcription factor active in other stress pathways) remains diffuse through the cells, rather than relocating to the nucleus, as it does under starvation or stress conditions⁸⁸ (Figure 2.2A). For more details on the quantities measured in Figure 2.2, refer to the sections on Experimental Set-Up and Data Acquisition and Analysis.

Microfluidics devices are produced using a process known as soft lithography. Soft lithography exploits the properties of SU-8 photoresist, an epoxy-based, negative photoresist that absorbs light in the UV region. Under normal light conditions, SU-8 is a viscous liquid, but when exposed to UV radiation and then baked, its epoxy groups cross-link, forming a solid. By covering a thin layer of SU-8 with a photomask that only allows UV light to penetrate certain regions, selective hardening can occur, creating patterns of solidified SU-8 structures which have nearly vertical side walls¹⁰⁶. While this technology was originally developed for use in the microelectronics industry for the fabrication of semiconductor devices^{196,79}, it is now also used for the construction of microfluidics devices for use

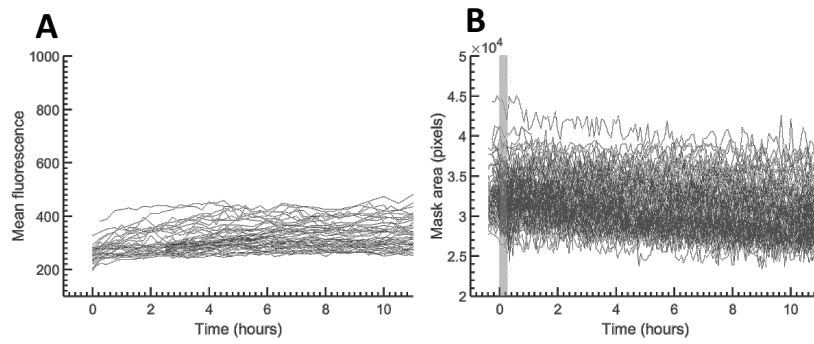


Figure 2.2: (A) Example mean GFP fluorescence (HSP-16.2p::GFP) response curves for individual worms in microfluidics device with no heat shock pulse applied. (B) Example mask size (pixels) from image analysis pipeline as measure of worm body size throughout experiment for worms exposed to a 15 minute heat shock pulse at 31°C or 34°C .

in biophysics and biochemistry^{141,63}. Complex photomask designs can be created using AutoCAD software to make microfluidics environments on the nano- and microscales.

To create a microfluidics device using soft lithography, a silicone wafer is coated with a thin layer of SU-8, ranging from 10s of microns to 100s of microns thick. The wafer is then soft baked for a short period of time to remove the solvent. This bake is usually performed with a hot plate and is a two step process, starting at 65°C before ramping up to 95°C to create an even bake with a slow and smooth transition of temperature. After the soft bake, the designed photomask is placed on top of the layer of SU-8 on the wafer and the wafer is exposed to UV radiation. After exposure, the photomask is removed, and the SU-8 undergoes another heat treatment, known as the hard bake. The baking times for both the soft and hard bakes depend on the thickness of the SU-8 layer¹⁰⁶. Following the hard bake, the cross-links have fully developed, and the unexposed portions of SU-8 are chemically removed from the wafer, leaving what is essentially a “mold” behind^{196,147,85}. These molds then have a silicon-based polymer, usually polydimethylsiloxane, or PDMS, poured onto them. After the PDMS cures and hardens, it can be peeled off of the mold as a single piece and sealed to a glass slide¹¹³. The locations of the SU-8 structures then become empty spaces sealed between glass and PDMS that can hold a variety of organisms of interest, from *Drosophila* embryos^{107,183} to *E. coli*^{160,4,19} to *C. elegans*^{9,97,124}. Not only is PDMS biologically inert¹⁸, so it does not interfere with biological experiments, but it is also easy to manipulate these structures and control the environment of the microfluidics devices.

The microfluidics devices I used were extended two-layer versions of the previously published WormSpa⁸⁸. The bottom layer was a 50 μm thick WormSpa layer made with SU-8 3050 (Microchem)

spin-coated onto a 3-inch wafer (Silicon Quest). It was first baked at 65°C for 2 minutes and 95°C for 20 minutes, then exposed to UV radiation at around 200 mJ/cm² for 30 seconds. Post-exposure, the wafer was baked for 1 minute at 65°C and 4 minutes at 95°C, then developed with propylene glycol methyl ether acetate (PGMEA). The top layer is a 300 μm water channel used for temperature control during the heat shock pulse. After spin coating with SU-8 2150, the wafer was soft baked for 8 minutes and 75 minutes at 65°C and 95°C respectively, then UV exposed for 45 seconds. The post exposure bakes were 5 minutes and 25 minutes respectively at 65°C and 95°C. Both masks were coated with heptadecafluoro – 1, 1, 2, 2 – tetra-hydrodecyl trichlorosilane by soaking for 10 minutes in a 0.1% solution with Novec 7100 (HFE) to prevent the PDMS from sticking to the wafer in later steps.

The water channel was made with PDMS mixed in a 10:1 ratio of Sylgard 184 silicone elastomer base to silicone elastomer curing agent (Dow Corning) and separated from the worm channels by a 100 μm membrane. The membrane was created by spin coating the worm channel wafer with two very thin layers of PDMS – the first was a 10:1 PDMS mix spin coated at 150 rpm for 5 seconds followed by 300 rpm for 60 seconds, and the second was a 5:1 PDMS mix spin coated at 375 rpm for 5 seconds followed by 750 rpm for 60 seconds. The worm channel wafer was baked for 30 – 60 minutes at 65°C to partially cure the PDMS, then the water channel was plasma bonded on top. After allowing the whole device to cure for 2 – 12 hours at 65°C, it was carefully removed from the wafer and plasma sealed to a cleaned glass slide (1" X 3"). Holes were punched in the device with a 1.07 mm dermal punch (Harris Uni-corn 0.75) prior to bonding to a glass slide to allow the introduction of fluids and worms into the device during experiments.

2.2 EXPERIMENTAL SET-UP

All *C. elegans* strains were maintained under standard conditions on nematode growth medium (NGM) plates seeded with *E. coli* strain OP50 at 22°C¹⁷¹. The N2 Bristol strain was used as wild type. Other strains used in this work include: TJ3001 [zSi3001 [hsp-16.2p::GFP::unc-54 + Cbr-unc-119(+)] II], ERL35 [ttx-1 (p767) V; zSi3001 II], ERL36 [ttx-3 (ks5) X; zSi3001 II], PR767 [ttx-1 (p767) V], and FK134 [ttx-3 (ks5) X]. TJ3001 was obtained from the Caenorhabditis Genetics Center. ERL35 and ERL36 were generated in-lab through crossing strain TJ3001 with strains PR767 and FK134 respectively, as described in Appendix A, Section 3.

Prior to each experiment, age-synchronized worms were obtained by letting 20 – 40 gravid adults lay eggs on a seeded plate for an hour, then removing the adult worms. Plates were incubated at 22°C for 60 - 70 hours. The evening before an experiment, an overnight culture of *E. coli* OP50-1 was grown in LB media with streptomycin for around 12 hours. The culture was then washed, centrifuged, and re-suspended in S-medium¹⁷¹ in one or two 50 mL Falcon tubes to an optical density of 5 at 600 nm (OD₆₀₀ = 5), as measured by a spectrophotometer. Next, the bacteria was heat killed for 35 minutes in a 65°C water bath, as previously described^{64,167} and cooled to room temperature, either on the benchtop or in a 15°C refrigerator. Heat killed bacteria created less build-up in the microfluidics device than live bacteria¹⁵, which improved the fluid flow. This method of heat killing was confirmed by plating bacteria and verifying no colony growth. Optical density was measured prior to heat treatment to ensure consistency of food density between experiments. The bacterial suspension was then filtered with a 0.5 μm syringe filter into a new Falcon tube to remove any large

particulates that could clog the microfluidics channels.

Next, the device to be used was mounted on a motorized stage on a Zeiss Axio Observer Z1 inverted microscope. The water channel was flushed out with a 70% ethanol solution, then it was connected to a reservoir of 2.5% pluronic solution with medical grade polyethylene microtubing with an inner diameter of 0.86 mm and an outer diameter of 1.32 mm (BB31695-PE/5, Scientific Commodities, Inc.). Pluronic solution was used rather than water alone to help prevent the collection of bubbles in the water channel which disrupt imaging. As needed, the pluronic reservoir was filtered with a Corning 500 mL 0.22 μm filter system prior to connecting, and the associated microtubing was rinsed with 70% ethanol. A SF-28 in-line heater (Warner Instruments) was placed directly over the tubing right before entry into the water channel to provide the heat shock, as controlled by the TC-324C temperature controller (Warner Instruments). Temperature in the water channel was measured with a Physitemp Instruments IT-24P insulated, Type T, Copper-Constantan thermocouple with a polyurethane insulated wire connected to the EXTECH Instruments Process PID Controller 48VFL. The tip of the thermocouple was enclosed in a combination of a syringe tip, a 200 μL pipette tip, and a small piece of BB31695-PE/1 tubing in order to be inserted into the microfluidics device and stay watertight. Once all of the tubing was connected fully, pluronic was allowed to flow through the channel (driven by gravity flow) until all bubbles were gone, then the flow was blocked until the start of the experiment.

Next, the worm chambers were also rinsed with 70% ethanol, then flushed thoroughly with S-medium. All other tubing and syringes associated with the worm chambers were also rinsed and flushed as such. For the experiment, the heat killed bacterial solution was contained in 10 mL male

luer lock syringes equipped with 20 gauge, ½” length, blunt tip, stainless steel industrial dispensing tips from CML Supply (Item 901-20-050) which were connected to the microfluidics device with the same medical grade polyethylene microtubing as described above. Input and output syringes were carefully loaded with solution, taking care not to introduce bubbles, then attached to the device. Flow of bacterial suspension was controlled with an in-lab developed custom LabView script and the New-Era NE-501 OEM syringe pump, and the syringes and syringe pump were kept on a VWR Standard Analog Shaker at speed setting 5 throughout the experiment to help prevent settling of the bacteria to the bottom of the syringe and ensure fixed density throughout the experiment. After the syringes were attached, bacterial suspension was driven through the device at 300 $\mu\text{L}/\text{min}$ until the worm chambers were cleared of any bubbles.

Next, 32 – 35 worms (early adult hermaphrodites) were picked from the aforementioned plate and gently transferred into 100 μL of the prepared bacterial suspension in a 600 μL , non-stick tube. The 100 μL of solution and the worms were then drawn into the input microtubing using the input syringe and injected into the device using the syringe pump. The pump was run at 300 $\mu\text{L}/\text{min}$ until the first worm appeared in the device, then the flow was slowed to 100 - 150 $\mu\text{L}/\text{min}$. The worms were given 5 - 10 minutes to enter the device and align in the chambers with this constant flow, then the syringe pump was stopped. The input and output syringes were then manually controlled to gently align any misaligned worms within the chambers. Worms were given two hours post-loading to adjust to the device before data acquisition began. During these two hours and during the experiments, a continuous bacterial suspension at fixed density was delivered to the worms at a rate of 5 $\mu\text{L}/\text{min}$, with 10 second pulses at 200 $\mu\text{L}/\text{min}$ applied every 20 minutes to clear bacterial buildup

and eggs away from the worms.

Data acquisition protocols are detailed below. After each experiment, all tubing was disconnected and cleaned with 70% ethanol. The worm chambers of the microfluidics device were filled with 70% ethanol as well, then left for 5 - 10 minutes to kill all worms. Worms bodies were then cleared from the device with a gentle flow of either water or S-medium, and the device could be reused for additional experiments. A more detailed protocol for the heat shock experiment set-up can be found in Appendix A, as well as in the previously published work on WormSpa⁸⁸.

2.3 DATA ACQUISITION AND ANALYSIS

Prior to starting the experiment, coordinates of each worm were programmed into the microscope control software, and focal planes for each chamber were set manually. The water channel flow was opened and allowed to stabilize. At the start of the experiment, four timepoints were taken before the application of heat shock to establish the basal level of autofluorescence for each worm. A heat shock pulse was then applied using the aforementioned in-line heater, and images were taken during the heat shock and up to 10 hours post exposure. Each worm was imaged every 2 minutes during the heat shock pulse and every 3 to 5 minutes before and after. Images were acquired using a Zeiss Axio Observer Z1 inverted microscope with a 10X objective and a Hamamatsu Orca II camera with 10 ms phase exposure and 50 ms GFP exposure from a Colibri LED light source. Image collection was automated throughout the experiment.

Data from an experiment was saved as a .czi file, then exported as uncompressed tiffs. The max-

imum image size that can be taken during the experiment is 400 X 1344 pixels, which covers about half of the worm body. Because of this, each worm was split into two images, and these images had to be stitched together with the worm halves aligned before further analysis could be performed. After stitching, any worms that were clearly unhappy or dead by the end of the experiment, as gauged by their motion (or lack thereof) in the device, were removed from further analysis. Similarly, any worms that escaped from their chambers before the end of the heat shock were also removed from analysis. Frames in which the worms misalign (by sticking their head between two columns, etc.), partially escape, escape, or return were all noted for removal from analysis in later steps. All image analysis was performed with in-lab developed, custom MATLAB scripts. The goal of the image analysis was to identify the worm in each frame, create a mask to isolate the image data from the worm only, then quantify the data contained therein.

First, to minimize image size during the initial analysis, each image was cropped to exclude the outer portions of the worm chamber. The bulk of the segmentation of the worm in each image relied on the fact that the pixels in the worm portion of the image are generally darker than the pixels in the rest of the image. A histogram of the pixel values in the image was produced and a thresholding value selected such that most pixels contained in the worm body were below the threshold. All pixels with values above the threshold were then set to 1, and all pixels with values below the threshold were set to 0. This produced a rough inverse mask of the worm, with most worm pixels black and the background white. The complement of the image was then taken, yielding a rough mask of the worm.

To improve the mask, first all “holes” in the worm portion of the mask were filled. Holes were

defined as a set of pixels that cannot be reached by filling in the background from the edge of the image – i.e. any groups of black pixels (value 0) located within the worm mask, which is mostly comprised of pixels of value 1. Next, the image was morphologically opened. To perform this operation, I made use of mathematical morphology, a technique for the analysis and processing of geometrical structures, usually used to analyze digital images. The basic operations of mathematical morphology are erosion, dilation, opening, and closing, and they all depend on the usage of structuring elements. For the purposes of 2D digital images, these structuring elements are matrices of pixels, each with a value of zero or one, that identify which pixel in the image is being processed and define the neighborhood used in its processing. I used a square structuring element with a width of 6 pixels as defined by the MATLAB command `strel`.

Morphological opening of an image is the erosion of the image with a structuring element, followed by the dilation of the image with the same structuring element. Morphological erosion of a binary image compares each pixel's surrounding pixels to the pixels surrounding the origin of the structuring element. If and only if all pixels in the neighborhood correspond between the two, the pixel is set to 1. This operation shrinks an image by removing a layer of pixels at all boundaries of regions (groups of ones). Dilation has the opposite effect, adding a layer of pixels at the boundaries of regions. Thus, opening removes any thin bridges of pixels connecting two regions, but keeps the regions themselves at their original size. Closing does the opposite, filling in any holes while keeping original region sizes. Because holes were already filled, I used morphological opening to remove any remaining strings of pixels and smooth the boundary of the mask.

Lastly, after this application of mathematical morphology, any small white regions left in the

background of the image are filled in (i.e. set to 0). Because the worm area is quite large, I defined “small” regions as anything less than 5000 pixels in area. Because this is a cropped version of an experimental image, the outer edges that were cropped previously were then reintroduced, with all pixels set to 0. This leaves a segmented mask of the worm, with all pixels in the worm body set to one, and all pixels in the background set to zero. When this matrix of values is multiplied with an experimental image, it produces an image that is all black except for the fluorescence data from the worm itself.

A unique mask was created for every worm in an experiment at every timepoint in the experiment. For each of these, then, the area of the worm (in pixels) can be calculated, as well as the total fluorescence, mean fluorescence, maximum fluorescence, and standard deviation. The total, mean, and maximum fluorescence values were all normalized by the average background fluorescence of the experiment, as measured in a sample image for each experiment. The quantity of interest, which in most cases was mean fluorescence, was then plotted against time, producing a series of individual worm response curves for each experiment. Since this considers the mean fluorescence averaged over the whole worm body, for further spatial analysis, the worm mask can also be split into three equal parts. When combined with data indicating which side of the image each worm’s head is on (as determined manually), these masks can be used to calculate mean fluorescence data for the anterior and posterior thirds of each worm as well.

In order to characterize the observed response dynamics quantitatively, I then fit each individual

worm response curve (mean fluorescence) to the generalized logistic function:

$$G(t) = A + \frac{K - A}{(C + Qe^{-Bt})^{1/\nu}} \quad \text{with } C, Q = 1. \quad (2.1)$$

In Equation 2.4, A is the lower asymptote, K is the upper asymptote, B is the growth rate, and ν affects near which asymptote the maximum growth occurs. I used this generalized logistic fit to extract four phenomenological quantities of interest (see Figure 2.3): the time from the start of the heat shock pulse to observed HSR activation (“time lag”); the rate of fluorescence accumulation (“rate”); the fluorescence level at saturation, adjusted to account for the basal level of autofluorescence (“magnitude”); and the rate of decline of GFP fluorescence at the end of an experiment (“decline”). The fit was applied to a domain from the start of the experiment to around an hour after the maximum fluorescence was achieved. The end region of the experiment where rate of decline came into play was excluded for fitting purposes, and rate of decline was calculated separately with a linear fit.

I then used the fitted parameters to estimate the *Rate of response* as

$$\text{Rate} = \max(G'(t)) = \max\left[\frac{B(K - A)e^{-Bt}(e^{-Bt} + 1)^{-\frac{1}{\nu}-1}}{\nu}\right] \quad (2.2)$$

and the *Magnitude of response* as

$$\text{Magnitude} = \max(G(t)) - \min(G(t)) = K - A. \quad (2.3)$$

and the *Time lag* as

$$\text{Time lag} = \frac{d^4 G(t)}{dt^4} = 0. \quad (2.4)$$

In addition, the parameters K and A were also calculated by linear fit to the start of the curve and the region of maximum fluorescence. These linear fit values were used to corroborate the magnitude as estimated from the logistic fit. Similarly, the time lag was also selected manually for each curve and compared to the estimate from the logistic fit. Rate of decline was calculated as a linear fit of the mean fluorescence curve between hours 8 - 10 post heat shock. The logistic fit and extraction of all four phenomenological quantities of interest were also applied to all anterior and posterior mean fluorescence curves.

2.4 QPCR EXPERIMENTS

In addition to the gene induction dynamics described above, which rely on transgenic worms carrying a transcriptional reporter that drives the expression of GFP from the promoter of a small heat shock protein, I also measured the transcription dynamics directly using qPCR. qPCR, also known as quantitative PCR, or Real-Time PCR is a variation on the basic lab technique of the polymerase chain reaction (PCR). PCR is used to amplify a small number of copies of a segment of DNA by several orders of magnitude. These quantities of DNA can then be used for sequencing and other molecular biology techniques. While this is useful in determining certain characteristics of the DNA, it does not provide quantitative information about numbers of copies of DNA or RNA in the cell.

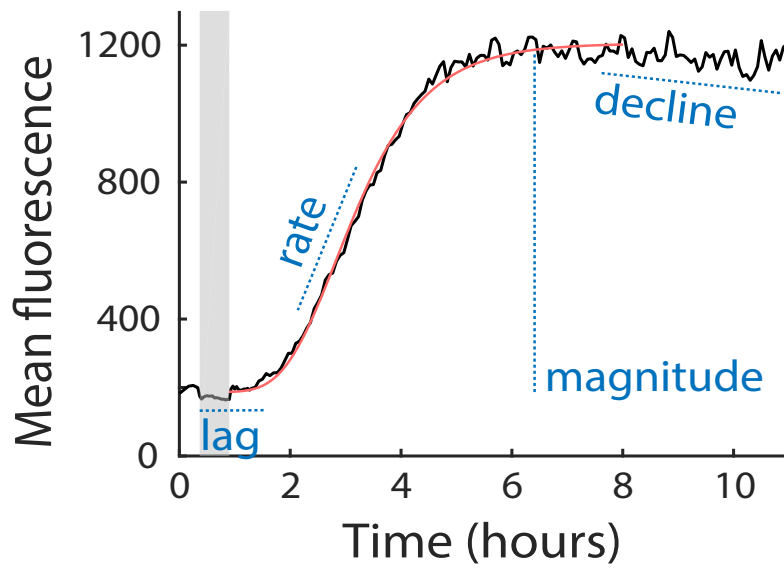


Figure 2.3: Quantification of HSR dynamics on an example individual worm response curve for a 15 min, 34°C HS. Response is measured as mean fluorescence over whole worm body. Curve is fit to generalized logistic function, then time lag, rate of response, magnitude of response, and rate of decline are analytically calculated.

In qPCR, samples are taken at multiple timepoints, at which the concentrations of the segment of DNA or RNA of interest are expected to be different. These quantities are then amplified and compared to the concentration of a housekeeping gene, which is expected to have the same concentration at each time point. In this way, relative changes in amounts of DNA or RNA can be detected across a time course. qPCR is mostly used to study gene expression by measuring the concentration of an mRNA of interest.

For our qPCR experiments, worms were synchronized by bleaching, as previously described¹⁷¹, plated 12 - 16 hours after the bleaching, and allowed to grow for 55 - 60 hours at 22°C before starting the heat shock. Around 300 worms were grown on each 10 cm plate.

To heat shock the worms, the plates were sealed with parafilm and placed in a 33°C water bath, which was determined to qualitatively approximate the fluorescence response from a 34°C HS in the microfluidics chip. Plates were weighted down with empty 250 mL glass bottles to ensure they were fully submerged for the length of the heat shock. The plates were then given 2 minutes to equilibrate to the temperature of the water bath, heat shocked for 15 or 60 minutes, and then moved immediately to a room temperature water bath to cool for 5 minutes. The first time point after the heat shock for a 60 minute heat shock was taken immediately after removal from the room temperature water bath.

At each time point, exactly 50 worms were picked into TRI-reagent to ensure an equal starting sample for each timepoint. RNA was extracted and treated with DNase I (NEB) to remove all DNA from the sample. cDNA was synthesized using the ProtoScript® First Strand cDNA Synthesis Kit (NEB). Lastly, qPCR was performed using the KAPA SYBR FAST qPCR Kit. Results were normal-

ized to the housekeeping gene *snb-1*. A more detailed protocol can be found in Appendix A.

It's not stress that kills us, it is our reaction to it.

Hans Selve

3

Quantifying the *C. elegans* HSR

TO QUANTIFY THE *C. ELEGANS* HEAT SHOCK RESPONSE, I measured the induction dynamics of HSP-16.2, a downstream heat shock protein, in individual worms under well-defined stress conditions. HSP-16.2 is part of the family of small heat shock proteins in *C. elegans* and is expressed mainly in the intestine of the worm²⁵. The dynamics of HSP-16.2 expression were tracked, as de-

scribed in Chapter 2, by imaging transgenic worms carrying a transcriptional reporter that drives the expression of GFP from the promoter of this heat shock protein, *hsp-16.2p::gfp*, integrated into the worm genome in a single copy¹¹⁵. At room temperature (22°C), worms expressed a basal level of GFP fluorescence that then increased on the timescale of hours during and after of a period of elevated temperature (“heat shock pulse”) (Figure 3.1).

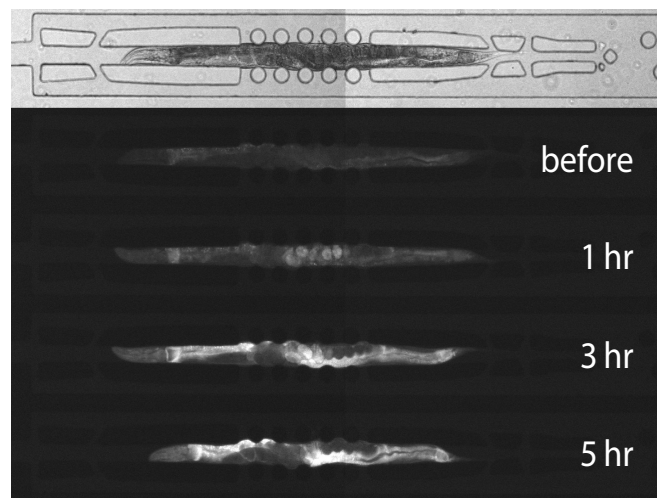


Figure 3.1: GFP fluorescence measured in *hsp-16.2p::gfp* *C. elegans* maintained in microfluidics devices. Example worm from top to bottom: phase image before heat shock (HS), GFP fluorescence image before 60 minute heat shock pulse at 34°C, GFP 1 hour after HS, GFP 3 hours after HS, GFP 5 hours after HS.

3.1 BASIC EXPERIMENTAL RESULTS

I applied heat shock pulses of different durations (15, 30, or 60 minutes) and temperatures (28°C, 31°C, or 34°C) and tracked the increase in fluorescence from individual worms for up to 10 hours following the heat shock pulse. For each experimental condition, I imaged worms from two or more independent repeats (See Table 3.1 for the number of worms per experimental condition). Fluo-

rescence measurements in WormSpa following a heat shock pulse were consistent with previously reported measurements performed by the traditional approaches described in Chapter Two¹⁰⁵.

Experiment	WT	ttx-1	ttx-3
15' @ 31°C	38	-	-
15' @ 34°C	51	30	-
30' @ 31°C	42	28	42
30' @ 34°C	41	28	48
60' @ 28°C	34	-	32
60' @ 31°C	37	-	29
60' @ 34°C	83	-	30
2X 30' @ 31°C	55	-	-
2X 30' @ 34°C	38	-	-
Anterior 30' @ 31°C	19	21	12
Posterior 30' @ 31°C	15	6	30
Anterior 30' @ 34°C	16	19	13
Posterior 30' @ 34°C	21	13	24

Table 3.1: Number of worms of each strain used in every experimental condition. Experiments for each condition had at least 2 independent repeats each.

I first considered the dynamics of the total fluorescence signal from each worm under each stress condition (Figure 3.2), deferring its distribution across the animal body to later sections. Each curve in Figure 3.2 corresponds to the mean fluorescence measured from a single animal throughout the experiment. These data suggest that the response dynamics depend on both the temperature and the duration of the heat shock pulse.

While the response dynamics I observed were highly consistent and reproducible across worms and across experimental repeats, I did observe a clear worm-to-worm variability that was more pronounced at higher temperatures. I hypothesized that this variability may come from higher sen-

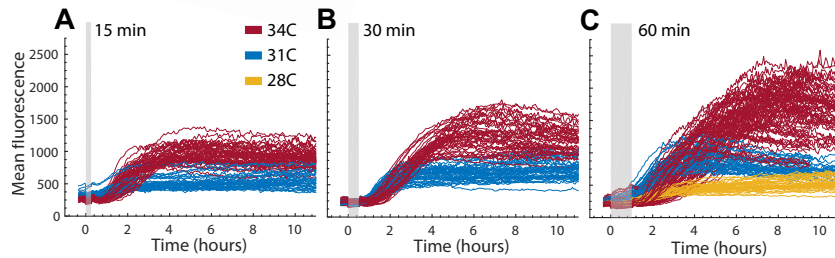


Figure 3.2: Worms subjected to heat shock pulse in microfluidics device for (A) 15 minutes, (B) 30 minutes, or (C) 60 minutes at 28°C (yellow), 31°C (blue), or 34°C (red). Each line indicates the mean fluorescence of an individual worm. Heat shock pulse is indicated by grey bar. Mean fluorescence was tracked for 10 hours following heat shock pulse.

sitivity to small changes in temperature across the device. Indeed at 34°C, I observe a significant correlation between the position of a worm in the device and the amplitude of its response which is not observed at lower temperatures (Figure 3.3). These data suggest an increased sensitivity to small temperature fluctuations at high temperatures.

3.2 TEMPERATURE- AND DURATION-DEPENDENT DYNAMICS

Both the rate and magnitude of response increased with increased duration of the heat shock pulse at lower temperatures (Figure 3.4). However, the changes in the rate were more significant at lower temperatures, whereas the changes in magnitude were more significant at higher ones. In Figure 3.4 and all further figures with boxplots, each box indicates the range from the 25th percentile (q_1) of the data set to the 75th percentile (q_3). The whiskers include the data points outside of this range that

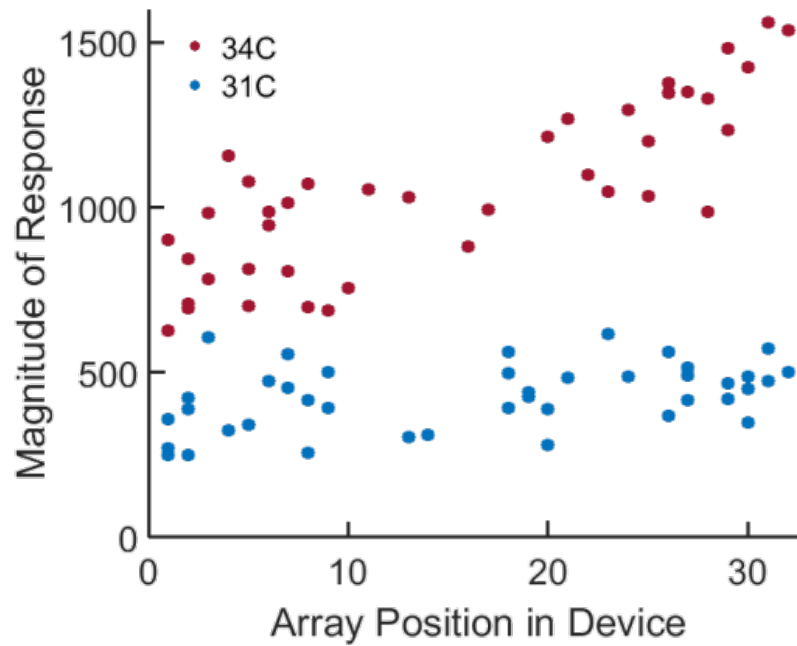


Figure 3.3: Magnitude of response for a 30 minute heat shock pulse at 31°C (blue; $R^2 = 0.29, p - value = 0.07$) or 34°C (red; $R^2 = 0.81, p - value < 10^{-9}$) plotted against position of worm in microfluidics device, where 1 is farthest from in-line heater, and 32 is closest to in-line heater.

are not considered outliers, where outliers are defined as being greater than $q_3 + 1.5 * (q_3 - q_1)$ or less than $q_1 - 1.5 * (q_3 - q_1)$ and are indicated by red asterisks.

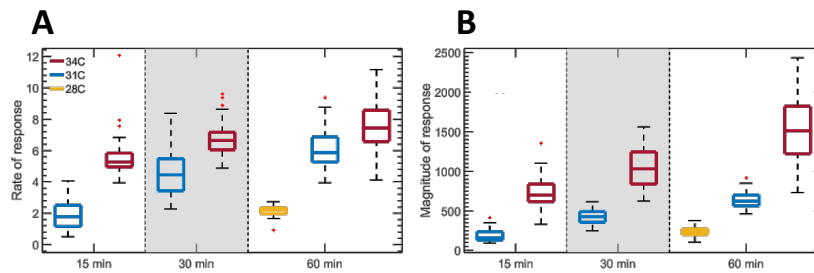


Figure 3.4: (A) Rate of response (mean fluorescence/minutes) for all single heat shock pulses, as indicated in legend. Rate is maximum response rate along logistic fit curve. (B) Magnitude of response (mean fluorescence) for all single heat shock pulses, as indicated in legend. Magnitude is normalized to basal autofluorescence of each worm at the start of each experiment.

To explore these differences, I plotted the rate against the magnitude of response for individual

worms (Figure 3.5). The “data collapse” observed in Figure 3.5, namely the fact that measurements from different animals under different experimental conditions fall on a single curve, suggests that the response dynamics are dictated by a “stress intensity”. This intensity can be described as an integration of both duration and temperature of the heat shock pulse, such that the same intensity leads to the same response. Moreover, this figure suggests that over a range of heat shock pulses (termed “low intensity”), there is a strong linear correlation between the rate and magnitude. Pulses with higher temperature or longer duration (“high intensity”) break from this pattern. Linear correlation between the rate at which fluorescence accumulates and the accumulated magnitude suggests that in the low intensity regime, the duration of accumulation is fixed.

Under most stress conditions, I observed that accumulation of fluorescence commenced after the end of the heat shock pulse. This time lag between the environmental shift and the response was temperature- dependent. At lower temperatures (28°C, 31°C), the time lag lasted around 30 - 40 minutes, as measured from the start of the heat shock pulse (Figure 3.6), irrespective of the duration of the pulse. Contrastingly, at 34°C the time lag was longer for a longer pulse. Interestingly, I observed that at this temperature, the time lag ended 40 minutes *after* the end of the heat shock pulse, irrespective of its duration. (Figure 3.7).

Previous reports suggest that translation may be inhibited during heat shock^{161,173}. I therefore asked if the observed lag in HSP-16.2p::GFP accumulation reflects a post-transcriptional block or a delay in the accumulation of hsp-16.2 transcripts. To address this question, I quantified the total concentration of hsp-16.2p::gfp mRNA using qPCR (Figure 3.8). Worms were subjected to a 15 or 60 minute heat shock pulse in a 33°C water bath, which qualitatively approximated the fluorescence

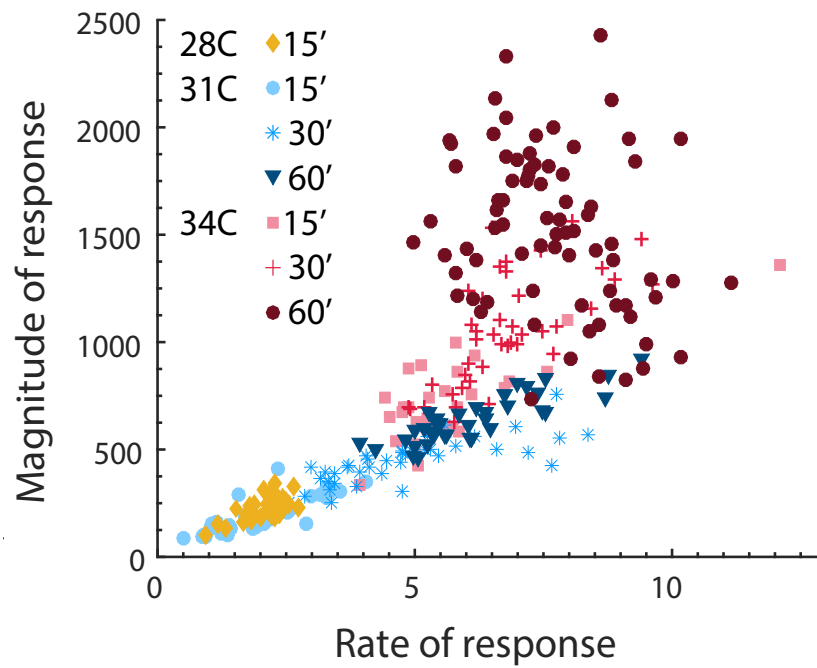


Figure 3.5: Magnitude of response (mean fluorescence) plotted versus rate of response (mean fluorescence/minutes) for all single heat shock pulses, as indicated in legend. Magnitude is normalized to basal fluorescence for each individual worm at the start of each experiment; rate of response is maximum response rate along logistic fit curve. Points represent individual worms.

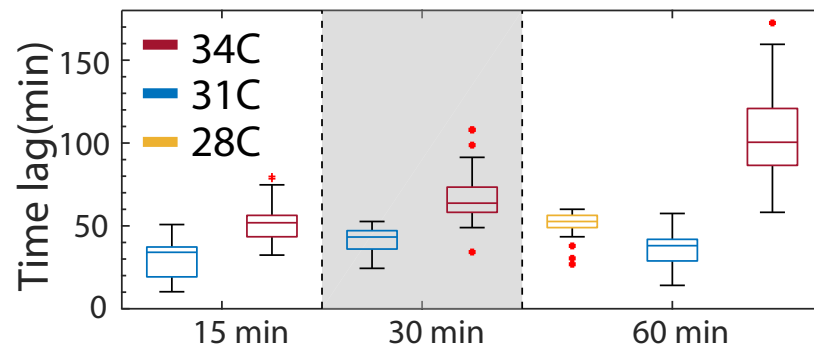


Figure 3.6: Time lag of response in minutes, calculated from start of heat shock pulse for all single pulses.

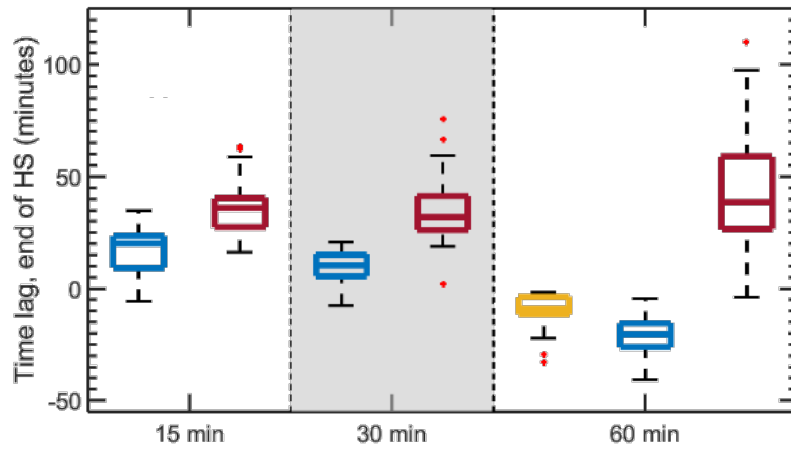


Figure 3.7: Time lag of response in minutes, calculated from *end* of heat shock pulse for all single pulses.

response from a 34°C heat shock pulse in the microfluidics chip. No time lag was observed in the accumulation of mRNA, even for a high intensity stress. Similar results were obtained for the mRNA of hsp-70 (Figure 3.9), suggesting that the accumulation dynamics of the hsp-16.2p::gfp transcripts are not dominated by its fusion to the GFP-coding region. While I cannot rule out GFP maturation as the cause of the 30- to 40-minute time lag at low temperatures, I consider below the possibility that the additional lag at 34°C lasting the length of the heat shock is due to translational pausing.

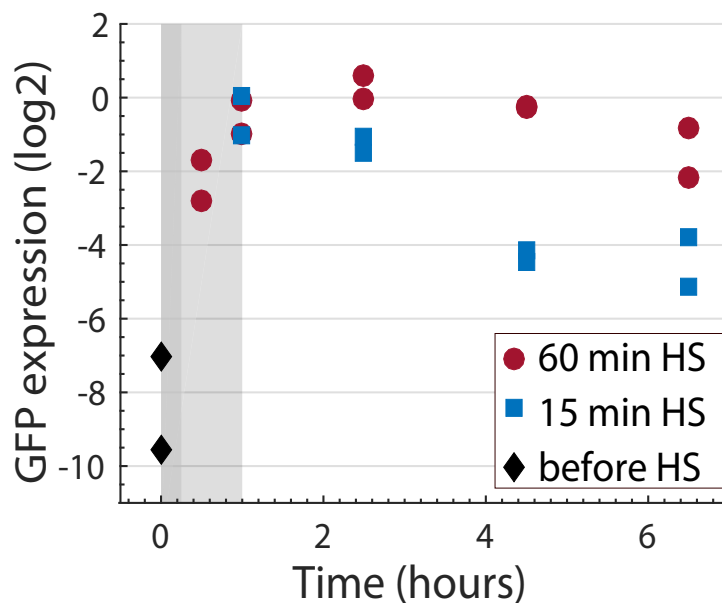


Figure 3.8: \log_2 GFP mRNA expression for hsp-16.2p::gfp expressing worms as measured by qPCR after 15 minute (blue) or 60 minute (red) heat shock pulse via plates placed in 33°C water bath. Each point is N = 50 worms. Results are normalized to the housekeeping gene snb-1.

Heat shock is known to evoke transcriptional pausing of non-heat-shock genes. HSPs, in contrast, are actively transcribed, and their mRNAs are preferentially processed and bypass some stages

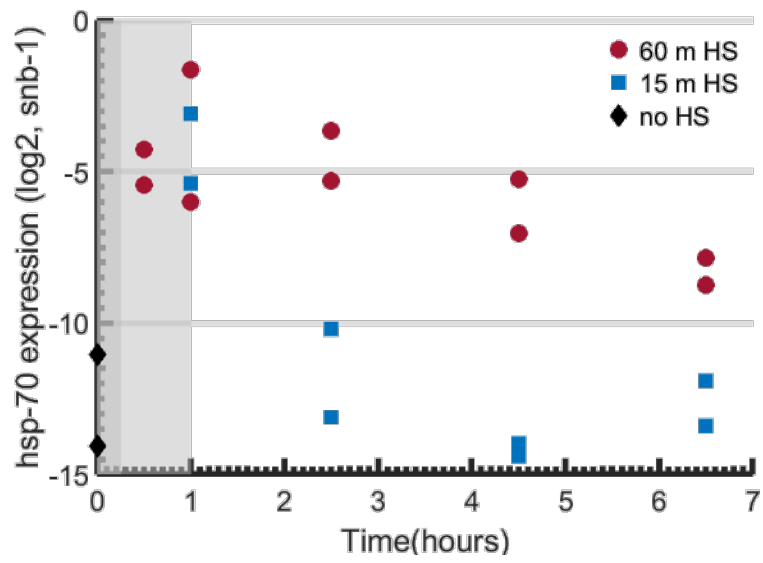


Figure 3.9: \log_2 hsp-70 mRNA expression as measured by qPCR after 15 minute (blue) or 60 minute (red) heat shock via plates placed in 33°C water bath. Each point is $N = 50$ worms. Results are normalized to the housekeeping gene snb-1.

of quality control for quicker export from the nucleus^{201,128,111}. In addition, translation is known to be inhibited during heat shock as well through elongation pausing^{22,104,168,161}. Consistent with these findings, our data confirm quick transcription of HSPs under high stress intensities but suggests a significant delay in translation of GFP from these promoters. Interestingly, we find that at lower temperatures, these proteins accumulate already during the heat shock pulse, while at higher temperatures translation only significantly resumes after the return to normal temperatures (Figure 3.2).

HSP-16.2::GFP is expected to be highly stable in the worm intestine⁴². This was reflected in the data for low intensity stresses, where I observed little to no decline in the GFP signal up to 10 hours following a heat shock pulse. In contrast, a significant decline in the fluorescence signal was observed in the hours following a pulse classified as a high intensity (60 minute HS at 31°C or a 34°C HS of any duration) (Figure 3.10).

3.3 SPATIAL CHARACTERISTICS

So far I ignored potential differences among the cells of the intestine and considered the level of HSP-16.2::GFP to be its average expression throughout the entire intestine. However, HSP-16.2 is expressed in 20 different cells of the worm intestine, which are organized into 9 rings that form the intestinal tube. I next sought to characterize possible differences among these cells and how these differences might depend on the stress intensity. The distribution of the fluorescence signal in two worms, each exposed to a single 60 minute heat shock pulse at either 28°C or 34°C, is depicted in

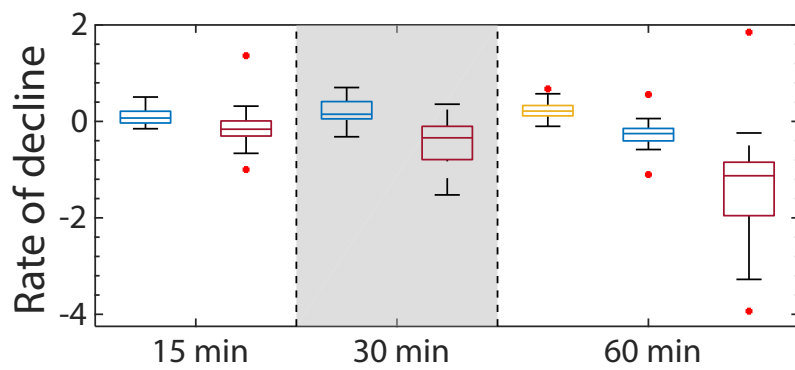


Figure 3.10: Rate of decline (mean fluorescence/minutes) calculated 8 – 10 hours after the heat shock pulse for all single heat shock pulses.

Figure 3.11 (and is representative of the dynamics observed in most worms). Every row in this figure comes from a single frame from the experiment, ordered in time from top to bottom. The intensity of each pixel corresponds to the observed fluorescence at the related position along the long axis of the worm, averaged over the lateral axis. Lines were aligned by performing spline interpolation and aligning the leftmost and rightmost peaks in intensity. Close inspection of these figures suggests that at 34°C, the farther a cell is from the anterior end, the more delayed is the increase in fluorescence. In contrast, at lower temperatures fluorescence is seen to increase simultaneously at all positions along the worm.

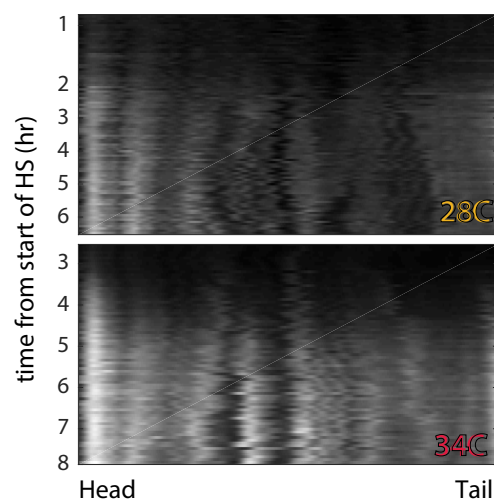


Figure 3.11: Example time courses for a worm exposed to a 60 minute heat shock pulse at 28°C (top) or 34°C (bottom). Each horizontal row is the mean fluorescence of the worm body at a single time point, collapsed to a single row of pixels. Time is zero at the top.

To test this proposition and quantify it, I partitioned the intestine into three equal parts: anterior, middle, and posterior. The middle section of the intestine overlaps with the uterus, and in some cases, embryos *in utero* express a fluorescent signal which interferes with the signal from the

intestine of the mother. I therefore ignored the middle third and focused on the differences between the anterior and posterior cells instead. To do this, I plotted the mean GFP fluorescence in each part separately (Figure 3.12) and parameterized these curves using a logistic fit, as above. I then asked if the dependence on the stress intensity of the magnitude of response and rate of response is the same in both parts (Figure 3.13).

To factor out differences in the measured fluorescence that come from the variable numbers and sizes of cells in each third, I normalized the measured fluorescence by its maximum observed value in the anterior or posterior, respectively. After normalization, I found that both the magnitude of response and rate of response are highly correlated between the two sections ($R^2 = 0.88$ and 0.84 , respectively, Figure 3.14A - B). This was also the case for the time lag of the activation for a heat shock pulse at 28°C or 31°C (Figure 3.14C). In contrast, the time lag after a 34°C heat shock pulse was significantly longer in the posterior for all pulse durations (Figure 3.14D, Figure 3.15). This suggests that the observed propagation of the fluorescence signal from head to tail (Figure 3.11) is due to a delay in the initiation of response, and not a modulation of its dynamics once it starts.

The observation that cells at the anterior end of the worm respond to stress earlier than cells at the posterior end can be interpreted in several ways. It is unlikely that this result is an experimental artifact: the heat-carrying fluid flows in a direction transverse to the head-tail axis, and worms can be situated in the device with their head either along or opposite the flow of food, with no noticeable effect on the HSR dynamics. It is possible that cells respond autonomously to the change in temperature with time lags that increase along the intestine, although it would be unclear what causes such a position-dependent time lag. Alternatively, it is possible that a small temperature gradient

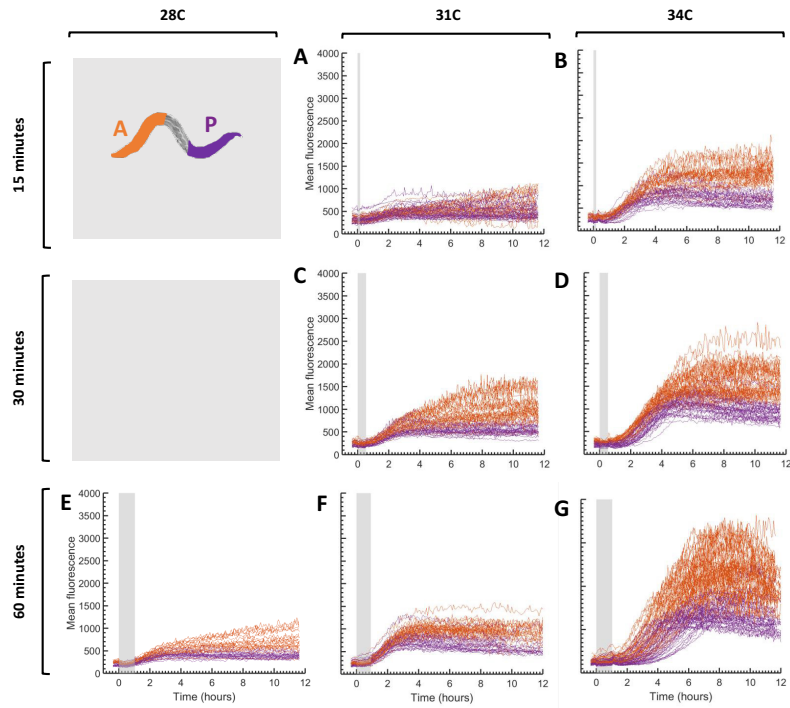


Figure 3.12: Individual response curves for anterior (orange) and posterior (purple) thirds of worm body for worms subjected to a whole body heat shock pulse of (A) 15 minutes, 31°C; (B) 15 minutes, 34°C; (C) 30 minutes, 31°C; (D) 30 minutes, 34°C; (E) 60 minutes, 28°C; (F) 60 minutes, 31°C; and (G) 60 minutes, 34°C.

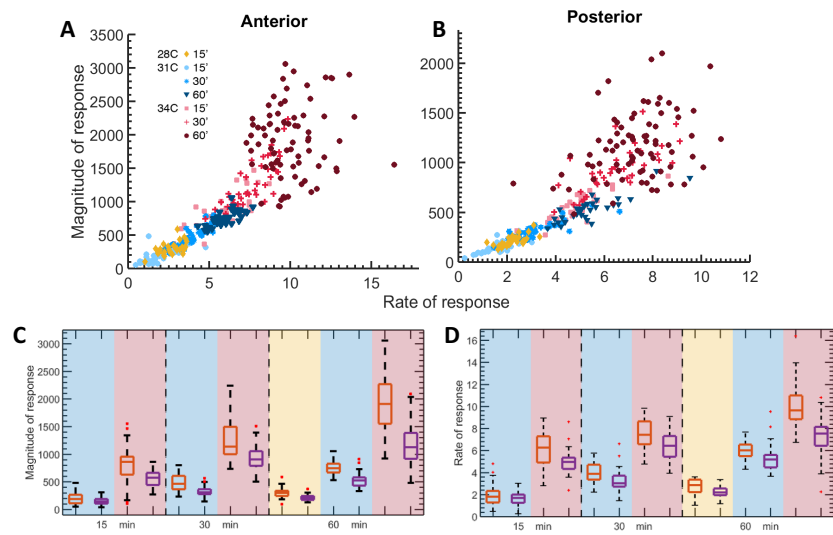


Figure 3.13: (A,B) Magnitude of response (mean fluorescence) plotted versus rate of response (mean fluorescence/minutes) for (A) anterior or (B) posterior third of worms subjected to a whole-body, single heat shock pulse; duration and temperature as indicated in legend. (C,D) (C) Magnitude of response (mean fluorescence) and (D) rate of response (mean fluorescence/minutes) of anterior (orange) and posterior (purple) thirds for 15, 30, or 60 minute heat shock pulse at 28°C (yellow), 31°C (blue), or 34°C (red).

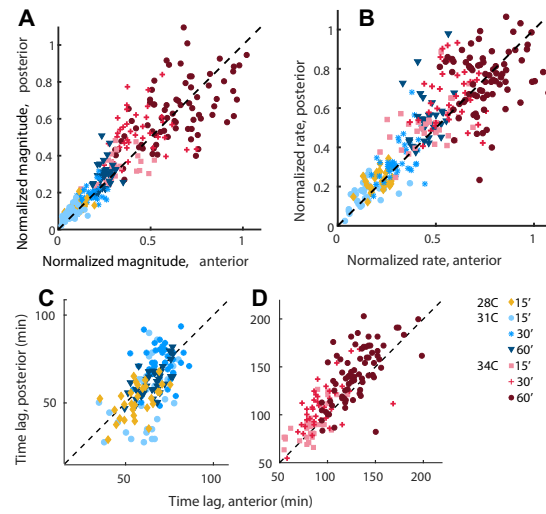


Figure 3.14: (A) Magnitude of response (mean fluorescence) and (B) rate of response (mean fluorescence/minutes) of posterior third of worms plotted versus anterior third of worms subjected to a whole-body, single heat shock pulse, duration and temperature as indicated in legend. Dashed black line is $y = x$. Magnitude and rate are both normalized to their respective maximum values in the anterior and posterior thirds. (C,D) Time lag (minutes) from start of heat shock pulse for posterior third of worms plotted versus anterior third of worms subjected to a whole-body, single heat shock pulse at a (C) low temperature or (D) high temperature; duration and temperature are as indicated in legend. Dashed black line is $y = x$.

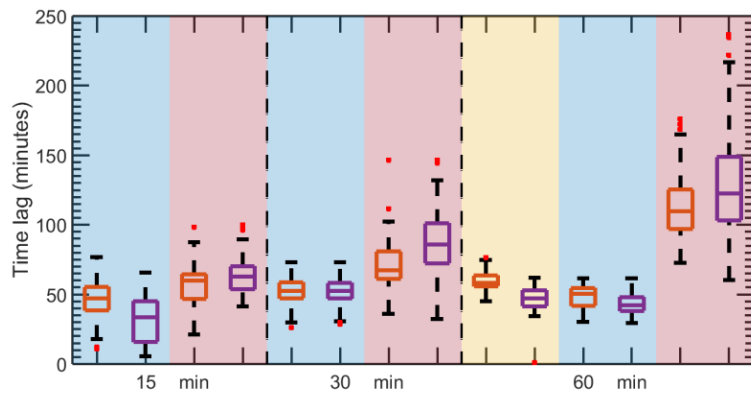


Figure 3.15: Time lag (minutes) from the start of the heat shock pulse of the anterior (orange) and posterior (purple) thirds for a 15, 30, or 60 minute heat shock pulse at 28°C (yellow), 31°C (blue), or 34°C (red).

develops in the intestine from the flow of ingested liquid. Another possible explanation is that some intercellular molecular signal propagates in the same direction and contributes to these activation dynamics. An interesting possibility for cell-to-cell communication is the transmission of proteins between cells^{129,206}. Movement of HSPs between cells has been detected in *Drosophila* and could play a role in *C. elegans* as well¹⁷⁶. I investigate the potential existence of this signal in the context of the thermosensory neurons in Chapter Five.

Modeling can be a bit brain damaging.

Kate Moss

4

Modeling the single cell HSR

PREVIOUS MODELS OF THE HEAT SHOCK RESPONSE at the cellular level aimed to characterize the importance and functionality of different aspects of the control circuit, including transcriptional, post-transcriptional, and post-translational regulation, as well as the feedforward and feedback loops^{28,144,135,1,91,95,67,46}. These models vary significantly in the level of detail at which different pro-

cesses are modeled. In bacteria and yeast, the resolution of available experimental data allowed for assignment of functional roles to different branches of the regulatory circuits^{46,95} and the association of HSP-70 with HSF-1⁹¹, respectively. In contrast, models of the heat shock response in HeLA cells rely on lower resolution experimental data¹⁴⁴. While some of these models explicitly account for multiple processes in great detail¹, it has been argued that a minimal model that limits the number of degrees of freedom and uses a coarse-grained description of the underlying molecular processes suffices to explain these data¹⁶⁴.

4.1 DEVELOPMENT OF MATHEMATICAL MODEL

To interpret my results in the context of the regulatory dynamics of the HSR, I developed a minimal mathematical model of the cellular HSR. Beyond technical benefits, such as reduced computational effort and lower risk of spurious parameter fitting, minimal models have a clear advantage in that they are more readily interpretable. In addition, complex models with many fitting parameters may be capable of reproducing the observed data even if their underlying assumptions are wrong. Under such conditions, the failure of a minimal model is more evident, and it may also be simpler to identify the incorrect assumption, and then formulate and test alternative hypotheses. My model was inspired by previously published models of HSR in mammalian cells^{144,156,135}, but it was considerably simplified even further, as discussed below, to a form that can faithfully describe my experimental observations. This model describes the dynamics of three main groups of molecules in the HSR pathway: the heat shock transcription factor (HSF), the heat shock proteins (HSP), and the

misfolded or unfolded proteins (Figure 4.1).

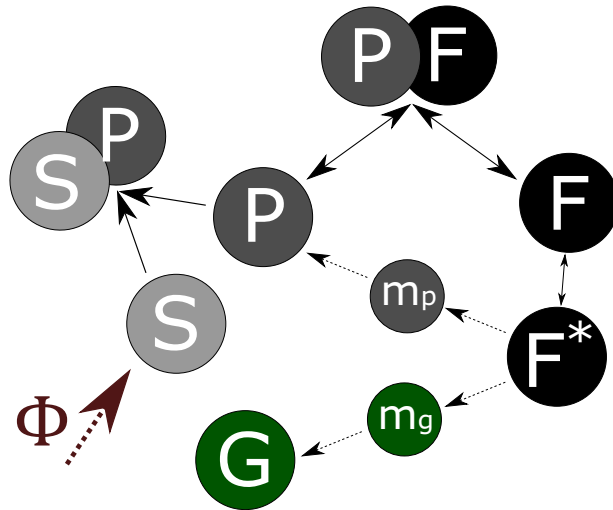


Figure 4.1: A simple model of the cellular HSR. F is inactive HSF; F^* is active HSF; m_p and P are mRNA and protein of HSP, respectively; m_g and G are the same for GFP; and S is misfolded protein. P and F exist both in a complex and separately. F^* generates m_p and m_g , which then generate P and G . A stressor, Φ , generates S , which is sequestered by P .

In this model, under non-stress conditions HSF is located in a complex with a single HSP. After the introduction of a stressor, this complex dissociates, and the HSF becomes active through a series of post-translational modifications including trimerization, translocation into the nucleus, and binding to a heat shock element on the DNA. For simplicity's sake, I considered this activation process as a single step. Active HSF is then used to induce transcription of the different HSP genes, which are accounted for collectively in my model. In order to account for my experimental set-up, I also considered a separate GFP mRNA, which was transcribed in a similar manner to the HSP genes. The mRNA are then translated into either HSPs or GFP. HSPs can be “used up” by forming a complex with a misfolded protein, which is produced during stress, while unused HSPs go back into complex

with the HSF molecules, providing a negative feedback loop. While the model assumes that the total concentration of HSF in all its forms does not change during the experiment, all other molecules undergo degradation. This behavior can be described with the following eight equations:

$$\frac{dC}{dt} = k_+PF - k_-C \quad (4.1)$$

$$\frac{dF}{dt} = -k_+PF + k_-C - 3l_+F^3 + 3l_-F^* \quad (4.2)$$

$$\frac{dF^*}{dt} = l_+F^3 - l_-F^* \quad (4.3)$$

$$\frac{dm_p}{dt} = A(F^*) - \gamma m_p \quad (4.4)$$

$$\frac{dm_g}{dt} = A'(F^*) - \gamma m_g \quad (4.5)$$

$$\frac{dP}{dt} = \lambda m_p - k_+PF + k_-C - \gamma_P P - cPS \quad (4.6)$$

$$\frac{dG}{dt} = \lambda m_g - \gamma_G G \quad (4.7)$$

$$\frac{dS}{dt} = \Phi - cPS \quad (4.8)$$

In this system, C is the complex of HSF:HSP, F is free and inactive HSF, and F^* is active HSF (trimerized, etc.). m is the mRNA concentration of a representative HSP (m_p) or GFP (m_g), whose concentrations are P and G respectively. All parameters are defined in Table 4.1. I assumed my reporter gene is transcribed identically to the HSP itself after a heat shock pulse, but has no basal transcription level. The concentration of misfolded proteins is denoted by S . Misfolded proteins are generated in the cell with rate $\Phi(T, t)$, which reflects the temperature and duration of the heat shock pulse. In my experiments, the ambient temperature changed with time, which I took into account in the model by considering $\Phi(T, t)$ to be a time-dependent function. Specifically, I modeled $\Phi(T, t)$ as a step function whose amplitude reflects the temperature of the heat shock pulse and whose width reflects the duration. This is comparable to the heat shock pulses experienced by worms in the microfluidics devices, as temperature changed at the start and end of the heat shock pulse within 1 - 2 minutes.

Given the experimental observation of a significant time lag between the accumulation of HSP mRNA and proteins, the model explicitly considers the concentrations of both types of molecules, m_p and P , separately. I captured this observation through the simplified assumption that translation of HSPs starts only after the heat shock pulse ends. To do this, I set the temperature-dependent translation rate, $\lambda(T)$, to some constant at innocuous temperatures and zero under stressful temperatures. While the data showed more complexity than this in terms of how long the blockage of

translation lasts - for example, translation starts during the 28°C 60 minute heat shock pulse - I did not aim to capture this feature or account for the mechanism behind this behavior.

Parameter	Definition
k_+	Association rate of the HSF:HSP complex
k_-	Dissociation rate of the HSF:HSP complex
L_+	Activation rate of HSF
L_-	Inactivation rate of HSF
γ	mRNA degradation rate
A	Transcription rate of HSP (basal & stress)
A'	Transcription rate of GFP (stress only)
γ_P	Protein degradation rate of HSP
γ_G	Protein degradation rate of GFP
λ	Translation rate
c	Association rate of HSP and a misfolded protein
Φ	Stress “temperature”

Table 4.1: Model parameter definitions.

I also assumed all mRNA have the same degradation rate, whether they are transcribed from hsp-16.2 or hsp-16.2p::gfp. In addition I assumed they have the same transcription rate as well, since they have the same promoter, though I assumed basal transcription for HSP only – not for GFP. Finally, I assumed that the misfolded proteins are cleared through a process that both requires and titrates heat shock proteins (the terms proportional to the parameter c in Equation 4.6 and Equation 4.8). While HSPs are thought to be reused *in vivo*, adding this into the model just renormalized some of the parameters, so I ignored it for simplicity’s sake. In addition, this assumption could be weakened without changing my results.

The behavior of the model can then be described as follows. At steady state, a basal level of HSPs

exists. If a stress dosage is low enough, this basal level will not be depleted, and the heat shock response will not be activated. Dosage of the stress experienced by a cell is a function of both the length and temperature of the heat shock pulse. For a slightly higher stress dosage, the basal level of HSPs will be used up entirely, and HSPs that are in complex with HSF will be titrated away, leaving HSF free to generate HSP mRNAs. Translation of the HSPs starts at the end of the heat shock, and the translation rate depends on the maximum production of mRNA. The length of the response once translation starts is a fixed time period. For a high stress dosage, the maximum number of mRNA possible will be produced, leading to the highest translation rate, and therefore the highest magnitude of response, measured by concentration of HSPs created. For a high enough stress, in order to deal with all of the misfolded proteins, the length of the response must then be increased. As the response ends, the HSP concentration will decrease back down to its basal level.

4.2 SIMPLIFICATION OF MODEL

To simplify the model, I first assumed that the kinetics of binding of HSF to its various partners is rapid compared with other kinetic rates in the system, and that the overall cellular concentration of HSF in its different forms is conserved¹⁵³. This is an acceptable assumption because the biochemical processes of HSF:HSP complex association and dissociation happen on the order of seconds, whereas the molecular biology processes of transcription and translation are on the order of minutes, and degradation is on the order of hours. By taking a quasi-steady state approximation, I deter-

mined a relationship between C , P , and F :

$$C = \frac{k_+}{k_-}PF = \frac{PF}{K} \text{ with } K = \frac{k_-}{k_+} \quad (4.9)$$

This allowed me to reduce the system to 7 equations and simplify my equations for F and P . The terms involving PF and C cancel, leaving these simplified versions:

$$\frac{dF}{dt} = -3l_+F^3 + 3l_-F^* \quad (4.10)$$

$$\frac{dP}{dt} = \lambda m_p - \gamma_p P - cPS \quad (4.11)$$

I then made the same quasi-steady state approximation, but with regards to the speed of HSF activation and inactivation, which produces a relationship between F and F^* that can be used to further simplify the equations:

$$F^* = \frac{l_+}{l_-}F^3 = \frac{F^3}{L} \text{ with } L = \frac{l_-}{l_+} \quad (4.12)$$

Note that for the dimensional analysis to work out, L must have units of concentration squared, unlike the previously defined ratio K , which is in units of concentration. In order to reduce the system to 5 equations, I assumed that F_{total} , the total concentration of HSF in all its different forms (F , F^* , and the HSF:HSP complex), is conserved¹⁵³, which allowed me to write an equation relating F_{total} to the concentrations of free and inactive HSF, free and active HSF, and HSF in a complex

with HSP. Using Equations 4.9 and 4.12 to write this relationship in terms of F only gives:

$$F_{total} = F + \frac{3}{L}F^3 + \frac{1}{K}PF \quad (4.13)$$

From this equation, I solved for the steady state value of HSF. Additional parameters from Equation 4.9 through Equation 4.13 are defined in Table 4.2.

Parameter	Definition
F_{total}	Total concentration of HSF – including free and inactive HSF, active and trimerized HSF, and HSF in a complex with HSP
L	Ratio of inactivation rate of HSF to activation rate
K	Ratio of dissociation rate of the HSF:HSP complex to association rate

Table 4.2: Model simplification parameter definitions.

4.3 DERIVATION OF TRANSCRIPTIONAL TERM

To determine the form of $\mathcal{A}(F^*)$, I first considered a few basic assumptions. I assumed there is no leakage in the transcription – so the function should start at zero, and it needs to level off at some point because of the limit of transcription rate. From these assumptions, I produced a basic form:

$$\frac{e^x}{1 + e^x} - 1 \quad (4.14)$$

For a more technical derivation of the exact form, I considered a polymerase, (P), with binding energy ϵ_P and a single binding factor, (\mathcal{A}), with binding energy $\epsilon_{\mathcal{A}}$, interacting with energy $\Delta\epsilon$. I

then wrote the probability that the polymerase is bound as:

$$P_{bound} = \frac{[P]e^{-\beta\epsilon_P} + [P][A]e^{-\beta(\epsilon_P+\epsilon_A+\Delta\epsilon)}}{[P]e^{-\beta\epsilon_P} + [P][A]e^{-\beta(\epsilon_P+\epsilon_A+\Delta\epsilon)} + [A]e^{-\beta\epsilon_A} + e^{-\beta(0)}} \quad (4.15)$$

This probability includes the polymerase bound alone or with the binding factor bound as well. The denominator contains all possible configurations (P alone, P and A , A alone, or nothing bound). The first two terms in the denominator can be neglected because the concentration of the polymerase is very low compared to the concentration of the binding factor. I then simplified the probability to:

$$P_{bound} = [P]e^{-\beta\epsilon_P} \frac{1 + [A]e^{-\beta(\epsilon_A+\Delta\epsilon)}}{[A]e^{-\beta\epsilon_A} + 1} \quad (4.16)$$

Next, the following terms were redefined:

$$e^{-\beta\epsilon_A} = k_A \text{ and } e^{-\beta\epsilon_P} = k_P \quad (4.17)$$

k_A and k_P have units of concentration and set the respective scales for the concentrations of P and A . I then rearranged and simplified the equation:

$$P_{bound} = \frac{[P]}{k_P} \frac{1 + \frac{[A]}{k_A} e^{-\beta\Delta\epsilon}}{1 + \frac{[A]}{k_A}} \quad (4.18)$$

Next, I generalized the equation by redefining terms again:

$$\frac{[P]}{k_P} = \alpha \text{ and } e^{-\beta\Delta\epsilon} = w \text{ and } k_A \rightarrow k_o \quad (4.19)$$

This then allowed me to simplify the equation to a more general form:

$$P_{bound} = \alpha \frac{1 + w \frac{[A]}{k_o}}{1 + \frac{[A]}{k_o}} \quad (4.20)$$

From this, I noted that if the interaction energy, $\Delta\epsilon$, is negative, meaning a positive interaction between the polymerase and binding factor, then $w > 1$ (an attractive interaction – the binding factor is an activator). If $\Delta\epsilon$ is positive, meaning a negative interaction between the polymerase and binding factor, then $w < 1$ (the binding factor is a repressor).

To generalize this form further to include systems with multiple binding factors, I simply added in another binding factor, again with energy ϵ_A , and followed the same steps as in the above derivation. The interaction energy of 2 A s is $\Delta\epsilon_A$. This produced eight possible configurations in the denominator of the equation for the probability that the polymerase is bound. This derivation is left as an exercise for the reader, but gave the following equation:

$$P_{bound} = \alpha \frac{1 + 2w \frac{[A]}{k_o} + w' \left(\frac{[A]}{k_o} \right)^2}{1 + 2 \frac{[A]}{k_o} + \left(\frac{[A]}{k_o} \right)^2} \quad (4.21)$$

All terms with a single $[A]$ were neglected because protein-protein interactions ($[A]^2$) are much stronger than protein-DNA ($[A]$) interactions. Substituting in F^* as the binding factor, I then

ended up with a final form for the transcription term in my model:

$$P_{bound} = A(F^*) = \alpha \frac{1 + w' \left(\frac{[F^*]}{k_o} \right)^2}{1 + \left(\frac{[F^*]}{k_o} \right)^2} \quad (4.22)$$

Additional parameters introduced in Equation 4.22 are defined in Table 4.3. The form of $A'(F^*)$, the transcription term for GFP, is the same, but without the basal transcription.

Parameter	Definition
w'	Maximal fold of activation
k_o	Affinity of activated HSF to the hsp promoter
α	Basal transcription level

Table 4.3: Transcription term parameter definitions.

4.4 TUNING OF PARAMETERS

Estimates and units of all parameters can be found in Table 4.4.

First, I considered the concentrations of each molecule. I set the total HSF concentration (F_{total}) to 1 and measured all other concentrations in comparison. In yeast and *Drosophila*, literature shows the steady state concentration of HSP is around 10 – 100 times larger than total HSF concentration^{135,185,52}. Parameters were thus adjusted to set the steady state ratios of molecular concentrations to this order of magnitude.

Interestingly, the binding efficiency of HSPs to HSF (or its functional homologs) was identified as the key determinant of the sensitivity and robustness of the system in gram-positive and gram-negative bacteria⁷⁸, in the fungus *Candida albicans*¹⁰⁰, and in HeLa cells¹⁴⁴. These results are puz-

Parameter	Value	Units
L	0.1	unitless
K	1	unitless
F_{total}	1	[F]
γ	$\log(2)$	hours ⁻¹
γ_P	$\frac{\log(2)}{100}$	hours ⁻¹
γ_G	$\frac{\log(2)}{50}$	hours ⁻¹
$\lambda(T)$	1 at RT, 0 otherwise	[P][m] ⁻¹ hours ⁻¹
c	1	[P] ⁻¹ hours ⁻¹
$\Phi(T, t)$	2 ⁷ at 31°C, 2 ⁹ at 34°C	[S]hours ⁻¹
w'	1000	unitless
k_o	0.1	[F] ³
α	0.15	[m]hours ⁻¹

Table 4.4: Estimated values of model parameters, as well as their units.

zling in the context of a multicellular organism: while the level of non-sequestered HSPs can be very different in different cells, the affinity of HSPs to HSF-1 cannot be independently tuned in each cell type. Indeed, our model suggests that this parameter has little effect on the dynamics of HSR given the observed fact that at homeostasis, the concentration of HSPs is high enough to titrate all HSF-1¹⁸⁸.

The order of magnitude of k_o was set by considering the activation dynamics I was looking for. Because of the form of the transcriptional term, $k_o L$ needed to be a little smaller than $\frac{1}{3}$ in order to be able to activate transcription. Similarly, K and L could be defined relative to one another by considering the equilibrium state of HSF. To determine ranges of the translation and basal transcription rates, I considered the behavior I saw in my data. I wanted to saturate the rate of translation by saturating the number of mRNA made, not saturate the rate of transcription by saturating the number

of active HSF. Next, activation of the HSR leads to the induction of HSPs by 10 – 1000 fold in human cell lines^{188,153}, depending on the chaperone that is being considered. I thus chose $w = 1000$ to reflect the high end of this range.

The rate constant c , which governs the kinetics of association between HSPs and misfolded proteins, only affected the degree at which HSP production overshoot its steady state level, with significant overshoot only for $c > 1$. Since my data indicated that such overshoot is only observed at high stress, I chose a marginal value of $c = 1$. The level of Φ at different temperatures determined the rate of response and magnitude of response. I picked the two values in Table 4.4 to best describe the experimentally measured kinetics at 31°C and 34°C, including ratios of GFP saturation between different length and temperature heat shocks and the timescale at which the GFP fluorescence saturates. Once fixed, these values were kept fixed for my entire analysis.

Lastly, I considered degradation. mRNA molecules in animals decay over a time scale of hours¹⁸⁸. Consistently, my qPCR results suggested a decay rate of 1 - 2 hours. GFP, contrastingly, is known to be very stable in the worm intestine⁴², and my data suggested a half-life of several days⁴². The HSP degradation rate had very little effect on the dynamics of my model as long as it was fairly small, and even at larger values it only affected the steady state value of HSPs. I therefore took the half-life of both GFP and HSP to be on the order of 10s of hours.

4.5 FINAL MODEL

Combining my reduced system of 5 equations, my forms of $\mathcal{A}(F^*)$ and $\mathcal{A}'(F^*)$ from Equation 4.22, and my relationship between F and F^* from Equation 4.12, I get the following final form of my model:

$$\frac{dm_p}{dt} = \alpha \frac{1 + w \left(\left(\frac{1}{Lk_o} \right) F^3 \right)^2}{1 + \left(\left(\frac{1}{Lk_o} \right) F^3 \right)^2} - \gamma m_p \quad (4.23)$$

$$\frac{dm_g}{dt} = \alpha \frac{w \left(\left(\frac{1}{Lk_o} \right) F^3 \right)^2}{1 + \left(\left(\frac{1}{Lk_o} \right) F^3 \right)^2} - \gamma m_g \quad (4.24)$$

$$\frac{dP}{dt} = \lambda(T) m_p - \gamma_P P - cPS \quad (4.25)$$

$$\frac{dG}{dt} = \lambda(T) m_g - \gamma_G G \quad (4.26)$$

$$\frac{dS}{dt} = \Phi(T, t) - cPS \quad (4.27)$$

4.6 CONNECTION TO EXPERIMENTAL RESULTS

The results of my model provide an interpretation of my experimental results. Full activation of the HSR requires exhausting the basal concentration of HSPs that exists in the cell under normal condi-

tions, followed by activation of HSF and accumulation of the HSP transcripts synthesized *de novo*. The time required to exhaust this initial pool of HSPs and the time required to activate all HSFs are both shorter at higher temperatures (Figure 4.2). In my model, accumulation of HSPs only starts when the heat shock pulse ends. The concentration of HSP mRNAs at that time sets the rate at which new HSPs are synthesized and accumulated, i.e. the measured rate of fluorescence accumulation. The concentration of misfolded proteins at the same time sets the total number of HSPs to be consumed throughout the remaining response, which (due to the stability of GFP) corresponds to the measured fluorescence magnitude.

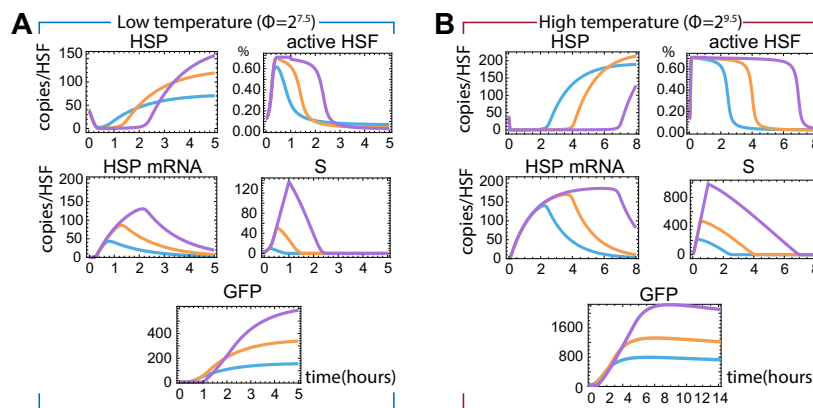


Figure 4.2: Kinetics of P (HSP), p (HSP mRNA), F^* (active HSF), S (misfolded proteins), and G (GFP) as predicted by model for a 15 (blue), 30 (orange), or 60 (purple) minute heat shock pulse at (A) a low temperature ($\Phi = 2^{7.5}$) and (B) a high temperature ($\Phi = 2^{9.5}$). All concentrations are proportional to the total concentration of HSF, which is set to 1.

Thus, when the heat shock pulse is in the low intensity range, both concentrations are proportional to the level of stress at the end of the pulse, which results from a combination of temperature and duration of the pulse. This means that the rate and magnitude of response are linearly propor-

tional to each other (Figure 4.3), as observed experimentally (Figure 3.5). This linearity breaks if the heat shock pulse falls in the high intensity range, where the rate of accumulation of HSP mRNAs is saturated before translation begins. In this case, the rate of HSP accumulation takes its maximal value (Figure 4.3), and synthesis of the required number of HSPs necessitates a prolonged period of response.

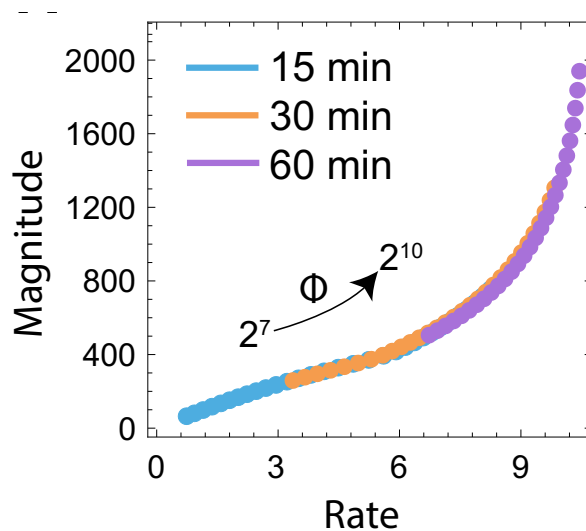


Figure 4.3: Magnitude of response (mean fluorescence) plotted versus rate of response (mean fluorescence/minutes) as predicted by model for single heat shock pulses of 15 (blue), 30 (orange), or 60 (purple) minutes over a range of temperatures ($\Phi = 2^7 - 2^{10}$).

Further confirmation for this mechanism comes from observed long-term decline of the fluorescent signal at high intensities. HSP transcription terminates only when there are enough free HSPs to titrate all HSFs, namely when HSPs are no longer required. Following a strong heat shock pulse, the level of HSP mRNA at this point may still be considerable, leading to further production of HSPs that are no longer needed. This “overshoot” is followed by a noticeable decline despite the fact that the half-life of these proteins may be considerably longer than the duration of the experiment

(Figure 4.2B). Higher induction of the HSR is thus linked to a higher rate of decline, as observed experimentally (Figure 3.10).

4.7 TEST OF MODEL'S APPLICATIONS

To test the limits of my single negative feedback model further, I asked what it predicts about habituation and memory effects. I exposed worms to two 30 minute heat shock pulses, both at either 31°C or 34°C, separated by a 90 minute rest (Figure 4.4). For the number of worms per experimental condition, refer to Table 3.1. While previous studies have compared mathematical modeling with experiments that exposed *C. albicans*¹⁰⁰ or mammalian cells²⁹ to two consecutive heat shock pulses, it has not been studied in *C. elegans*. Both studies in other model organisms found that the response to the second pulse depended on the properties (temperature and duration) of the first. While we performed two-pulse experiments mainly as a tool for validating our mathematical model, which was constructed based on our single-pulse data, multiple pulses are also interesting to consider due to hormesis effects. Small doses of heat shock can have a beneficial effect on the exposed worm, including longer lifespan and higher stress tolerance for both heat shock and other stressors like pathogens¹³⁹.

I found that at both temperatures, the magnitude of the response was additive, resembling that of a single 60 minute pulse (Figure 4.5). At 31°C, the rate of response to the second pulse was similar to that of the first, and both were comparable to the rate of response to a single 30 minute pulse at 31°C. In contrast, at 34°C the rate of response to the second pulse was significantly larger than that

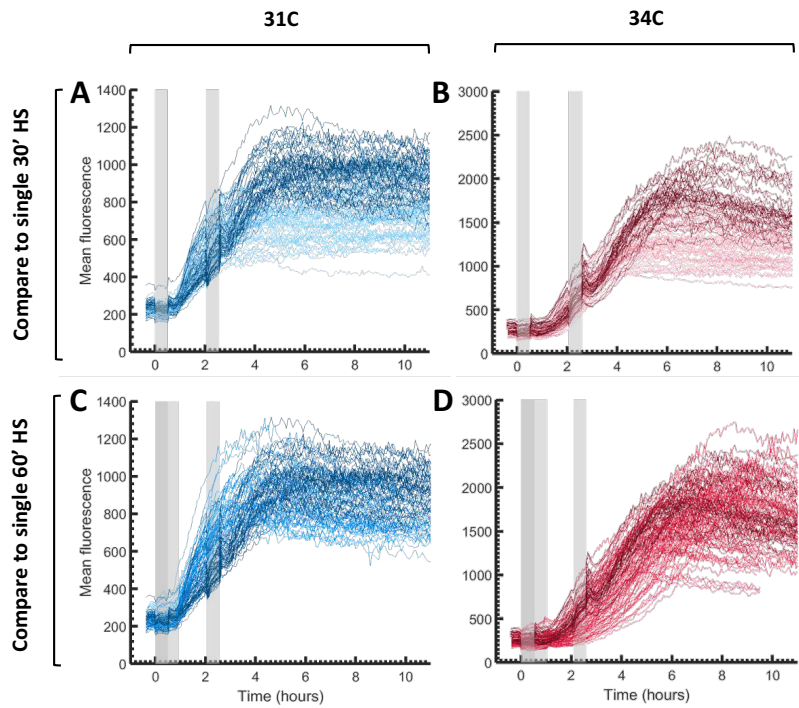


Figure 4.4: (A,B) Individual response curves for worms subjected to a single 30 minute heat shock pulse (light color) or two consecutive 30 minute heat shock pulses separated by a 90 minute rest (dark color) at (A) 31°C (blue) or (B) 34°C (red). (C,D) Individual response curves for worms subjected to a single 60 minute heat shock pulse (medium color) or two consecutive 30 minute heat shock pulses separated by a 90 minute rest (dark color) at (C) 31°C (blue) or (D) 34°C (red). Heat shock pulse(s) indicated by grey bar(s).

of the first pulse in most animals, reaching rates comparable to those of a single 60 minute pulse at 34°C (Figure 4.6A, Figure 4.7). These results confirm the predictions of my model, which predicts similar rates in response to two low temperature heat shock pulses and an increase in rate between consecutive pulses at higher temperatures (Figure 4.6B).

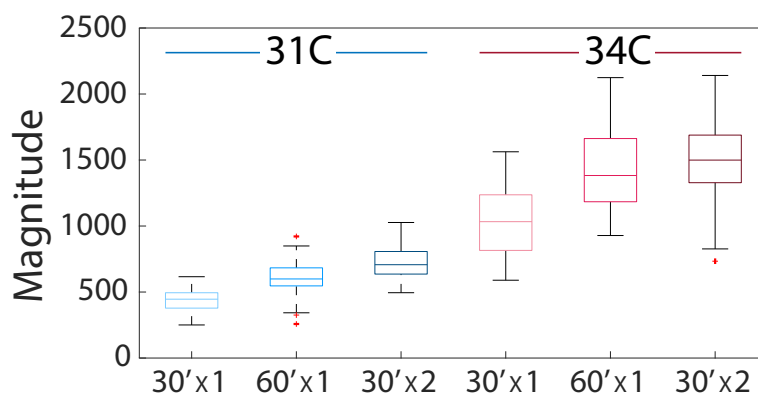


Figure 4.5: Magnitude of response (mean fluorescence) for two consecutive 30 minute heat shock pulses separated by a 90 minute rest (dark color) compared to one single 30 minute (light color) or 60 minute (medium color) heat shock pulse at 31°C (blue) or 34°C (red).

The model can be used to provide an interpretation of these results via the predicted kinetics of different molecules. At lower temperatures, the 30 minute pulse is short enough to avoid saturation of active HSF, and consequently the 90 minute interval between pulses permits complete annihilation of misfolded proteins and a reset of the active HSF to basal level. In contrast, at higher temperatures, HSF remains active in the interval between pulses, continuing production of HSP mRNA and saturating the rate of HSP mRNA production during the second heat shock pulse (Figure 4.8).

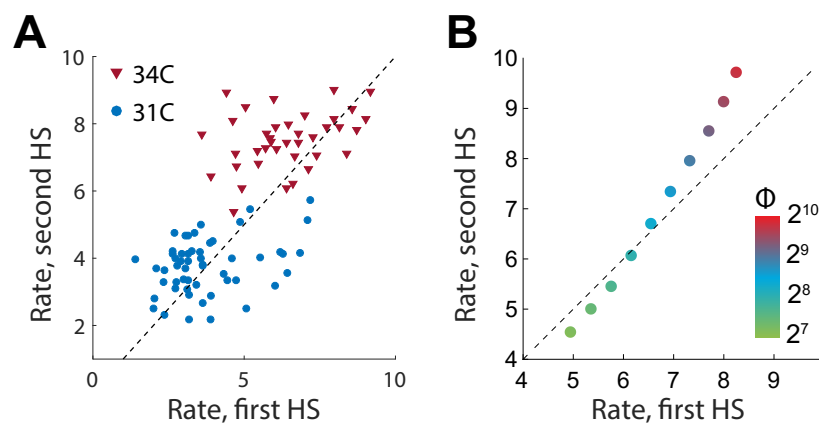


Figure 4.6: (A) Rate of response (mean fluorescence/minutes) for two consecutive 30 minute heat shock pulses separated by a 90 minute rest. Rate of response to second HS plotted versus rate of response to first at 31°C (blue) and 34°C (red). Dotted line indicates first rate of response equal to second rate of response. (B) Rate of response (mean fluorescence/minutes) as predicted by model for two consecutive 30 minute heat shock pulses over a range of temperatures ($\Phi = 2^7 - 2^{10}$) separated by a 90 minute rest. Dotted line indicates first rate of response equal to second rate of response.

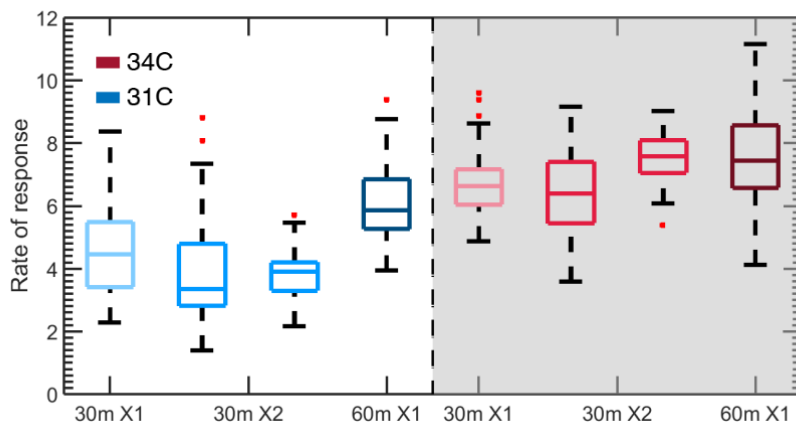


Figure 4.7: Rates of response (mean fluorescence/minutes) for the first and second pulse of two consecutive 30 minute heat shock pulses (medium color) compared to the rate of response for one single 30 minute (light color) or 60 minute (dark color) pulse at 31°C (blue) or 34°C (red).

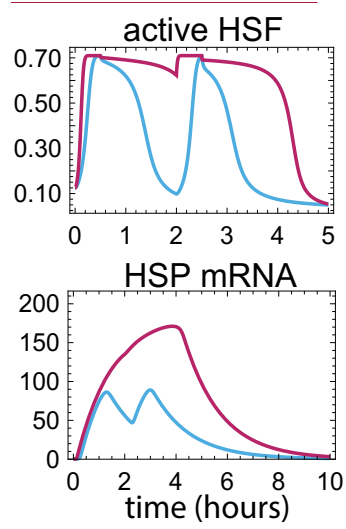


Figure 4.8: Kinetics of active HSF and HSP mRNA as predicted by model for two consecutive 30 minute heat shock pulses at 31°C (blue) and 34°C (red), separated by a 90 minute rest.

The sky was filled with worms. The worms were tornadoes.

Kurt Vonnegut

5

Systemic HSR in *C. elegans*

SO FAR, I HAVE ONLY CONSIDERED the whole animal response and its spatial components, as well as their role in the context of a cellular level mathematical model that I developed. To probe the systemic level of regulation of the HSR in *C. elegans*, I turned to its neuronal circuitry. The observation that the HSR starts earlier in the anterior of the intestine than in the posterior raises the hy-

pothesis that some signal propagates from the head to the tail. One possible source of such a signal is a pair of thermosensory neurons located in the head. Two likely candidates are the AFD thermosensory neurons and AIY interneurons, both of which have been previously implicated in thermotactic behavior¹²⁰ and linked to the HSR¹³⁷.

5.1 THERMOSENSORY NEURONS IN THE HSR

To investigate the potential roles of these two pairs of neurons, I characterized the HSR dynamics in mutant worms carrying either the *ttx-1* (p767) allele, which prevents terminal differentiation of the AFD neurons¹⁵⁴, or the *ttx-3* (ks5) allele, which ablates the functionality of the AIY interneurons⁷⁴. The dynamics of HSP-16.2p::GFP accumulation in individual mutant worms under a variety of stress conditions are shown in Figure 5.1. The number of worms per experimental condition can be found in Table 3.1.

I found that neither the activation of the HSR nor the linear relationship between the magnitude and rate of response at low intensity stresses required either the AFD or AIY neurons (Figure 5.2). However, while the break in linearity at high intensity stresses in the AIY-ablated mutants was similar to that in wild type animals, in the AFD-ablated animals, it occurred at lower stress intensities. At the higher intensities, these worms responded with only a minimally higher rate than wild type but reached a higher magnitude that was not within the linear regime. These observations also hold separately for the anterior and posterior thirds of the intestine in both mutants (Figure 5.3).

Lastly, I asked if the thermosensory neurons are involved in the observed propagation of HSR

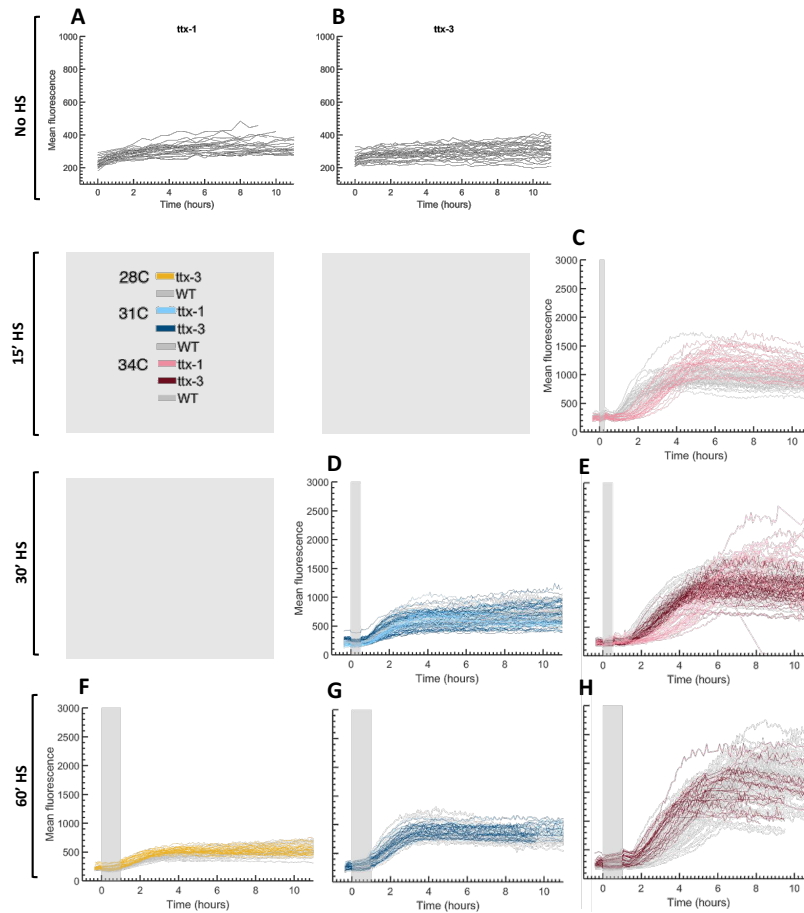


Figure 5.1: Individual response curves for mutant worms subject to a whole body heat shock pulse of (A) no heat shock (*ttx-1*); (B) no heat shock (*ttx-3*); (C) 15 minutes, 34°C (*ttx-1*); (D) 30 minutes, 31°C (*ttx-1* & *ttx-3*); (E) 30 minutes, 34°C (*ttx-1* & *ttx-3*); (F) 60 minutes, 28°C (*ttx-3*); (G) 60 minutes, 31°C (*ttx-3*); and (H) 60 minutes, 34°C (*ttx-3*). Wild type worm curves for the same experimental conditions are indicated in grey for comparison. Each line indicates the mean fluorescence of an individual worm. The heat shock pulse is indicated by a grey bar. Mean fluorescence was tracked for 10 hours following the heat shock pulse.

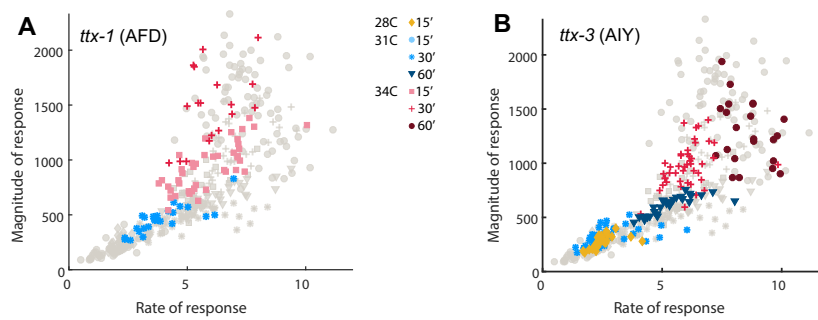


Figure 5.2: Magnitude of response (mean fluorescence) plotted versus rate of response (mean fluorescence/minutes) for all single heat shock pulses, duration and temperature as indicated in legend, for (A) *ttx-1* (AFD) mutants and (B) *ttx-3* (AIY) mutants. Magnitude is normalized to basal autofluorescence of each individual worm at start of each experiment; rate of response is maximum response rate along logistic fit curve. Points represent individual worms. Light grey points are wild type worms of the corresponding experimental condition.

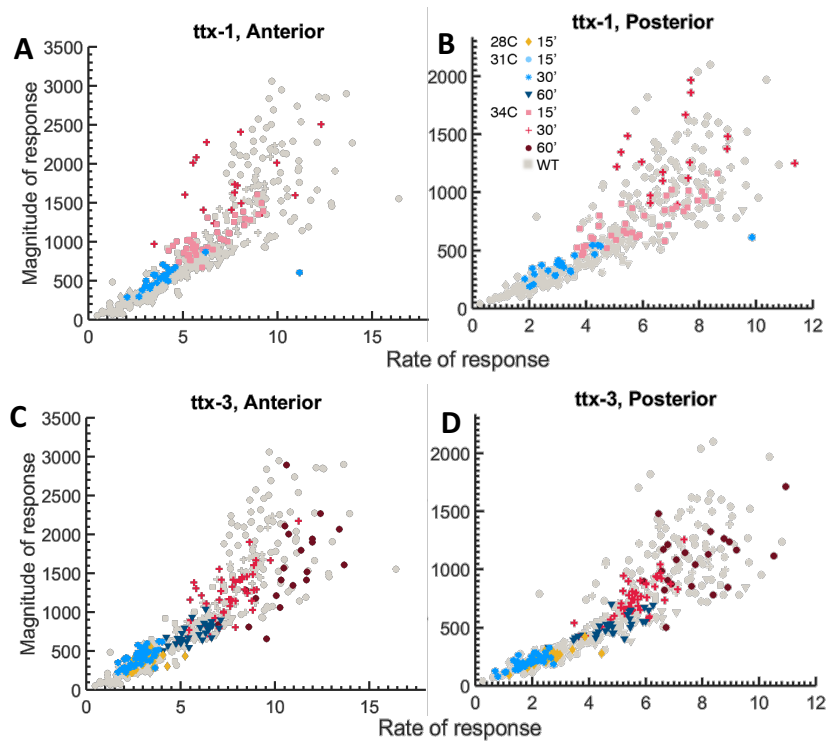


Figure 5.3: Magnitude of response (mean fluorescence) plotted versus rate of response (mean fluorescence/minutes) for (A,C) anterior or (B,D) posterior third of (A,B) *ttx-1* (AFD) or (C,D) *ttx-3* (AIY) mutant worms subject to a whole-body, single heat shock pulse; duration and temperature are as indicated in legend.

activation from head to tail. The dynamics of response, i.e. rate and magnitude, to heat shock in the anterior and posterior thirds were highly similar, as seen in wildtype animals (Figure 5.4). Moreover, my data did not implicate these neurons in generating the time lag difference between the anterior and posterior parts, which still exists in both mutant strains (Figure 5.5).

5.2 SYSTEMIC REGULATION IN A CELLULAR MODEL

In search of possible mechanisms behind the observed change in dynamics in AFD-ablated animals, I turned to my model to test the hypothesis that AFD-dependent signaling alters one or more of the kinetic rates of this model. However, the relationship between magnitude and rate, as depicted in Figure 4.3, turned out to be robust to changes of parameters within the relevant range (as described in Chapter 4 and Table 4.4). This comes from the fact that parameter changes lead to proportional effects on both the level of HSP mRNA, m_p , (which in turn sets the rate) and on the demand for HSPs by S (which sets the magnitude). Thus, if the two were linearly proportional with the original set of parameters, they remained proportional after the parameter change for a given stress intensity.

Looking for an alternative explanation for the modified relationship between magnitude and rate, I considered the time lag before activation. While the qualitative time lag associated with the observed propagation of HSR activation from head to tail was observed, I noticed quantitative differences from the wild type time lags. At low stress intensities, the time lag of both the *ttx-1* and *ttx-3* mutants was similar to that of the wild type animals. At high intensities, however, the time lag was significantly prolonged (i.e. the response was delayed) in AFD-ablated mutants but not in

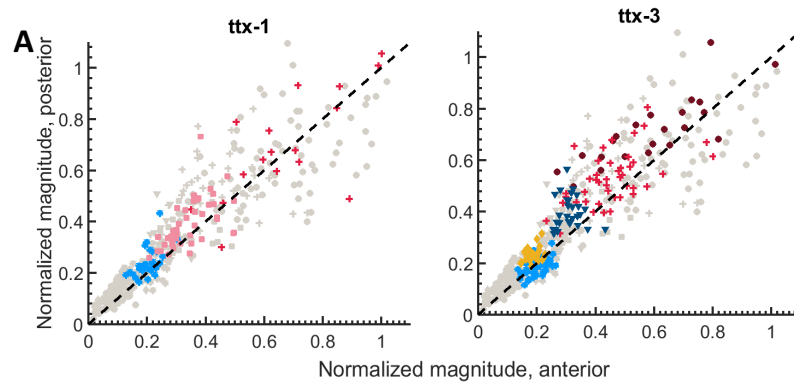


Figure 5.4: Magnitude of response (mean fluorescence) of posterior versus anterior of (A) *ttx-1* (AFD) and (B) *ttx-3* (AIY) mutant worms subject to a whole-body, single heat shock pulse; duration and temperature are as indicated in legend. Magnitudes are normalized to their respective maximum values in the anterior and posterior parts.

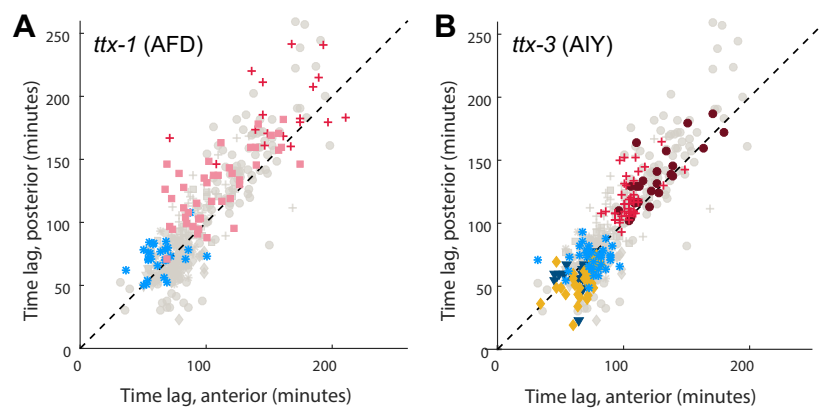


Figure 5.5: Time lag (minutes) from start of heat shock pulse of posterior part versus anterior part of (A) *ttx-1* (AFD) and (B) *ttx-3* (AIY) mutant worms subject to a whole-body, single heat shock pulse; duration and temperature are as indicated in legend.

AIY-ablated mutants, particularly in the posterior portion of the animals (Figure 5.5, Figure 5.6). For example, the time lag for a 30 minute heat shock pulse at 34°C in an AFD-ablated worm surpassed that of a 60 minute heat shock pulse at 34°C in a wild type worm.

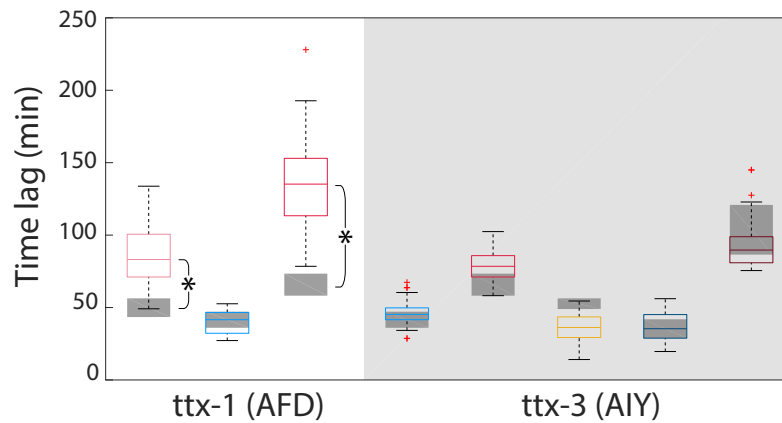


Figure 5.6: Time lag of response in minutes, calculated from start of heat shock pulse for all single heat shock pulses for *ttx-1* (AFD) and *ttx-3* (AIY) mutants. Temperature and duration are color-coded, as indicated by the legend. Grey boxes represent the corresponding experiment in wild type worms. Statistically significant differences between mutant and wild type worms are indicated by an asterisk: for 15 and 30 minute HS at 34°C, p-values are $<10^{-11}$ and $<10^{-14}$, respectively.

Motivated by this observation, I used my model to test the hypothesis that a delayed start of the response may lead to the observed increase in magnitude with a lower increase in the rate. Biologically speaking, the AFD neurons have ciliated dendrites that are exposed to the outside of the animal, so it is possible that sensing temperature directly from the environment accelerates the response to temperature changes in the intestine. To explore this in my model, I introduced a time delay be-

tween the emergence of misfolded proteins and their detection by HSPs. This time lag was taken to be one hour to match the observed data, and Equation 4.25 was replaced with:

$$\frac{dP}{dt} = \lambda(T)m_p - \gamma_P P - cPS(t - dT) \quad (5.1)$$

A direct consequence of this change to the model is a prolonged time lag before activation. This leads to a delay in the kinetics of HSF and HSP during heat shock but does not change the accumulation of active HSFs or the level of HSP mRNA (Figure 5.7). The observed rate of HSP accumulation is therefore unaltered. In contrast, as the accumulation of misfolded proteins is unchanged by HSP sequestration for a longer period of time, the demand for HSPs, which sets the magnitude of the response, is increased (Figure 5.7). Thus, this model predicts that a delay in the time of detection results in an increased magnitude of response with minimal effect on its rate, as observed experimentally.

As discussed in the previous chapter, my model links high stress intensities with the observed late decline in fluorescence. Given the overshoot in the response in the AFD-ablated mutants, the model predicts an increase in the rate of decline in these worms as compared with wild type animals. This prediction is confirmed by the experimental data, which shows a higher rate of decline for the AFD-ablated mutants but not for the AIY-ablated mutants (Figure 5.8).

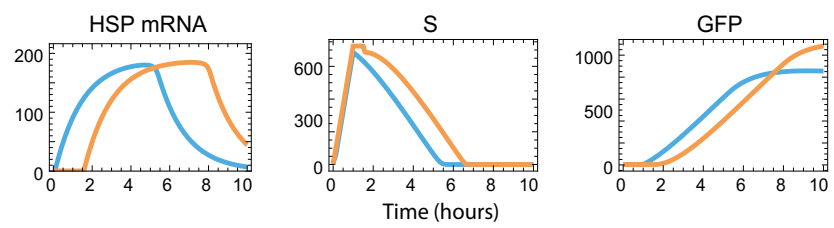


Figure 5.7: Kinetics of HSP mRNA, misfolded proteins (S), and GFP as predicted by model for a single 60 minute heat shock pulse at 34°C (blue) and the same heat shock pulse with a one hour delay (orange).

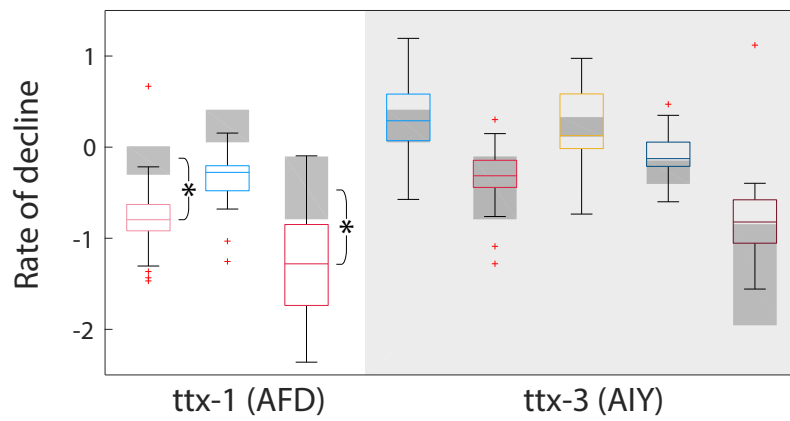


Figure 5.8: Rate of decline (mean fluorescence/minutes) calculated 8 – 10 hours after heat shock pulse ends for all single heat shock pulses, duration and temperature as indicated in legend, for *ttx-1* (AFD) and *ttx-3* (AIY) mutants. Grey boxes represent wild type worm data. Statistically significant differences between mutant and wild type worms are indicated by an asterisk: for 15 and 30 minute HS at 34°C, p-values are $<10^{-3}$ and $<10^{-10}$, respectively.

5.3 NON-LOCAL ACTIVATION OF HSR

Finally, to explore the interplay of systemic and spatial regulation of the HSR, I asked how activation of the HSR in one part of the intestine depends on the stress experienced by another part of the intestine in both wild type and mutant worms. To address this question, I modified my microfluidics device to have two water channels, each perpendicular to the worm chambers and positioned above about one half of the worm body, instead of a single water channel covering the whole worm chamber (Figure 5.9). The temperatures of the two channels were controlled independently, allowing me to apply a 30 minute heat shock pulse at either 31°C or 34°C to one half of the worm body while maintaining the other half of the worm at room temperature. Localized thermal stimuli such as this have been studied in the zebrafish embryo previously using the application of a localized infrared laser pulse¹⁸⁶. In addition, they have also been studied in *C. elegans* previously, however only the behavioral responses were considered¹¹⁸.

I then used these devices to track the dynamics of HSP-16.2p::GFP accumulation in both halves of the worms (Figure 5.10). The number of worms per experimental condition can be found in Table 3.1. Data analysis was performed in a similar manner to my previous spatial analyses, however the locations of the water channel edges were used to select the worms' anterior and posterior portions. Surprisingly, I found an increase in fluorescence in both halves of the worm, including the half that was maintained at room temperature.

When wild type animals were heat shocked in the anterior, the response in the anterior was substantially suppressed as compared with its response to a similar heat shock pulse to the entire body

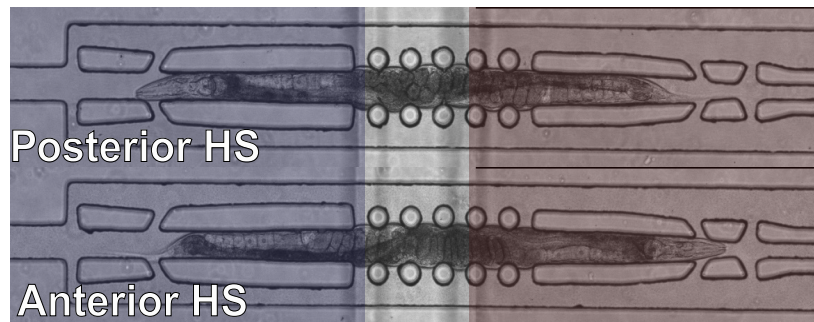


Figure 5.9: Variation on microfluidics device from Figure 2.1 with an additional water channel ; each channel's temperature can be maintained separately. Water channels are positioned over the body of the worm such that one part of the worm – either the anterior or the posterior – can be heat shocked (red) at a time, while the rest of the worm is maintained at room temperature (blue).

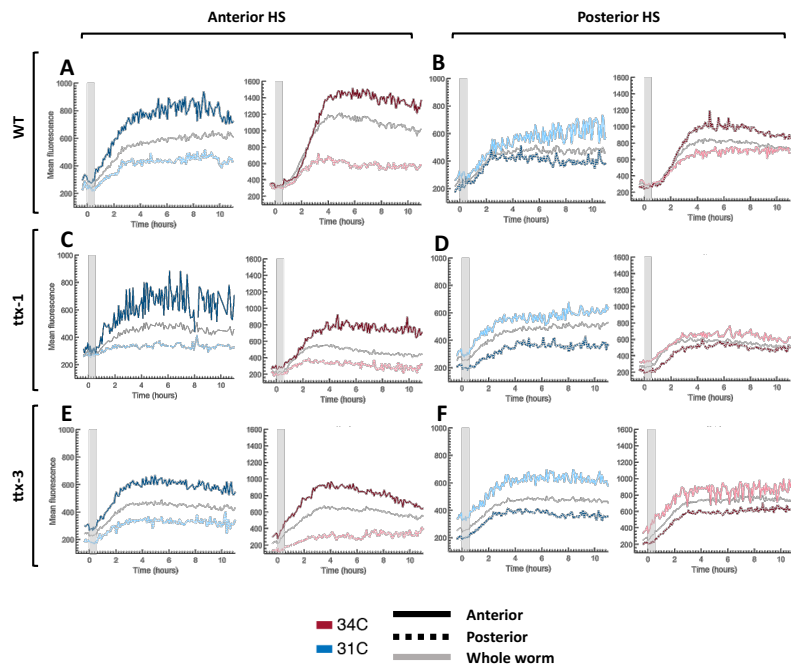


Figure 5.10: Example individual worm response curves for (A, B) wild type, (C, D) *ttx-1* (AFD) mutant worms, and (E, F) *ttx-3* (AIY) mutant worms subjected to a 30 minute (A, C, E) anterior-third-only or (B, D, F) posterior-third-only heat shock pulse at 31°C (blue) or 34°C (red). Grey line indicates mean fluorescence response averaged over the whole body. Darker line is mean fluorescence response averaged over the part of the body that was heat shocked. Lighter line is mean fluorescence response averaged over the part of the body that was kept at room temperature. Solid colored line is anterior; dashed colored line is posterior. Four columns going left to right are: (1) Heat shock anterior, 31°C; (2) Heat shock anterior, 34°C; (3) Heat shock posterior, 31°C; and (4) Heat shock posterior, 34°C.

of the worm. The response in the posterior, while even smaller, was still significant (Figure 5.11A). In both cases, the relationship between magnitude and rate of response was found in the linear regime of the same “universal” curve observed in Figures 3.5 and 3.13. Heat shock applied to the posterior half of the worm resulted in a corresponding pattern: a suppressed response in the posterior and an even more suppressed - but still significant - response in the anterior (Figure 5.11B). These results suggest that induction of the HSR in a cell results not only from the stress on its own proteome, but also from heat-triggered signaling from other cells.

5.4 SYSTEMIC REGULATION OF NON-LOCAL ACTIVATION

To test the involvement of the thermosensory neurons in this cell-non-autonomous response, I repeated these experiments with the two mutant strains from above. When either the AFD or AIY neuron was ablated, the response to a partial heat shock was highly suppressed in both the heat shocked and room temperature parts of the animals (Figure 5.11C - F). This suggests that the thermosensory neuronal circuit is required for cell-non-autonomous triggering of the HSR and that some properties of the cellular HSR depend on external signals that integrate information from multiple loci in the body.

I also previously observed that for a 34°C full-body heat shock pulse, the time lag to activation of the response is longer in the posterior than in the anterior and is prolonged in AFD-ablated worms. Both of these features were not observed when the heat shock pulse was applied to only part of the worm (Figure 5.12). The time lag observed following either a 31°C or 34°C heat shock pulse was

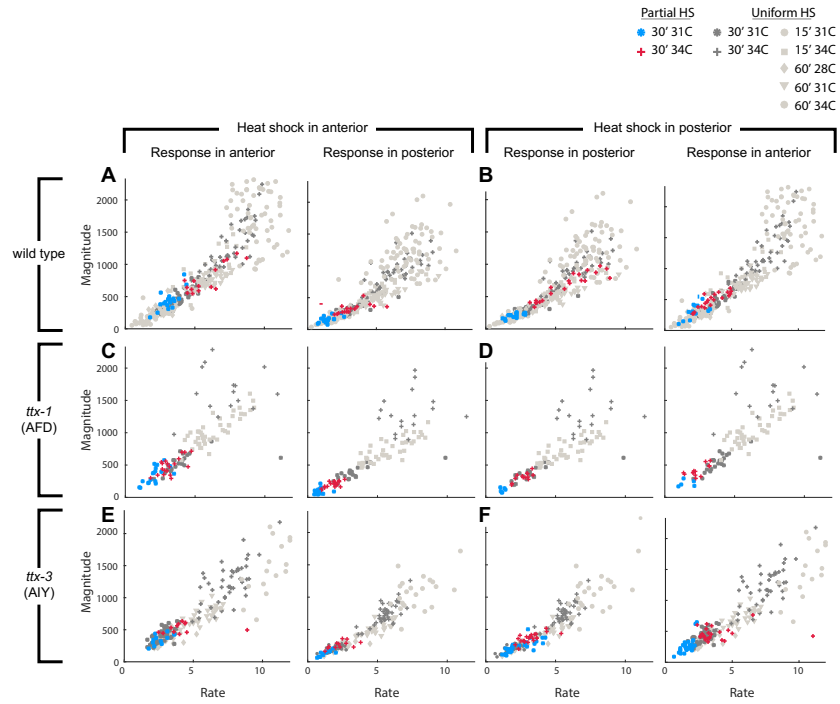


Figure 5.11: Magnitude of response (mean fluorescence) plotted versus rate of response (mean fluorescence/minutes) for (A,B) wild type worms, (C,D) *ttx-1* (AFD) mutants, and (E,F) *ttx-3* (AIY) mutants for single 30 minute heat shock pulse at 31°C (blue) or 34°C (red). Four columns from left to right are: (1) Heat shock anterior, measure anterior; (2) Heat shock anterior, measure posterior; (3) Heat shock posterior, measure posterior; (4) Heat shock posterior, measure anterior.

Points represent individual worms. Dark grey points are relevant wild type, *ttx-1*, or *ttx-3* data points for anterior or posterior third of a whole-body heat shocked worm. Light grey points are data points from other non-relevant wild type, *ttx-1*, or *ttx-3* experiments, heat shock pulse and duration as indicated in legend.

always around 30 - 40 minutes in both the anterior and the posterior, irrespective of temperature, the part of the body that was heat shocked, or the ablation of the thermosensory neurons. This data implicates the nervous system in regulating the signals integrating spatial information and systemic temperature sensation. Identifying these putative signals is an important question for a future study. In this context it may be relevant to note that several signaling systems have been shown recently to be involved in regulating activity of HSF-1 and its downstream targets, including insulin-like signaling³⁰, integrin signaling⁹⁴, and serotonin¹⁷⁷.

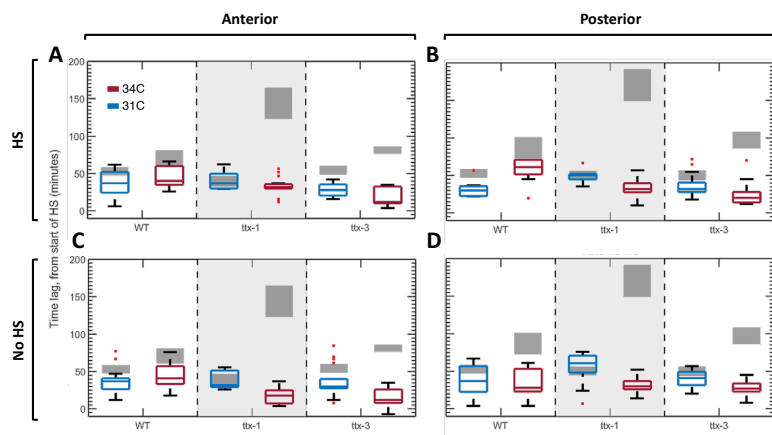


Figure 5.12: Time lag in minutes from start of heat shock pulse for wild type, *ttx-1* (AFD) mutant, and *ttx-3* (AIY) mutant worms subjected to a 30 minute anterior- or posterior-third-only heat shock pulse at 31°C (blue) or 34°C (red). Lag is for (A) heat shock anterior, measure anterior; (B) heat shock posterior, measure posterior; (C) heat shock posterior, measure anterior; and (D) heat shock anterior, measure posterior. Grey boxes represent comparable anterior or posterior data for equivalent worm strain, given a full body heat shock pulse at the same temperature and duration.

*You have evolved from worm to man, but much within
you is still worm.*

Friedrich Nietzsche

6

Conclusions and Outlook

IN THIS DISSERTATION, I demonstrated that a favorable combination of high-resolution experimental data and a minimal mathematical model can help in uncovering the essential determinants of the HSR dynamics in a multicellular organism. Because organisms must be able to continue functioning in both low and high stress conditions, stress response must be modulated and tuned to the

level of stress experienced. While a lot of single cell data on stress response exists, I aimed to close the gap in organismic stress response data.

The nematode *Caenorhabditis elegans* provided an ideal opportunity to study how multicellular organisms integrate external stimuli to coordinate disruptions to proteostasis on multiple levels. While it displays stress responses similar to those in mammals, it is a much simpler organism to work with. In addition, the use of microfluidics was instrumental for this study. The WormSpa device permitted the administration of precise heat shock pulses to either all or only part of the animal body, as well as longitudinal observation of individual animals before, during, and after the perturbation. By following individual animals, I was able to relate the rate and magnitude of response, which led me to observe the universal behavior that defines low intensity stress and the deviation from this behavior at higher intensities. Longitudinal imaging also allowed me to observe the progression of activation of the fluorescence signal along the intestine, from the anterior to the posterior end.

These quantitative observations drove the development of my minimal mathematical model of the cellular heat shock response, as well as the integration of my systemic data into its interpretation. I found two regimes, low- and high-intensity stress, that I observed both experimentally and in my model. I found that the classification of a heat shock pulse to one of these regimes depends both on its temperature and on its duration. Most importantly, these are global, dynamical properties of the response that the classification depends on, rather than on instantaneous properties, such have been identified in previous models' regimes, which relate to normal, acute, and chronic stress conditions^{164,169}.

While I have made great strides in the study of organismal regulation of the HSR, many avenues

remain open for exploration. These can be subdivided into smaller experiments that my research did not cover, updates to the experimental set-up, and questions that this research has brought to light.

SMALLER EXPERIMENTS

On a smaller scale, there are several experiments of interest that I did not get to during my research tenure. Firstly, an experiments of biological interest would be repeating the experiments with a *ttx-1;ttx-3* double mutant in order to further characterize the thermosensory neuronal circuit in regards to HSR. For completion's sake, it would also be relevant to repeat key experiments using a different manner of ablation of the AFD and AIY neurons to confirm the efficacy of the mutations I used. To this end, I generated mutants carrying KillerRed⁸⁷, a phototoxic fluorescent protein, selectively expressed in the AFD neurons and AIY interneurons for future use in the lab.

Another experiment of interest would be studying the effect of an extended heat shock (e.g. a five hour heat shock pulse) on both wild type and mutant worms. This would, by necessity, be at a low temperature, as a long duration, high temperature heat shock would have serious deleterious effects on the health of the worms, which would preclude any conclusions I could make about the regulation of the HSR. This experiment could provide insight into hormesis effects and also produce further experimental data that could be used to test my model. In addition, previous research has discussed how regulation becomes more unpredictable for prolonged activations (on the order of several hours or longer) of stress response pathways⁹⁸, and it would be interesting to see if I observe that in this system as well.

Lastly, while I measured the temperature in the water channel using a thermocouple during ex-

periments, I would also like to be able to make temperature measurements in the worm chambers. Previous lab members have tried using rhodamine B¹⁵⁰, a temperature-dependent fluorescent dye, to do this, but ran into problems with the dye being absorbed into the PDMS that made up the device. In addition, the dye was not easily sensitive within the range of temperatures I used in my experiments. I also tried using thermochromic liquid crystals¹⁵⁸, but had no luck with that method either due to complexity of imaging. Finding a way to conduct this temperature measurement would be useful to be able to draw further conclusions about the spatial response and regulation of the HSR within worms in the microfluidics device.

UPDATES TO EXPERIMENTAL SET-UP

The main updates to the experimental set-up that would be most helpful relate to the microfluidics device and its operation. Firstly, the water flow through the water channel(s) on the device was driven by gravity flow for all of my experiments. The addition of something that could control the water flow, such as a peristaltic pump, would introduce increased consistency of heat shock pulses between experiments. Also, despite the usage of a shaker to help maintain the suspension of the bacteria in the syringes through the experiment, some settling still occurred. The addition of a small stir bar into the syringe could help increase consistency of the concentration of bacterial suspension between experiments. Lastly, the loading of worms into the current microfluidics device design is more of a learned art form than a scientific protocol. As such, it would be useful to develop a design for which the loading of worms is easier (and perhaps which would hold more worms as well).

REMAINING QUESTIONS

One remaining question, which is likely the most simple to answer, is the source of the 30 - 40 minute time lag I observed in my experiments. One obvious source of this could be GFP maturation time, meaning that the heat shock proteins themselves are translated immediately during a heat shock. In order to disprove this, a western blot could be performed to detect the levels of both GFP and HSP-16.2 proteins during a heat shock pulse. Another considered source of at least part of the time lag is some other systemic control or signal. Other sources of interest include both the *daf-16* signaling pathway as well as serotonin signaling, both of which could be tested by running the experimental conditions with the most obvious time lags in *daf-16* and *tph-1* mutant backgrounds.

Lastly, many unexplored questions remain in regards to signaling at both the tissue and systemic levels. To this end, I generated a strain carrying *hsp-16.1p::mCherry*. HSP-16.1 is a small heat shock protein from the same family as HSP-16.2, however it is expressed mainly in the muscle cells of the worm, as opposed to the intestine. Running key experimental conditions with this strain could reveal if the dynamics of regulation remain the same in other tissues of the worm. As for possible sources of signaling, worms have also been shown to transmit certain proteins between tissues^{129,206}. In addition, movement of HSPs between cells has been detected in *Drosophila* and could play a role in *C. elegans* as well¹⁷⁶. Finally, microRNAs have also been shown to regulate heat stress in *C. elegans*¹²⁶ and remain another avenue of interest to explore in HSR regulation.



Extended Protocols

A.1 HEAT SHOCK EXPERIMENT

A.1.1 PRIOR TO DAY OF EXPERIMENT

1. *Synchronize worms.*

Pick 20 - 40 gravid worms of the strain you want to use in your experiment and transfer them to a fresh OP50 or RNAi plate for 30 - 90 minutes (the more worms, the less time you need). After the 30 - 90 minutes, remove the adult worms. You should be able to count at least 35 eggs on the plate, preferably more. Put the plate in the room in which you will be running

your experiment to ensure worms are growing at the temperature that will be considered “room temperature” during the experiment. (NOTE: It is important to put the plates at whatever will be considered “room temperature” for your experiments once you’ve removed the adults so the heat shock is really a heat shock with the expected temperature change.) Use the worms anywhere from around 60 - 70 hours after the adults are taken off the plate for them to be the right size for the device. Different strains will grow at different rates, and you may need to adjust the time for certain strains. Optimally, pick the worms sometime in the evening (4 PM - 8 PM) and run an experiment three days later in the morning or afternoon (10 AM - 4 PM) (“64 hr”).

2. *Grow bacteria to feed the worms.*

Take an aliquot of OP50 (see below) from the -80°C freezer and an aliquot of streptomycin from the -20°C freezer out to thaw. Make an overnight culture: 50 mL LB, 35 μL OP50, 150 μL Strep60 (see Appendix B) in an autoclaved 250 mL flask, taking care to flame the opening before replacing the aluminum foil top. Label the aluminum foil top and place on the shaker at 37°C overnight - no more than 16 hours. NOTES: Try to be consistent with your timing (within several hours) of how long you’re growing the bacteria at 37°C , as you don’t want to introduce inconsistencies into your experiment.

Make bacterial aliquots.

In a Falcon tube, add 2.5 mL (2500 μL) (from a culture grown only 8 - 12 hours) of OP50 to 0.5 mL (500 μL) of 80% - 100% glycerol and vortex to mix. Aliquot 36 μL of this mixture to individual PCR tubes, shaking the Falcon tube every 10 - 20 aliquots because the glycerol will settle. Store in the -80°C freezer.

3. *Calibrate the thermocouple (optional).*

Place the thermocouple tip in the 25°C incubator and shut the door on the wire. Wait 10 - 15 minutes, then check the readout of the thermocouple. Can also compare in a water bath - on a shelf somewhere in the experiment room, compare the readout from a glass thermometer to the thermocouple tip. To calibrate the in-line heater’s thermistor, after calibrating the thermocouple, place the thermocouple and thermistor in the same location and find the measurement difference.

A.1.2 DAY OF EXPERIMENT

1. *Remove OP₅₀ from incubator.*

- Take the flask of OP₅₀ out of the 37°C incubator and put it on the bench.

2. *Measure optical density (OD).*

- Make a diluted 1 mL sample for each overnight culture you made (900 μ L LB + 100 μ L overnight culture) in a 1.5 mL tube, vortexing to mix.
- While spinning down your overnight cultures to wash them (see next step), measure the OD₆₀₀ for each overnight culture from the 10:1 dilution.
- Turn the spectrophotometer on - the switch is on the back left side.
- Wait for the main screen to appear (nm, #), and make sure it's set to 600 nm, which is standard for measuring bacterial suspensions. If it isn't, adjust with the nm up and down buttons as needed.
- Fill a cuvette with 1 mL of the solution your bacteria is suspended in - this is your "blank". Place it in the cuvette holder, making sure not to touch the sides the light will pass through, and hit "blank / Abs".
- NOTE: The number next to 600 nm should now read "0" as you essentially just "zeroed" the reading.
- Remove the cuvette and fill a new cuvette with 1 mL of your sample. The number next to 600 nm is your OD. Anything around 2 and less can be trusted - ODs higher than that (3 and above) tend to be erroneous readings and can't be trusted; dilute your suspension 10:1 again and measure that instead. Remember to write down the OD, taking into account all dilutions performed.

3. *Wash and heat kill the OP₅₀.*

- Split each 50 mL of OP₅₀ into two 50 mL conicals - 22.5mL in each. Spin down at 4000 rpm for 10 minutes.

- Pour out the LB and refill with 20 mL of s-medium per conical. Vortex to resuspend. Spin down at 4000 rpm for 10 minutes (wash step).
- While spinning, calculate the amount of s-medium to add to get an $OD_{600} = 5$ concentration.
- Pour out the s-medium and refill both conicals with the calculated amount of s-medium. Vortex to resuspend. Combine each overnight culture into 1 conical – should have 15 - 20 mL in it.
- Place all conicals in the 65°C water bath for 35 minutes. After 35 minutes, take them out and allow them to cool at room temperatures. If needed, you can place them at 15°C to cool faster, just make sure it's not too cold when you load it.
- After it is cooled, connect a 30 mL syringe to a 0.5 μm syringe filter, pour the OP50 into the syringe, and filter the OP50 into one new (labeled) conical.

4. *Turn all parts of microscope set-up on.*

- If needed, restart the computer and log on with your RC account.
- Start up the microscope set-up if not already on - i.e. turn on the power strip, turn on the camera (ORCA, hold down the button to get a green light), turn on the microscope itself (button is on the body of the microscope behind the camera), and turn on the stage controller (power switch on the back right).
- NOTE: These three devices are the only ones that are separately turned on/off. Everything else is controlled through the power strip, though you can also turn everything off and keep the power strip on. Turn them off in reverse order after experiments.

5. *If necessary, filter the pluronic and clean the pluronic tubing.*

- If the pluronic is no longer transparent (you can see junk in it), it needs to be filtered with a 500 mL filtering system. If still clear and uncontaminated, skip this step.
- Connect the system to the vacuum with the enclosed white connector piece.
- Pour the pluronic in the top, close the lid, and turn the vacuum on.

- Turn the vacuum off when all the liquid has gone through the filter.
- Disconnect the vacuum, take the bottle of pluronic off the bottom, and cap it. Everything else (filtering portion) gets thrown out (in normal trash).
- Use a heated nail to poke two holes in the lid of the pluronic container.
- Run ethanol through both the input and output tubing for the pluronic to clear them.
- Before placing the end of the input tubing into the filtered pluronic, wipe it down well with ethanol and make sure you're wearing gloves - the pluronic will get contaminated much less quickly if you do this. Make sure the other end of the input tubing is blocked off so it will not start flowing.

6. *Optional: Measure the flow rate before the device.*

- Once the input pluronic tubing is connected to the pluronic source (but not connected to a device yet), unblock the end of the tubing and place it in a 15 mL conical. Measure how long it takes to fill 3 mL, then calculate the flow rate. Repeat as desired for a more accurate measurement.
- It can be best to let the pluronic flow for a bit before you measure the flow rate to make sure it is at steady state flow.
- If the flow is slower than expected (should be between 1.0 - 1.5 mL/min or faster), try:
 - Filtering the pluronic if you haven't yet.
 - Cleaning the tubing if you haven't yet. Can also soak in ethanol overnight.
 - Replace tubing as last resort.

7. *Pick a device and clean both the water and worm channels.*

- Choose a device that is clean and free of most defects. It's always best to use a new device, but used devices are fine if they have been cleaned and are still moderately clear.

- If this is a new device: run ethanol through the worm channels from the bottom outlet first, then the top outlet, then the bottom outlet again. If this is a used device, run ethanol through the worm channels from the bottom outlet, then let soak 5 minutes or longer.
- Run ethanol through the water channel from one end, then the other to test the flow. You should see ethanol coming out of all of the holes punched in the water channel, more from the closer holes, but still some from the other end of the channel.
- After soaking (if it was needed), use water, s-medium, or M9 to rinse the ethanol out of the worm channels. Rinse at least twice.
- NOTE: Bleach is not good for the PDMS - it messes up the devices - so don't use bleach unless utterly necessary. Ethanol is also not the best, so always rinse the device after flowing ethanol through it. If bleach *is* used, it causes metal to rust, so thoroughly clean and take apart the syringe you're using with the bleach after every usage to avoid rust particles in the bleach. Use new bleach every time.

8. *Set up the tubing for the water channel, including the in-line heater.*

- Flow ethanol through the two small connector tubings before attaching them to the device.
- Place the small connector tubings into the proper holes in the water channel for the device you are using and block the other two - one of these blocks will be removed to place the thermocouple.
- Try flowing water from the input connector tubing and see that there are no impediments to the flow out of the output connector tubing. This should be easy - you shouldn't have to push very hard to get it flowing.
- Right before moving the device to the microscope, wipe the bottom of the slide (what you will be imaging through) off with ethanol and a Kimwipe, then dry it.
- Move to the microscope - place the device on the stage and go to "live imaging".
- Try to align the device so that the worm channels are straight in the images - the edge of a worm channel should align with the edge of the imaging field. When you've got this aligned as much as possible, carefully tape the device onto the stage.

- Connect the output tubing to the small connector tubing on the output side of the device and unblock the flow.
- Carefully place in line heater over the small connector tubing on the input side of the device and secure it in the holder. Turn the in line heater on, but keep it at room temperature.
- Connect the input tubing to the small connector tubing on the input side of the device - you should see a flow start.

9. *Remove bubbles from the water channel and place the thermocouple.*

- Attach a syringe to the far end of the output tubing and use it to clear bubbles out of the water channel (they mess up imaging). You may also need to press on the water channel some to get some of the bubbles to move - only use a *very* light pressure.
- Remove the block from the thermocouple hole and place the thermocouple tip in the device. Bubbles may collect around the tip of the thermocouple - try to move the tip around gently to get rid of them. Optimally, you should be able to see the tip of the thermocouple in the water channel using the microscope, but it can be hard to see.
- Let the pluronic flow for around 10 minutes to clear any remaining bubbles, then block the flow.

10. *Optional: Measure the flow rate after the device.*

- If desired, before you block the flow, use the same techniques as before to measure the flow rate after the device.
- Because of the added friction of the device and output tubing, it will be slower than you measured before the device - you are still aiming for 1.0 - 1.5 mL/min.
- This step isn't super necessary - as long as you see a steady drip from the output tubing, you should be okay to just run your experiment.

11. *Clean the worm syringes and tubing.*

- Keeping the tubing connected, rinse the worm syringes with ethanol twice - around 2 - 3 mL per rinse each time. After getting the ethanol into the syringe, pull it out of the tubing by pulling air into the syringe, then disconnect the syringe tip and push the ethanol out of the syringe.
- Fill the syringes completely with ethanol. When they are halfway filled, you will need to disconnect the syringe tip and clear extra air out of the syringe. At this point, use an ethanol spray bottle to get ethanol in the connecting region between the tip and syringe as well. Reconnecting, leave the ethanol in the tubing as well and let the tubing and syringes soak for at least 10 minutes.
- Rinse three times with water - making sure to pull all liquids up through the tubing and empty the syringe itself to clear the ethanol more thoroughly - again, 2 - 3 mL each time.

12. *Fill the worm syringes with OP₅₀ and connect them to the device.*

- Fill the output syringe first, pulling 3 mL of OP₅₀ into the syringe. Turn the syringe upside down and tap/flick it with your finger until all (or almost all) of the bubbles are at the tip of the syringe. Placing the end of the tubing into a trash conical, push the bubbles out of the syringe - you should end up with about 2 mL of filtered OP₅₀ in the output syringe.
- Fill the input syringe next, pulling 5 mL of OP₅₀ into the syringe, then clearing the bubbles in the same way. There will be more bubbles in this syringe since the tubing is longer. Fill slightly more than 10 mL - to the base of the rectangle on the outside of the syringe.
- Disconnect the tip from the input syringe, screw on a 0.5 μ m syringe filter, and attach 4 screwed-together luer tips to the end of the filter.
- Push OP₅₀ out of the input syringe until you see it dripping from the end of the luer tips - there should be no bubbles in the input syringe or the filter (hopefully) at this point.
- Taking care not to introduce bubbles into the input syringe tip, connect the luers to the syringe tip. You should have about 10 mL of OP₅₀ left in the input syringe.

- Moving to the device, connect the output syringe first. After attached, push some OP₅₀ through with fairly high pressure to try to get rid of some of the bubbles in the device. Leave a small drop of OP₅₀ at the input hole.
- Making sure there is a drop of OP₅₀ on the tip of the input syringe, attach it to the device as well - try to avoid introducing bubbles when you do this.
- Place the input syringe in the pump system.
- Turn the pump on at 300 $\mu\text{L}/\text{min}$ and watch through the eyepiece until you see all of the bubbles are cleared out of the worm channels, then pause the pump.

13. *Pick and load worms into the device.*

- Pipette 100 μL of the remaining filtered OP₅₀ into a small, non-stick tube and pick 32 - 35 worms (for a 32-channel device) into the tube.
- Moving to the microscope, take the input syringe out of the pump, then disconnect the OP₅₀ input tubing and small connector tubing (attached to the device), leaving the metal connecting piece on the side of the small connector tubing.
- Place the end of the input tubing into the non-stick tube. Use the input syringe to fill the tube with OP₅₀, then pull it almost all up - repeat this 5 times, then pull all of it up into the input tubing.
- Making sure there are no bubbles at the end of the input tubing, reconnect it to the small connector tubing, and thus to the device.
- Replace the input syringe onto the pump, then run it at 300 $\mu\text{L}/\text{min}$. Once you see the first worm enter the device, change to 100 - 150 $\mu\text{L}/\text{min}$.
- Continuing to look through the microscope eyepiece, track the worms as they enter the device. Give the worms 5 - 10 minutes to get in on their own (unless two try to go into the same channel, then try to use the output syringe to gently push one back out, with the pump still running), then turn the pumps off and play with the input and output syringes to get the worms loaded as well as possible. There is no real technique here, it is more of an art form...
- Once they are in the channels as good as it's going to get, replace the input syringe and restart the pump at 5 $\mu\text{L}/\text{min}$ (for 20 minutes) with a pulse of 200 $\mu\text{L}/\text{min}$ (lasting 10 seconds).

14. *Two hour de-stress break for the worms.*

- After loading, give the worms 2 hours to de-stress in the device. This is generally a good break to do anything else you need to do in lab or take a lunch break!
- It's generally good to start your experimental log after the worms have been loaded/while they are de-stressing, e.g. general information about imaging conditions, when the worms were synchronized, loading notes, etc.
- If you're on a tight schedule, make sure you start setting up the imaging conditions about 1.5 hours into this break!! Open the water flow at the same time that you start setting imaging conditions.

15. *Set up imaging conditions for the experiment.*

- Zen should already be open, but if it isn't - use the Zen icon on the desktop and choose "Zen PRO" when it opens. You should always have all of the microscope parts turned on before opening this.
- Check that the settings are correct for your experiment:
 - (a) Correct light paths (check under Light Path Settings, set under Light Path → Settings Editor). Always want "After (blank)" to be "Closed".
 - (b) ROI: 400 x 1344, centered
 - (c) Binning: 2 x 2
 - (d) Model Specific: mirror at -45
 - (e) Focus strategy: local updated by definite focus
 - (f) GFP: 50 ms exposure
 - (g) TL Phase: 10 ms exposure
 - (h) Experiment Designer: don't use
 - (i) Time Series: generally 12 hours, time points every 5 minutes for the start of your experiment.
 - (j) Auto Save: turn on, select correct folder, correct name (don't use automatic sub-folder!)

- *Tiles:* You want to define 3 corners of the device so Kyung Suk's MATLAB program can determine the tiles' locations. The program is called EditZeissTiles. Find the top of the last worm channel, focus, then "add single position." Move to the bottom of that worm channel, making sure there is some overlap in the images, then "add single position." Find the top of the worm channel on the other side of the device and "add single position." File → Tiles → Export Tiles → tiles.czsh → Yes, replace.
- Moving to MATLAB, call up EditZeissTiles. For a typical experiment, your entries are 400, 1344, 10. (For the size of the image and magnification - change as needed for other experiments.)
- Enter "o" and select the first point you added. Enter "a" and select the second point you added, then 2. Enter "d" and select the third point you added, then 32. Enter "c." These can all be adjusted for different experimental set-ups.
- Back in Zen PRO... File → Tiles → Import Tiles → tiles_edit.czsh.
- Delete all single positions, but leave all of the position arrays.
- Making sure only the TL Phase channel is selected, go to Tiles → Advanced Setup → Start Preview.
- Use the preview images to delete any arrays that you don't want (don't have a worm, have poorly loaded worms, etc.), then close out of the picture without saving.
- In your log, note all of the array positions with worms and how the worms are doing - egg laying, pumping, movement, etc.
- Place a sign on the door, turn the lights out, and close the curtain in 256.10 (not in 256.20 - traps heat near the microscope).
- Make sure both TL Phase and your fluorescence channel of interest are both checked under Channels.
- Turn on live image, go to each array position in TL Phase, and check that the focus looks okay for each worm.
- Stop live image and start the experiment!

16. *Run the experiment.*

- Run 3 - 4 timepoints to get a “pre-HS” set of data. You can run this longer if you have the time, but it isn’t necessary.
- After timepoint 4, pause the experiment and set it to take pictures every 2 minutes, then restart the experiment. Turn the in-line heater “set point” up to the appropriate temperature for the experiment you are running. Log the temperature changes in your experimental log, i.e. how long it takes to change each degree. When the appropriate temperature is reached, the heat shock “starts” - make note of this time.
- Watch to make sure the temperature is stable during the heat shock and adjust the set point on the in-line heater as necessary to keep it stable, keeping track of any changes.
- At the end of the heat shock, pause the experiment and turn the lights on.
- Working fast, disconnect the input water tubing and block it, then remove the in-line heater and turn it off.
- Reconnect the input water tubing and use a syringe at the end of the output water tubing to clear bubbles from the water channel, as when you first set up the water channel.
- Once all bubbles are cleared, turn the lights off and restart the experiment. Make sure you’re logging the time it takes as the temperature drops back down to RT.
- Around 5 minutes after the temperature is stable at RT (or longer), pause the experiment again and turn on the lights.
- Close the water flow. Reset the experiment to take pictures every 3 - 5 minutes, as desired.
- Stop the pumping and remove the input syringe, refilling it to 10 mL. To do this, disconnect it between the syringe and the filter, making sure to leave OP50 in the input to the filter so you don’t get bubbles when you reattach. Replace the input syringe, then remove the output syringe and empty it. Replace the output syringe, then restart the pump. NOTE: You can replace the OP50 later in the day if you will be around longer, it doesn’t have to be immediately following the heat shock.
- Turn the lights back off and restart the experiment.
- You can watch longer if you want, but the experiment should be good to run without you now.

17. *Post-experiment, clean up.*

- When the experiment stops, clean all syringes, tubing, and the device.
- Check on your worms before you do anything else - pumping, movement, number of eggs in body (and developmental stage they're at), eggs they laid during the experiment, etc. Log this!
- Disconnect the input and output water tubing and close both of them off.
- Untape the device, remove the input OP50 syringe from pump, remove the thermocouple from the device, then move the device and all remaining attachments to your bench.
- Remove the OP50 syringes and tubing - do at least 1 ethanol rinse and then leave ethanol in the syringe to soak. The filter can be thrown out, but the luer connectors should be rinsed with ethanol and kept to reuse.
- Clear the worm channels in the device - rinse with ethanol once, let soak 5 minutes, rinse with ethanol again, then rinse with water two times. You should see the most worms come out of the device with the water rinse. Try to get as many worms as possible out of the device so you can reuse it.
- Also rinse the water channel in the device and the small connector tubings with ethanol. You can leave the tubings to soak until the next experiment, but rinse the ethanol out of the water channel with water.

A.2 MICROFLUIDICS DEVICE CONSTRUCTION

A.2.1 SINGLE LAYER DEVICES

1. *Design your device and create the mask design.*
 - (a) Create a design for the microfluidics device using CAD software. Kopito recommends DraftSight - a free program downloadable online. Multiple designs are made and placed on the same page to save money and time for an order.

- (b) The designs get printed by a special printing company. Once the sheet with the designs has been received, make sure it gets opened in the cleanroom ONLY. Cut the sheet into separate devices, and label the “top” of the sheet with a sharpie arrow for future reference.

2. *Spin coating.*

- (a) Take a silicon wafer out and test it in the spinner on the program you are going to use. To create your own program, go to program 5 and press the “mode” button. Use the left and right arrows to switch between parameters and the up and down arrows to change parameters. Press enter after you have changed a parameter to save the change. Make sure all steps after the ones you want are null. Press mode to stop editing.
- (b) The main error you might need to troubleshoot is “check vacuum”. Try cleaning the center piece (chuck and axle) with some PGMEA on a cleanroom wipe. Note, after you get an error message, you have to press “clear error” before you can try running the program again.
- (c) Add about a quarter-sized dollop of the appropriate SU8 on top of the wafer. Try to spread it a little bit by tilting the wafer before you run the program to make spreading more even. For 50 μm thickness, use SU8 3050 and spin coat on program 3 (preset). This is the thickness of the worm channels in normal devices.
- (d) If you get uneven spin coating, there are several possibilities. You may not have used enough SU8, and also make sure to partially coat it as specified above to help. A wafer can be spun more than once if needed (and more SU8 added).

3. *Soft bake.*

- (a) Remove the wafer and place it on the 65°C hot plate for the first step. For SU8 3050, leave it there for 2 minutes.
- (b) Move the wafer to the 95°C hotplate. For SU8 3050, leave it there for 20 minutes. For other SU8s or thicknesses, refer to the chart for the specific soft baking times at both temperatures.

4. *Exposure.*

- (a) Remove the wafer from the 95°C hotplate and let it cool to room temperature.
- (b) Sign in to the log book for the UV machine - make sure to sign out at the end as well, and turn the machine off if you are the last user.
- (c) Place the wafer on the metal piece, place the mask on top (making sure the side with the arrow is facing up), then the glass, then the top, which gets tightened on. Make sure not to over-tighten the screws, as this can cause the glass to break (you should see a bit of rainbow pattern; as soon as you see some, stop tightening). Irradiate for about 30 - 32 seconds for the regular 50 μm thick cover of SU8 3050. Cover the front of the machine with one of the placemats while the machine is running.
- (d) To determine the time you need to irradiate, take the exposure energy you want (from the chart) and divide by the energy the machine is producing (as currently measured). For SU8 3050, 50 μm thickness, we want 150 - 250 mJ/cm².
- (e) To run the machine, there are two on switches, one on the main machine and one on the smaller machine with the timer. If you're turning it on, you should also press the start button on the main machine and wait for the V/I values to stabilize (record these in the logbook). Adjust the exposure time, and press "expose" to run the machine - it will stop by itself.

5. *Post exposure bake.*

- (a) Remove the irradiated wafer from the device, and place it on the 65°C hotplate again (around 1 minute for SU8 3050, 50 μm).
- (b) Move it to the 95°C hotplate for 4 minutes for SU8 3050, 50 μm.
- (c) If wrinkles form, try cooling the wafer on the tabletop after the prescribed time, then placing it back on the hot plate for a few minutes. Repeat this a couple times and that supposedly will help with wrinkles.

6. *Develop.*

- (a) Remove the wafer from the hotplate and allow it to cool to room temperature. Rinse the wafer with PGMEA and place it in a glass container (smallest size) filled with PGMEA.
 - (b) Sonicate the container for 10 - 20 seconds (place the entire glass container in the mesh basket, press the start button, time it yourself, then press stop and remove it. You will see bubbles in the PGMEA if the sonicator is working properly (and you should hear the machine).
 - (c) Move the glass container to the shaker and leave it there for half of the prescribed amount of time. For SU8 3050, 50 μm , try 8 minutes. After you've waited the appropriate time, remove the wafer, rinse with PGMEA, replace the PGMEA in the glass container and repeat the sonication and shaker time.
 - (d) After waiting the second half of the prescribed amount of time, remove the wafer, rinse with PGMEA, rinse with isopropanol, and dry it with the air gun. Check under the microscope light to see if it looks clean. You don't want to see any sort of film or rainbow effect on the surface. If it is not clean, replace the PGMEA and repeat the sonication and shaker steps until the wafer appears clean.
 - (e) Note - it's important to be patient and repeat the development process as many times as possible to clean the wafer properly.
7. *When it is clean and dry, label the bottom of the wafer with a sharpie. Place it in a labeled petri dish with your initials, the date, and a description.*
8. *Silanation.*
- (a) In the lab, mix HFE and the appropriate silane in a 1000 : 1 ratio (e.g. 40 mL HFE, 40 μL silane) in a 50 mL tube. You will need about 8 - 10 mL of this mixture for each wafer you are coating. Close all lids immediately, as these chemicals evaporate quickly. Reseal the lids of the HFE and silane stock containers with parafilm. Shake the tube to mix and seal its lid with parafilm as well while you transport it to the cleanroom.
 - (b) In the cleanroom, working in the hood to the left of the solvent hood, pour about 10 mL of the mixture into each petri dish with the silicon wafer and cover the dishes. Let them sit for 10 minutes.

- (c) Remove each wafer and rinse it immediately with isopropanol into a glass waste container from the solvent hood. Air dry the wafer with an air gun.
- (d) Make sure the petri dish is dry. The solution will evaporate out, but it may not be quick enough, so you may have to dry it manually. Then, replace the wafers in their petri dishes.
- (e) The isopropanol used to rinse the wafers can be put in with the regular solvent waste (assuming there is a very small amount of HFE + silane in it). If there is any HFE + silane that didn't evaporate in the petri dishes, make sure to dispose of it properly.

9. *Adding PDMS.*

- (a) Mix a 10 : 1 ratio of bigger bottle : smaller bottle (of the 2 PDMS components) in one of the provided cups. You can mix it by hand with the provided stirrers. You will want about 30 g per petri dish. Pour it into the dishes once mixed, using the scale to make sure you're pouring an appropriate amount into each dish.
- (b) The PDMS can be degassed before or after filling the petri dishes, but regardless, it will always need to be degassed after filling the petri dishes, as pouring creates more bubbles. To do this, place the cup or petri dish into the aerator. To de-vacuum the chamber, turn the arrows so they are facing the chamber and one of the section of the PVC open to the outside air. Make sure to do this very slowly, or it will mess up stuff inside the chamber. To re-create the vacuum, turn the arrows to face the vacuum tube and the aerator. Leave it in the aerator until most of the bubbles are gone.
- (c) Note: if you mix the PDMS first thing when you get to the cleanroom and place the cup in the chamber to degas, you won't have to wait as long for the petri dishes to degas.
- (d) After degassing, use the football-shaped device to remove any bubbles on the surface, then place the devices in the oven to cure at 65°C. Cure them for 2 hours minimum, 12 hours maximum.

10. *Once the devices are baking, make sure to clean up. Replace the spinner foil, place all liquid waste in the appropriate disposal container beneath the solvent bench, dump any other waste in the appropriate trash can, and put any tools back in their proper places.*

11. *Prepping PDMS pieces for plasma.*

- (a) Remove the PDMS-filled petri dish from the oven and let it cool, then cut out the baked PDMS devices using the scalpel tool. Cut along the center between the two devices first, then all other sides. Make sure to leave enough space between the device and the edges so you can get a good seal to a slide. Also, it's best to make only one cut, so get the blade all the way down to the wafer and try to pull smoothly and cut straight.
- (b) Using the tweezers (preferably a pair with flat sides and no holes), slide one side into the cuts you have made and slowly leverage the device off of the mask (it peels off). GO SLOWLY! It's *very* easy to break or injure the device at this step or crack the wafer if you press too hard with the tweezers, so be careful.
- (c) Place the device channel side up on a mat and punch holes in the proper places (worm chamber inlets and outlets). Use the 0.75 punch, and make sure you are punching from the side that will be touching the glass. To use the punch properly, punch through the PDMS, twist the punch, push out the piece of PDMS, and then remove the punch. Cover the channels with tape once this is done.
- (d) Press firmly (but carefully) on the tape, then peel it off to try to remove any small bits of PDMS remaining. Repeat this at least once, until you are satisfied with the cleanliness of the channels.
- (e) Rinse the device with isopropanol (don't put it in an isopropanol bath!). Air dry it with the air gun, then re-cover the channels with tape and place it in the oven in a new (labeled!) petri dish to ensure all the isopropanol is evaporated.
- (f) The empty petri dishes with good masks can be refilled with PDMS at this point (10 g) to make more devices and put back into the oven to bake after they are degassed.

12. *Clean the slides and/or coverslips.*

- (a) Place each slide or coverslip in an isopropanol bath. Let them sit for a bit, then remove them one by one and wipe them down with a cleanroom wipe. Hold each slide up to the light while doing this to check how clean it is. It is important that it is as clean as possible to get the best possible bond.

- (b) Move each slide/cover slip to an ethanol bath for a bit.
- (c) Remove and air dry each slide/cover slip, then wrap it in aluminum foil and place it in the oven for around 5 minutes to ensure all the isopropanol evaporates.

13. *Plasma prep.*

- (a) Place one slide/cover slip and one device in the plasma machine at a time, with the sides you will be sticking together facing up.
- (b) Instructions for running the machine will differ depending on which machine you are using, but you want to first ensure you have the necessary vacuum, then start the oxygen flow and make sure it is stable. Then set the machine for the proper power and time (as recommended in the machine instructions and possibly in the log book) and run it for the specified time.
- (c) After the pump is stopped and the chamber is ventilated, you can remove the pieces.

14. *Construct the device.*

- (a) Line up the slide on the lined mat (plasma-exposed side up), and place the channel side of the device (also the plasma-exposed side) against the slide. Press firmly to seal the two together; you should be able to see if it is sealing or not. Make sure there is contact at all points between the slide and the PDMS. Again, the two sides that were facing up in the plasma machine should be the two sides touching. For coverslips, it's easier to place the coverslip on the device; for slides, place the device on the slide. It's also important to have the device straight so that it will be straight when you image it in an experiment.
- (b) Place each device (now attached to slide or coverslip) back into its respective petri dish and bake for 2 - 12 hours at 65°C. After baking, they can be taken back to the lab for use.

A.2.2 TWO LAYER DEVICES

1. *General notes.*

- (a) Water channels have their own sets of masks.
- (b) When making the masks on the wafers, it is key to have as much area that will not be exposed to the UV radiation around the design as possible, otherwise when you try to cut your device out, you may have multiple thicknesses of PDMS, which will make sealing things difficult.
- (c) You will only use one set of devices per wafer, instead of two (as you do for single layer devices). You can use spare pieces of the black paper that the designs are printed on to cover up the second device to make sure you can cut a large enough chunk of PDMS out with the water channel to cover all of the worm chamber parts.

2. *Making masks.*

- (a) The general procedure for making masks is the same - the same steps in the same order - however, some of the details are different.
- (b) The mask for the bottom layer of the device (the one with the worm chambers that will touch the glass slide) is made as per usual. Use SU8 3050 (to make 500nm thick channels), and the time and instructions for soft bake, exposure, post exposure bake, and developing are all the same.
- (c) For the mask for the top layer (with the water channels), use SU8 2150 to make a 250 - 300 μm thick layer. To spin coat, you can either use program two or make a new program that goes to 500 rpm for 5 seconds, then 1800 rpm for 30 seconds. Soft bake the wafer for 8 minutes, then 75 minutes at 65°C and 95°C respectively. Irradiate for 45 seconds. Post exposure bake the wafer for 5 minutes and 25 minutes respectively. Develop the wafer in 20 minute intervals and use the sonicator for 20 seconds (the sonicator is especially helpful for thicker layers like this).
- (d) The SU8 doesn't always spin coat as evenly for the water channel layer. It is essential to make sure the hot plates are even. Also, ripples form in the SU8 during the post exposure bake usually around the outer edges, which is also why it is important to have enough unexposed space around the water channels.

3. *Once the masks have been made, place each of them in a labeled petri dish and soak them in silane, as before.*
4. *Adding PDMS.*
 - (a) The top layer with the water channel can be made the usual way. Mix PDMS in a 10 : 1 ratio, pour about 30 mL into the petri dish, and let it cure 2 - 12 hours.
 - (b) When cured, cut the device out and punch four holes evenly spaced across the water channel. The two outer holes are labeled on the design; the spacing of the other two is estimated. The holes will be located on either side of the three devices when the layers are combined. Make sure these holes are punched at this step to give the air bubbles somewhere to go when you bond the two layers of PDMS together.
 - (c) After punching holes, clean this chunk of PDMS the usual way - tape, isopropanol, air gun, oven to evaporate remaining isopropanol.
 - (d) The top layer with the worm chambers has different steps:
 - (i) Mix PDMS in the regular ratio (10 : 1) (about 10 g per wafer) and degas it. All air bubbles must be gone when you pour this, so it is useful to make it in advance.
 - (ii) Make a new spin coat program - 150 rpm for 5 seconds, then 300 rpm for 60 seconds. Spin coat the degassed 10 : 1 PDMS on the wafer with this program and bake for 2 - 12 hours.
 - (iii) After this has cured, mix PDMS in the ratio 5 : 1 and degas it (again, do in advance if possible).
 - (iv) Make a new spin coat program - 375 rpm for 5 seconds, then 750 rpm for 60 seconds. Spin coat the degassed PDMS on the wafer with this program and bake for 30 - 60 minutes (45 is good). You can check with a scalpel when you take it out; the PDMS should be cured, but very soft.
 - (v) NOTE: The keys to having no bubbles in the PDMS when you pour it onto the wafer are to let the PDMS degas for an excessively long period of time and to pour it very slowly.
5. *First plasma bonding - PDMS/PDMS.*

- (a) At this point, if you stuck the top layer on the bottom layer, they should stick together without plasma because some of the PDMS is only partially cured. However, to be on the safe side we plasma them as well.
- (b) The water channel layer should be just a chunk of PDMS that has been cleaned with isopropanol and put in the oven for a bit. The worm channel layer is still on the wafer.
- (c) Plasma them as per the machine instructions (for PDMS/PDMS) and stick the two layers of PDMS together - there are marks on the masks that should aid with alignment. This is a tricky step, especially for the devices with two water channels; there is no real trick, just be very careful with the alignment.
- (d) Cure this (still on the wafer) for 2 - 12 hours.

6. *Second plasma bonding - PDMS/glass.*

- (a) Use a scalpel to cut the device off the wafer. Cut around the chunk of PDMS on top (this is where it is important that the piece is big enough, so you do not miss pieces of the worm chambers or have them too close to the edge of the PDMS). Be very careful when peeling the two layers off of the wafer; they can get stuck and you can very easily damage the device (and make the wafer unusable in the future). If the two layers of PDMS did not bond well, it will be obvious here, as they will start to separate when you try to peel the device off. No real tricks here either, just be very careful.
- (b) Punch the regular holes for the worm channels (inlet/outlet). Clean the device via the same procedure - tape, isopropanol, air gun, oven. Keep it in the oven slightly longer, as isopropanol gets stuck in the water channel and will not all come out with the air gun.
- (c) Clean slides as per the usual procedure as well. Plasma bond the slide and device together (regular PDMS/glass bonding). Again, it is important to try to align the device straight on the slide. Bake 2 - 12 hours.

7. *To reuse the masks, the water channel mask gets more PDMS poured in as usual. Peel the excess PDMS off the worm chamber mask and you can spin coat PDMS on again.*

A.3 C. ELEGANS GENETICS

A.3.1 CROSSING STRAINS

Parent generation (P₀):

Take 15 male worms of your first strain and 3 young L₄s of your second strain and place them on a plate together. Make sure to have a transfer plate for the males so as to not accidentally transfer hermaphrodites of the first strain to your P₀ plate. If you accidentally transfer hermaphrodites of the first strain, you may be picking self progeny when you pick the F₁s. When planning your cross, if you can use males from a strain that has a marker, you will know the cross has been started successfully if you see progeny with the marker; if you don't see progeny with the marker, it hasn't worked. It's beneficial to do two P₀ plates, in case one doesn't work, if you have enough males to do that.

First generation (F₁):

When the P₀ plates have a moderate number of worms on them (preferably before they are close to starving out because the plates become crowded and harder to pick a single worm from), pick five worms from one of the P₀ plates, putting each worm on a separate plate. Label these plates. Assuming the cross worked (check with a marker, if possible), these worms should all be heterozygous for the genetic qualities of both strains you are using.

Second generation (F₂):

When the F₁ plates have a moderate number of worms on them (defined the same way), pick one F₁ plate that looks the best and pick 50 worms, each to a separate plate. It is important to avoid

stragglers when transferring these as well, since you want to know the progeny came from only 1 parent worm. Each of these 50 worms you picked has the possibility of being homozygous for the traits you are interested in.

To test if these worms are homozygous for a genetic characteristic you want:

- If the characteristic includes a marker, all progeny on the plate should have the marker (if the parent is homozygous, all of the progeny will be homozygous as well. If heterozygous, the progeny will be a mix.)
- If the characteristic doesn't include a marker, PCR (polymerase chain reaction) can be used to confirm the parent worm's genetics. There are two options – PCR the parent worm only from each plate, or PCR multiple worms from each plate. If you PCR only the parent worm, you have to be careful not to let the progeny grow too much or you will be unable to distinguish the parent. You can also just PCR multiple worms - again, if the parent was homozygous, all progeny should be as well, which should be reflected in your PCR results.
- Once you have determined which plates are homozygous for the traits you're interested in, dump the rest and maintain those plates.
- It's best to double check for traits you PCRed for as well (and check that all progeny have the expected markers). Maintain the plates you believe are homozygous, then pick one plate and pick 10 worms from it to separate plates. Pick one of those plates and PCR five worms from it - if everything comes out homozygous again, you have successfully crossed your strains!

NOTE: Never use just one L4 for the Po plate - you might damage it when you're transferring it.

Also, a higher number of males is always better because of all the self-progeny. Lastly, always write out all details of the cross (including the expected genetics at each step) before starting your cross.

A.3.2 GENERATING MALES

Via N₂ males:

Pick 15 N₂ males (generated by heat shock, see below). Transfer them to an empty plate before you transfer them to the cross plate to avoid contamination by N₂ hermaphrodites. Pick 3 young L₄s of the strain you want to generate males for. Put the 3 L₄s and 15 males on a plate together, and check for males in several days among the progeny. All progeny should be heterozygous for wild type and the strain you are interested in. You can remate these males with hermaphrodites of your strain of interest to produce homozygous male worms for your strain of interest, but you will have to PCR to check which progeny are homozygous.

Via heat shock:

This is the preferred way to generate males. Make 6 - 10 plates with 5 late L₄s per plate of the strain you want to generate males for. Heat shock the plates 4 - 6 hours at 31°C, then keep the plates at room temperature when they come out (don't stick them immediately into 15°C). Check the plates several days later when there's a moderate number of progeny, and there should be at least 1-2 males/plate. It's important to propagate your males before you do crosses with them to guarantee that you'll have all the males you need for your cross (this saves time in the long run).

A.3.3 PCR (POLYMERASE CHAIN REACTION) AND GEL ELECTROPHORESIS TO CONFIRM GENETICS

1. Check that there's enough dry ice in the -80°C freezer; if not, go buy more.
2. Unfreeze lysis buffer (LB) and proteinase K (PK), which are located in Rack A of the -80°C freezer. They should be thawed on ice. It is convenient to store LB in 8 µL aliquots and PK in 6 µL aliquots because you want 8 µL of LB to every 2 µL of PK. You need 3 µL of the mixed solution per worm/tube that you'll be PCRing, so calculate how much you will need accordingly, and always make a bit more of the mixture than you need - it's easiest to round

up to the nearest factor of 10 ($8 \mu\text{L LB} : 2 \mu\text{L PK} = 10 \mu\text{L total}$).

3. When thawed, mix the proper amounts of LB and PK together (in the ratio described above in a 1.5 mL tube). Vortex the tube to mix it, then spin it down. Store this on ice as well.
4. Label your PCR sample tubes, then pipette $3 \mu\text{L}$ of the LB/PK mix into each.
5. Add 1 worm (or more, depending on your protocol) to each tube, dipping the tip of your pick into the solution to get the worms off of it. Make sure you can see the worm(s) in the solution, and that they're not stuck to the pick or on the side of the tube.
6. Place the sample tubes (in a holder) on dry ice. Close the Styrofoam container and leave the samples there for at least 5 minutes to fully freeze.
7. After 5 minutes, check that they are all frozen, then move container with the dry ice over to the PCR machine and start the SWPCR (single worm PCR) program. When the lid reaches 103°C , take the tubes off the dry ice, place them in the PCR machine, and let the cycle run. NOTE: The standard SWPCR cycle is 60°C for 60 minutes, then 99°C for 15 minutes. This breaks down the worms and exposes the DNA for the second PCR cycle.
8. While the SWPCR cycle is running, prepare the master mix for the second PCR step:
 - (a) If your primers are new (have not been used before), add the specified amount of water (on the information sheet packed with them) to dilute them down to $100 \mu\text{M}$. Vortex the tubes, then spin them down.
 - (b) If your primers are not new (you have used them before), thaw them (at room temperature, no ice necessary); they should already be at $100 \mu\text{M}$.
 - (c) Determine how much you need, and dilute a portion of the $100 \mu\text{M}$ primers down to $10 \mu\text{M}$ for use in the master mix (vortex, then spin after you mix). To dilute the primers down to $10 \mu\text{M}$, you want $1/10$ of the total volume of diluted primer to be the original $100 \mu\text{M}$ primer (e.g. $2 \mu\text{L } 100 \mu\text{M}$ primer + $18 \mu\text{L}$ water = $20 \mu\text{L}$ total diluted primer; $2/20 = 1/10$).
 - (d) Combine your forward and reverse primers and the water in a 1.5 mL tube.
 - (e) About 15 minutes before your SWPCR cycle is about to end, take the 2x one Taq MM out to thaw - thaw this on ice. You will add this to the master mix right before you use it. The master mix solution consists of:

0.5 μL forward primer (10 μM)

0.5 μL reverse primer (10 μM)

9 μL H_2O

12.5 μL 2x one Taq MM

This plus the 2.5 μL of DNA already in the tube after the first PCR step will yield 25 μL total per tube.

9. Also while the SWPCR is running, plan out your second PCR cycle if you are using a new primer:

94C for 2 minutes (initial melting)

94C for 30 seconds (melt DNA so can amplify) (*)

55C - 57C for 45 seconds (temperature based off of the primer info chart) (*)

68C for ?? (temperature based off of the Taq; time based off of the length of DNA) (*)

4C for infinity (keep refrigerated after done)

(*) These three steps are repeated 30 times to ensure maximum amplification. The length of the third repeated step is based off of the length of DNA that you're trying to replicate - it should be 1 minute for every 1k base pairs (e.g. 3500 base pairs - 3.5 minutes).

10. When SWPCR is done, keeping the tubes on ice, add the master mix. Using a new tip for each tube so as to not contaminate samples, pipette 22.5 μL of the completed master mix into each tube and mix it up (by pulling some into the pipette and releasing - repeat this several times).
11. PCR these tubes with the previously planned cycle for this primer.
12. Plan out the set-up for your gel while your samples are PCR-ing. The first column will be a DNA ladder, the second and third your positive and negative controls, and the rest your samples. NOTE: Larger combs have 16 wells, smaller combs have 10, and you can have two rows per gel, so plan accordingly.
13. Using 1% agar stock (made of TAE buffer and agar and stored at 65°C on the counter in the lab in a water bath - see Appendix B), put about 50 mL of the agar stock for the largest gel (or 35 mL for the smallest gel) into a 50 mL tube. Add 5 μL of ethidium bromide (or 3.5 μL) and

shake to mix (with the top on!). Make sure to have gloves on whenever you're working in the gel room and with ethidium bromide in general.

14. Put the rubber end pieces on a gel plate and add the combs (making sure everything is on tight and secure, and the combs are inserted the right direction for maximum distance between the top edge of the gel and the wells), then pour the agar mix in. Let the agar set for 20 - 25 minutes; it will no longer be clear when it is fully set.
15. Remove the rubber end pieces very carefully (peel off, trying to not break the wells). Place the gel plate into the TAE buffer in the electrophoresis machine. The TAE buffer should be covering the gel; add more if needed.
16. To load your samples onto the gel when the PCR cycle ends:
 - (a) The first column is for the DNA ladder - no loading dye needed. Pipette 10 μL of the DNA ladder very carefully into the first well.
 - (b) For every other column, combine 2 μL of loading dye with 10 μL of sample (mix with the pipette on a piece of Parafilm), then pipette all 12 μL into the wells in the proper order, as planned. You can pipette up to 10 drops of loading dye at a time without them drying out.
17. Once everything is loaded, place the cover on the machine and start it. The machine should always be at 110V. 15 - 25 minutes should be long enough if you're not looking for particularly distinct separations of your bands, otherwise let it run longer. Make sure it isn't running off the end of the gel! (NOTE: RUN TO RED - you should see bubbles coming up on the sides if it is running properly.)
18. Once you are satisfied with the length of the run, shut the machine off, carefully remove the gel plate, and transfer your gel to a box. Take it upstairs to image. Make sure when you image, you're using the correct filter or you won't see any results! There aren't really any rules for the imaging software - just play around until you get a good image that shows your PCR results.

A.3.4 SEQUENCING A SAMPLE

GENERAL NOTES:

- You generally only want to sequence one direction, so you only submit the fwd direction primer, though you can also submit the rev direction primer to sequence the other way as well.
- If you're sequencing with a primer you haven't sequenced with before (for a mutation you don't necessarily know well or anything), you should always also send in for sequencing both a positive and negative control - the mutant strain you used while doing your cross (positive control) and N₂ (negative control) - so you can make sure you see the mutation to begin with and can identify it/make it easier to identify it.
- Note that WormBase can on occasion be wrong about the sequence/mutation, so it always behooves you to check the paper in which they generated the strain if things aren't matching up between WormBase and your sequencing.

PROTOCOL:

1. SWPCR to get your starting DNA template - Use the usual 3 μ L LB/PK mix (2.5 μ L sample after SWPCR), but always use 5 worms per tube.
2. PCR on the appropriate program for your samples (50 μ L total):

2.5 μ L DNA from SWPCR
1 μ L fwd primer (10 μ M)
1 μ L rev primer (10 μ M)
25 μ L 2X one taq
20.5 μ L H₂O

3. Run 5 μ L of your PCR product out on a gel (use a 2% gel and a 2-log ladder if it's a small band you're looking for, for example the ttx-3 and ttx-1 PCR products). This is to make sure your primers worked well. You should have a nice, solid band for each sample.

4. Purify the other 45 μL (may be a little less than 45 μL in reality) using the purification kit (yellow box, says “Pur” on the side). Instructions are on the inside of the lid of the box:
 - (a) Pipette 225 μL ($5 \times 45 \mu\text{L}$) of the DNA binding buffer to a 1.5 mL tube. Add the 45 μL of your PCR product directly to that. Vortex to mix and spin down.
 - (b) Follow the rest of the instructions - change tubes, centrifuge, 2 rinses, etc. (see inside of kit lid).
 - (c) To elute, use 8 μL of “elution buffer” - you can just use autoclaved water (put some in a 15 mL or 50 mL conical that you’ll just use for sequencing so as to not accidentally contaminate your whole stock with random DNA - or contaminate your sequencing).

5. Put 4 μL of the purified product and 4 μL autoclaved water in a PCR tube - this is what you will submit for sequencing. Don’t worry about mixing the samples, as they’ll have to mix them after they pick them up anyways. If you’re submitting multiple samples, put them in a strip of PCR tubes. Label your tubes clearly on the side of the tube (not the top).

6. Primers should be submitted in separate PCR tubes (not part of the strip). They should be at 5 μM concentration, and you want to submit 5 μL /sample plus a little extra. For example, if you have 10 samples, submit 60 μL of your primer. Again, you generally only need to submit the forward primer.

7. Submit your order on the Eton Biosciences website:
 - (a) Genomics \rightarrow DNA Sequencing \rightarrow Place Order
 - (b) DNA Type \rightarrow PCR; Purified \rightarrow Yes; How \rightarrow Other
 - (c) Service Type \rightarrow SeqRegular; Mail in \rightarrow No; Ready Time \rightarrow by 6pm and they’ll pick up that day; Number of Samples \rightarrow self-evident; Vector Name \rightarrow e.g. pENTR5’-hsp-16.1
 - (d) Make sure to give your samples names that correspond between what you put on the tube and what you submit on the order form, but the order form can have more detailed names (e.g. on the tubes, just put the sample number/letter - 1, 2, 3, A, B, C, etc. - but on the order form, put the name of the strain and the sample number/letter - hsf1GFP;ttx3 - 1, etc.).

- Place the order in the proper box; there is one on the third floor of Northwest Labs near the Nanodrop.

FAQ for Eton Bio on sequencing: https://www.etonbio.com/sequencing/faq_seq.php

DNA sequencing order form: https://www.etonbio.com/sequencing/order_form.php

A.3.5 GATEWAY MOLECULAR CLONING WITH TOPO

PART I: PCR FOR DESIRED DNA (GENE, PROMOTER, ETC.)

General Notes:

Design and order the primers you will need to isolate the section of DNA you'll want to be incorporating into the genome (see Appendix B for protocol on designing and ordering primers). Use N2 (WT) genomic DNA in your reaction - no need to do SWPCR, just PCR.

Master mix for PCR (25 μ L total volume):

1 μ L DNA (100 ng/ μ L N2 genomic)

0.5 μ L 10 μ M fwd primer

0.5 μ L 10 μ M rev primer

12.5 μ L 2X one taq master mix

10.5 μ L H₂O

Instructions:

Run PCR with the temperature suggested by your primers for the length necessary for your elongation - as per usual PCR protocol. If you have a longer section of DNA, you may want to use HF

taq instead. Run 10 μL on a gel to make sure you have your product (if small, use a 2-log DNA ladder and 2% agarose). The other 15 μL can be stored at 4°C until you do your TOPO reaction.

PART 2: TOPO REACTION AND TRANSFORMATION INTO COMPETENT CELLS

General Notes:

If you have a new TOPO kit, write the received date on your kit. To do your TOPO reaction, you'll need the vector and the salt solution; these are stored at -20°C in the kit box when not in use. You'll also need one tube of competent cells, which are stored in Rack A of the -80°C freezer. Keep the vector, salt solution, and competent cells on ice at all times. The SOC medium can be kept at room temperature.

TOPO reaction and transformation:

These instructions can also be found in the TOPO manual.

1. Turn the heat block on and adjust it to 42.3°C (do this early in the day so it has time to heat up). Fill several holes with deionized water (including the one with the thermometer) - you will use this to heat shock cells.
2. In a PCR tube, mix by pipetting up and down:

4 μL PCR product
1 μL salt solution
1 μL TOPO vector

Keep at room temperature for 5 minutes, then place on ice. This can be stored overnight if needed and you can't do the transformation the same day.

3. Mix 2 μL TOPO product into the competent cells tube - don't pipette up and down (this will break the cells), but rather mix by rubbing the tube along the holes in the tube holder to gently mix.

4. Keep the TOPO and cells mix on ice for 20 minutes - while waiting, take 2 Kanamycin (Kan) plates out of the cold room (2 green stripes) and place at room temperature.
5. Heat shock the competent cells for 30 seconds at 42°C by placing the tube in the water bath on the heat block.
6. Move immediately back to ice for 2 minutes.
7. Add 250 μL RT SOC medium. Tape the tube horizontally onto a beaker holder in the 37°C incubator and shake for 60 minutes. 30 minutes into this incubation, place the 2 Kan plates at 37°C as well to prewarm.
8. Pipette 50 μL of transformation to one plate and 100 μL to the other plate. Spread it across entire plate with a spreader (as in inoculation). Incubate the plates overnight at 37°C; remove them the next morning and place at 4°C. You can use the plates for 1 - 1.5 weeks after this.

PART 3: OVERNIGHT CULTURE OF COMPETENT CELLS

Pick 5 single colonies from either plate and place one each in a Falcon tube with 3 mL of LB broth and 2.5 μL of Kanomycin (Kn50). Kn50 is likely to have precipitated out, so thaw it all the way out, vortex and spin it down, and then use it.

Put the Falcon tubes on the shaker in the 37°C incubator overnight. Remove them the next morning and place at 4°C until you are ready to do your miniprep.

PART 4: MINIPREP TO PURIFY PLASMID

General Notes:

Minipreps isolate the plasmid from your competent cells; the final products are stored in the -20°C freezer.

You will use 1.5 μL of your culture for the miniprep; the other 1.5 μL should be kept in the Falcon tubes at 4°C and will be used to make glycerol stocks of the plasmid.

Miniprep:

This protocol can also be found in the “E.Z.N.A. Plasmid DNA Mini Kit 1 Spin Protocol” manual that comes with the kit.

1. Pipette 1.5 μL of culture into new 1.5 μL tube. Centrifuge in the small centrifuge at 10,000 g for 1 minute to isolate your cells.
2. Take off the culture media with a pipette. It's okay to leave a little bit in order to not lose any of the pellet. Add 250 μL of Solution I/RNase (which breaks down the RNA we don't want, stored in the 4°C fridge) and vortex to mix it thoroughly. You don't need to worry about this being too rough. Spin it down after vortexing.
3. Move the solution to a new 1.5 mL tube; there will be around 270 - 275 μL to transfer.
4. Add 250 μL of Solution II (swirl the bottle before using). Very gently rotate your tubes several times. You are breaking down the cell walls in this step, but mixing too roughly will break the nucleus and contaminate your plasmid sample with chromosomal DNA. Do not let the reaction run > 5 minutes.
5. Add 350 μL of Solution III and immediately invert the tube 3 - 5 times. A white precipitate will form; you want to make sure it forms evenly throughout the solution, but you still want to try to be gentle in order to not get chromosomal DNA.
6. Centrifuge the tube at 13,000g for 10 minutes. While centrifuging, label your final 1.5 mL collection tubes (which will be frozen at -20°C) and put together the correct number of HiBind DNA Mini Column + 2 mL collection tubes (also label the Mini Columns).
7. After centrifuging, there will be a compact, white pellet - this is all the cellular debris you don't want. Carefully pipette the supernatant out and place it in the Mini Column. Centrifuge these at 13,000g for 1 minute.
8. Separate the collection tube and mini column. Discard the filtrate that is left in the collection tube. You can just shake it out into the sink (making sure to rinse the sink after) and replace

the mini column. Add 500 μL of the HBC buffer to the mini column. Centrifuge at 13,000g for 1 minute.

9. Discard the filtrate as before. Add 700 μL of the DNA wash buffer. Centrifuge at 13,000g for 1 minute.
10. Repeat the previous step.
11. Discard the filtrate and centrifuge at 13,000g for 2 minutes (adding nothing else). This is to remove the remaining ethanol in the membrane.
12. Move the mini column to your final labeled 1.5mL tubes. Add 30 μL - 100 μL of “elution buffer” (50 μL is the standard amount; less will give you a more concentrated end product). DO NOT use the elution buffer provided in the kit. You can either use sterile deionized water (but you should make sure its pH is around 8.5) or make your own elution buffer of 2 - 5 mM Tris in sterile, deionized water. This can be stored at -20°C and reused as long as you keep it clean.
13. Let the tubes sit for 1 minute once elution buffer is added, then centrifuge them at 13,000g for 1 minute.
14. Throw away the mini columns. The remaining 1.5 mL tubes are your final product. Store them at -20°C .

PART 5: SEQUENCING TO CONFIRM THE DIRECTION OF INSERTION IN THE PLASMID

General Notes:

TOPO plasmids have standard primers built in (pENTR 5'-TOPO has M13 fwd (-20) built in), so when you send it in for sequencing, you don't need to send in a primer with it.

Plasmids also don't need to be digested or purified or amplified or anything after being produced with a miniprep before sequencing. You just make sure they're at the right concentration (50 - 150 ng/ μL for Eton Bio).

Using the Nanodrop to find the concentration of your plasmids from the miniprep:

1. To bring up with you: 1 μL pipette and tips, all your samples, 1.5 mL tube of autoclaved water, sharpie.
2. The Nanodrop is located in NWL368 - go in, walk to the main room, turn right. Log in to the computer with your RC account. NOTE: You need to get trained by Claire Reardon to be able to use the Nanodrop.
3. Double click on ND - 1000 and select "Nucleic acid".
4. Clean the read point on the machine (the small circle). Make sure to clean both top and bottom, and always clean between samples.
5. Load a water sample first; samples are always 1 μL . Pipette it onto the small circle on the read point. (The program will prompt you to load a water sample first.)
6. After cleaning, load a blank of whatever you eluted your sample with. Place 1 μL on the same circle, close, and hit "Blank". If you eluted with the same thing for all samples, you don't need to redo the blank between samples.
7. Go through your samples one by one, making sure to clean the machine between samples. Load your 1 μL of sample and hit "Measure". You want to get a distinct peak that's above 1.8 (will see number on the right of the screen). The concentration will be displayed in the bottom right of the screen. 150 - 400 ng/ μL is expected for a miniprep; less than 150 can be caused by a variety of things, but sometimes indicates a plasmid that doesn't grow well in the cells (very big, etc.). It should still be okay even if your concentrations are lower (50 - 80ng/ μL).
8. Make sure to record the concentration (ng/ μL) on the side of your sample tube, as you can't print it off anywhere.

Sending for sequencing:

1. Eton Bioscience takes plasmids with concentrations anywhere between 50 - 150ng/ μL , so as long as you're within that concentration, you're fine. If you're not, you'll need to dilute your sample.

2. Fill out the order form on the Eton Bioscience website - sequencing a plasmid with SeqRegular. The primer should be one of the universal primers on your entry vector, so you don't need to send one in.
3. Pipette 8 μL of each sample (at the proper concentration) into a labeled PCR tube on a PCR tube strip, using only as many tubes as you need from a strip. Place the strip in a bag/envelope with the order number written on it place in the submission box. Results will be returned by email.

PART 6: MAKING A GLYCEROL STOCK OF YOUR PLASMID

NOTE: Only make a glycerol stock once you have confirmed which of your minipreps you want to use.

To a cryotube, add and mix by inverting the tube:

375 μL 40% glycerol

625 μL your overnight culture

Make two of these vials - one should go in the lab stock and the other should go in your personal stock, both in the -80°C freezer. It's also good to have an Excel spreadsheet to organize your personal boxes so you can keep track of what you have in them and find things easily.

NOTE: You don't want to let your overnight culture sit for more than 2 weeks before making the glycerol stock. Sooner than that is better, so do it as soon as you've confirmed your sequencing results.

PART 7: LR REACTION TO CREATE YOUR INJECTION PLASMID

General Notes:

Look at your sequencing results to confirm which plasmids have your insertion inserted in the correct direction. Pick one of the correct ones - generally go for the one which had the highest concentration from your miniprep - and make sure you note which one you are picking. You can dump the other 4 you aren't using.

Also, make sure to check that there are no wrong bases in the sequence of your gene/primer/etc. of interest!

1. Dilute your entry and destination vectors with autoclaved water to the proper concentrations (make an aliquot of the proper concentration in a 1.5 mL tube from your stock 1.5 mL tube). These can all be thawed at RT for the duration of use. The desired concentrations are:

5' entry vector: 20 ng/ μ L

Gene entry vector: 20 ng/ μ L

3' entry vector: 20 ng/ μ L

Destination vector: 50 ng/ μ L

2. In a PCR tube, combine and mix by pipetting (for a total of 10 μ L):

1 μ L 5' entry vector (20 ng)

1 μ L gene entry vector (20 ng)

1 μ L 3' entry vector (20 ng)

1.5 μ L destination vector (75 ng)

3.5 μ L TE buffer, pH 8.0

2 μ L LR Clonase II Plus

3. Incubate overnight at 25°C in a PCR machine.
4. The next morning, add 1 μ L of Proteinase K (found in the small BP clonase box) to your PCR tube and mix the same way you mix competent cells (rubbing along a tube holder).

Incubate for 10 min at 37°C (again, can use the PCR machine). You can then leave this at RT or on ice until you're ready to do the transformation.

PART 8: TRANSFORMATION OF FINAL INJECTION PLASMID INTO COMPETENT CELLS

1. Turn the heat block on and adjust it to 42.3°C (do this early in the day so it has time to heat up). Fill several holes with deionized water (including the one with the thermometer); you will use this to HS cells.
2. While your PK is incubating (see previous step), take out your competent cells from the -80°C freezer. You will use 10G cells, which come in a tube with a yellow lid. Thaw this tube on ice.
3. Once the competent cells are thawed, take 40 µL from the tube and put it in a 1.5 mL tube. The other 40 µL go back in the -80°C with an X on the top of the lid, so you know to use the other half of that tube first next time.
4. Mix all 10 µL of your LR reaction into the competent cells tube. Don't pipette up and down (this will break the cells), but rather mix by rubbing the tube along the holes in the tube holder to gently mix.
5. Keep the mix of LR and cells on ice for 30 minutes. While waiting, take 2 LB Carb plates out of the fridge (2 red stripes) and place at room temperature to warm up.
6. Heat shock the tube for 30 seconds at 42°C by placing it in the water bath on the heat block.
7. Move immediately back to ice for 2 minutes.
8. Add 250 µL RT SOC medium. Tape the tube horizontally onto a beaker holder in the 37°C incubator and shake for 1 hour. 30 minutes into this incubation, place the 2 LB Carb plates at 37°C as well to prewarm.
9. Pipette 50 µL of transformation to one plate and 100 µL to the other plate. Spread across the entire plate with a spreader (as in inoculation).
10. Incubate the plates overnight at 37°C. Remove them the next morning and place at 4°C. You can use the plates for 1 - 1.5 weeks after this.

PART 9: OVERNIGHT CULTURE OF COMPETENT CELLS

1. Pick 3 single colonies from either plate and place 1 each in a Falcon tube with 3 mL of LB broth and 2.5 μL of Carb (Cb100). Cb100 is likely to have precipitated out, so thaw it all the way out, vortex and spin down, and then use it.
2. Put the Falcon tubes on the shaker in the 37°C incubator overnight.
3. Remove them the next morning and place at 4°C until you are ready to do your miniprep.

PART 10: MINIPREP TO PURIFY PLASMID

1. Minipreps isolate the plasmid from your competent cells; the final products are stored in the -20°C freezer.
2. As before, you will use 1.5 μL of your culture for the miniprep; the other 1.5 μL should be kept in the Falcon tubes at 4°C and will be used to make glycerol stocks of the plasmid.
3. Follow the same protocol as in Part 4.

PART 11: RESTRICTION DIGEST TO CONFIRM YOUR LR REACTION

General Notes:

You can thaw the buffer and BSA at RT, but the enzyme should be kept in the blue shuttle box at all times that it's not in the -20°C freezer.

You want your enzyme to be 10% or less of your total digest, so you can use 2 μL max - for example, if you're doing a double digest with two different enzymes, you can use 1 μL of each.

1. Make up a master mix for your restriction digest - do $n + 2$ multiples of the single sample digest solution, n being the number of samples you have. The single sample solution is:

1.5 μL DNA (from miniprep, don't need to nanodrop first)
2 μL 10X buffer (for EcoRV, use NEB buffer 3)
1 μL 10X BSA (for EcoRV; not all enzymes require this)
1 μL enzyme (EcoRV for hsp-16.1)
14.5 μL H_2O (to bring up to 20 μL total)

2. Make your master mix in a 1.5 mL tube, mix by vortexing, and spin down.
3. Transfer 18.5 μL to a 1.5 mL tube for each sample you have and add 1.5 μL of your sample.
4. Mix by vortexing, then spin down.
5. Incubate the digestion for around 1 hour at 37°C (easiest to do in a water bath). NOTE: HF (high fidelity) enzymes will only take 5 - 20 minutes. Other enzymes can potentially be left for up to 5 - 6 hours, but if you see a lot of little bands showing up in your gel (called "starring"), you should just redo the digest - you left it too long.
6. Make your gel while you're waiting on the digestion so it has time to set.
7. Immediately after your incubation ends, load your samples onto the gel (10 μL sample + 2 μL loading dye, any color) and run for 20 - 30 minutes. Check for your bands!

PART 12: MAKING A GLYCEROL STOCK OF YOUR PLASMID

See Part 6.

PART 13: MOSSCI INJECTION

See next section for injection protocol.

FINAL NOTES:

At some point during this procedure, you also want to design your final plasmid in a program like ApE. This will allow you to figure out what restriction digest to run to confirm your final plasmid in addition to allowing you to have an easier time designing your plasmid and making sure it will work well.

A.3.6 INJECTION PROTOCOL

PRIOR TO DAY OF INJECTION:

INJECTION PADS:

1. Combine 1 g agarose + 50 mL H_2O in a bottle and microwave until it has dissolved completely to make 2% agarose. Place in a 65°C water bath or use immediately.
2. Set out 5 slides on your bench and wipe the top surface clean with ethanol to allow pads to transfer easier with less resistance.
3. Use a plastic transfer pipette to place EXACTLY one drop of the 2% agarose on the center of the slide and drop a 22 x 40 coverslip on top, perpendicular to the bottom of the slide. Only use one drop, and place the coverslip immediately, or you will end up with pads that are either too big or too thick. Aim for no bubbles in the pad, but they're not the end of the world.
4. Carefully slide the coverslip and pad off of the slide and place it onto an 8.5" x 11" sheet of aluminum foil with the pad side up. Let them air dry overnight; they should be clear when they're dry.
5. Store dried pads in an old 22 x 40 coverslip box. Pads can be used indefinitely; if they are over-dry because they've been there a while, you can blow on them to moisten them.

EYELASH PICK:

1. Pick an eyebrow (or eyelash) hair and place it on a small piece of Parafilm near a small dot of clear nail polish.
2. Roll the end of a toothpick in the nail polish and pick up the hair on the end. You want it to be attached on the end of the hair that's thinnest (not the end with the follicle that's attached to you, which is thicker).
3. Carefully dab the end of the toothpick into the nail polish dot to coat thoroughly (but don't lose the hair!), then sit it upright to dry.
4. After it's dry, use a razor to cut off the end of the hair until it's fairly short. Shorter, thicker hairs are easier to pick up worms with.
5. Eyelash picks can be reused if you wipe them with a kimwipe after an injection, or you can make a new one each time you inject.

INJECTION NEEDLES:

1. Remove the cover on the needle puller, turn it on (button on left side), select program 55, then hit enter.
2. Put the capillary tube (WPI 1BooF-4) into the grooves of the left side of the apparatus and slide it to the right, ensuring that it goes through the heat loop and aligns in the grooves of the right side. Tighten the left first, then slide the grooved holders to the middle of the apparatus, then tighten the right side. Try to have even lengths of capillary on either side.
3. Push the "Pull" button (green) and the capillary tube will heat up and separate into 2 sealed needles that should be identical.
4. Store pulled needles in a designated petri dish.

GLASS TO BREAK INJECTION NEEDLE TIP:

1. Turn a Bunsen burner on to the smallest flame you can get.
2. Hold a capillary over the flame with the middle of the capillary directly in the flame.
3. When the middle has started melting, remove it from the flame and pull the two sides apart to create a thin strand of glass, then bend the capillary to break it into two.

INJECTION MIX:

- This can be made prior to the day of injection and stored at -20°C .
- In a 1.5 mL tube, mix:

50 ng/ μL pCFJ601
10 - 50 ng/ μL transgene in targeting vector - start at 25 ng/ μL
10 ng/ μL pMA122
10 ng/ μL pGH8
2.5 ng/ μL pCFJ90
5 ng/ μL pCFJ104

- Make 60 μL of your injection mix – to calculate how much to add of your stock:

$$(\text{stock ng / } \mu\text{L}) \times \# \mu\text{L to add} = (\text{final ng / } \mu\text{L}) \times 60 \mu\text{L}$$

WORMS:

- Check the Wormbuilder website for the proper strain to use. For example, for Uni_III MosSCI injection, you want to use EG8080, which is unc-119.
- For best results, grow unc worms at 15°C or 20°C , never at 25°C .

- Grow unc worms on HB101 plates. To do this, make the usual NGM plates (see Appendix B) but with no streptomycin and inoculate them with HB101 instead. HB101 grows thinner, so it's easier for unc worms to maneuver on. Plates can be made ahead of time, inoculated, and stored at 4°C for about 4 months (or stored in the 15°C fridge). See the CGC website or the Wormbuilder site for more detailed instructions on the strain.
- Maintain your worms for a week before you attempt injection so that you have healthy, not starved worms. Maintaining unc worms includes both moving 2 - 10 worms to a new plate every day AND spreading worms on previously started plates around to prevent starvation from happening as quickly. This can take a fair amount of time, so plan it into your schedule for the week before injection.
- Pick late L4s to a plate around 20 hours before you're planning on injecting and leave the plate on your benchtop until you're ready to inject. For example, to inject from 11 AM - 1 PM, pick your late L4s at about 3 PM. Late L4s have a very distinct half-circle patch in their middle where the vulva is developing.

DAY OF INJECTION:

- Make the injection mix if it's not already made.
- Make sure to vortex your mix, then spin it down for 10 minutes at 15000 rpm.
- Only take mix to load into your needles from the top of the tube.
- Your mix can then be put back in the freezer and reused unless you need to change the concentrations of plasmids.
- To load the injection needles, pipette 1.5 μ L into a gel loading tip (Eppendorf E.P. tips, 20 μ L Microloader, 5242 956.003). This should be enough to inject at least 100 worms, including flooding them to rescue.
- Insert the pipette tip into an injection needle until it reaches the base of the needle.
- Release the injection mix. It may take a couple of seconds for it to come out of the pipette tip, so be patient. You should be able to see it in the needle after you remove the pipette.

- Load 2 - 3 needles total and place them in a humidity chamber. You'll also have a bubble at the very tip of the needle that you will need to push out before injecting.
- Gather your supplies, including picking your worms if you didn't do this the day before:

Injection pads
 Loaded injection needles
 Oil + a pipette tip to get oil out of container
 Eyelash pick
 Small petri dish to balance coverslips on
 Plate to put injected worms on
 Worms to inject

- NOTE: If you already picked L4s the previous day, check that your worms are of the proper age. You want young adults (vulva fully formed, single line of eggs, around 7 - 10 eggs or perhaps a bit older - single row plus a couple - gonad arms are larger in slightly older animals). If you are picking day of, pick young adults with the proper number of eggs onto a new plate.

INJECTION:

INJECTION SYSTEM & MICROSCOPE:

1. There's only one button to turn the injection machine on.
2. Make sure the middle number on the injection controller is set to 12-1300 and the numbers on the left and right are set to zero.
3. From left to right, the three controllers on the micromanipulator (the thing that holds the needle) are for z, y and x.

BREAKING THE NEEDLE:

1. Add a big drop of oil to the center of an injection pad.
2. Break off a piece of the hand-pulled capillary you made into the oil.
3. Place the injection pad on the injection scope and bring the capillary piece into focus, slightly to the left of center.
4. Start moving the needle into the plane of focus. You can move it back and forth in the y-direction, and you should see a shadow moving back and forth if you're close to being in the right plane of focus.
5. Bring the tip of the needle to where it's touching the edge of the capillary, then do some combination of hitting the clear button on the injection controller and tapping the table until the vibrations and friction break the tip.
6. Once you see liquid flowing from the tip (or think you have broken the tip - there may not be liquid flowing immediately if there is an air bubble), move the needle away from the capillary and check the flow.
7. You may need to hold the clear button down for a while to clear an air bubble if it's there, and you won't be able to inject until you get it out.
8. Note: a bigger opening in the needle leads to better flow of the injection mix and less needle clogs, but if it's too big, it's easy to poke a too-big hole in the worm and kill it.
9. When you're happy with the needle, move it to where the tip is in the center of the scope, and raise it enough to slide the injection pad off.

LOADING A WORM:

1. Before placing the first worm, put a line of oil across the center of your injection pad.
2. On the second scope, pick a worm with the eyelash pick.
3. Place the worm in the oil (which keeps it from drying out immediately) on the injection pad and get it off of the eyelash pick.

4. Move the worm out of the oil streak onto the side of the pad, and gently rub along the body of the worm with the eyelash pick to move oil out from under it and stick it down to the injection pad.
5. Move more oil on top of the worm once it is stuck down to prevent drying out.
6. Before moving to the injection scope, look for the 2 white patches on the sides of the worm that are the gonad arms so you can properly align your worm when you place the injection pad on the scope. You want the worm to be at a 90 degree to the needle, so the needle will be able to go directly into the gonad arm.

INJECTING A WORM:

1. Bring the worm into focus to the left of center on the injection scope and find the gonad arm you want to inject. The gonad arm will appear light colored, maybe slightly “fuzzy”/blurry/cloudy, and it is filled with small round circles (may just look like texture in the middle of the cloudy patch). It may be hard to pick out at first, but practice will help. Ask someone if you can’t find it, as you are unlikely to figure it out on your own... and this is key to getting a successful injection
2. Lower the needle slowly until it is in the same plane of focus as the worm.
3. Move the needle tip to where it is applying pressure on the side of the worm where the gonad arm is - you should see it creating a dip in the side of the worm.
4. Tap the table a couple of times until the needle pops through the worm cuticle. It may be harder to get it to punch through the more injections you do, as the needle tip gets blunter.
5. Hold the clear button down on the injection controller until you see the gonad inflating - if you don’t see it inflating, you’re either not in the gonad, or the needle is clogged.
6. NOTE: Try to inject about 25 worms, especially if you aren’t entirely sure you’re injecting properly. For MosSCI injections, inject at least 50.

RECOVERY OF WORM:

1. Remove the needle from the worm and hold the clear button down on the injector control. You should be able to see a steady stream of injection mix coming out.
2. Keep holding the clear button down until the worm starts coming up off the pad.
3. Raise the needle as little as possible to be able to remove the pad, and move the pad to the other scope. (If you move it up more, it will take longer for you to get it into the correct plane of focus for the next worm you inject.)
4. Use the eyelash pick to gently loosen the worm, pick it up, and move it to your injected worm plate.
5. Note: The worm should be off the plate/in the oil for 10 minutes maximum if you want to have any good chance of survival. Always rescue any worms, however, even if it takes you longer to inject one, but aim for maximum speed over being overly slow and cautious, as being too slow will kill all your worms before you even get them off the pad.

CLEAN-UP OF AREA:

1. All sharps (used needles, coverslips, etc.) go in a sharps container. Don't leave your needle in the needle holder.
2. Turn the microscopes and injector off.
3. Clean up any messes you made with the oil and make sure to take all your supplies with you.

POST-INJECTION GENERAL STEPS:

1. Allow the injected worms at least 1 hour after injection to recover, then separate them 1 - 2 to a plate.

2. Note: If you are using an antibiotic marker (NeoR, etc.) to confirm injection success, you can either put injected worms directly on plates with the selection marker (adults worms last several days on plates without resistance to the transgene, so they will have time to lay eggs), or you can add it 1 - 2 days after injection. NeoR works better on growing populations than starved populations.
3. Put the plates at 25°C for about a week until they are fully starved out.
4. If you injected into unc worms and you see dauers at this point, you know they're rescued for unc-119, as uncs don't form dauers. You should also see a lot of L1s that are moving (because the plate is starved out, but they are rescued).
5. To check for successful injections for worms with the hs:peel-1 marker, heat shock the plates for 2 - 3 hours at 34°C. Don't put the plates in a box, and make sure they aren't stacked more than 3 high to ensure they equilibrate to the temperature quickly.
6. Give hs:peel-1 at least 4 - 6 hours (or more) to kill off worms that have the injection mix, but haven't integrated it into the chromosome.
7. Pick for worms that move well (if you injected into uncs) and don't have any of the fluorescence coinjection markers (if you're looking for your insertion to be integrated into the chromosome).
8. If you're creating an extrachromosomal array, look for worms expressing the proper fluorescence or other markers. Note that you may have weird fluorescence expression in the first couple generations after injections, but you should move to the expected expression in later generations.
9. Pick as many possible successes as you can, 1 to a plate, and propagate them for a couple of generations to homozygose them (if you're making an integrated strain). Pick for worms that have the traits listed above (no uncs, proper antibiotic resistance, no weird fluorescence markers).
10. PCR for the MosSCI or miniMOS insertion arm markers to fully confirm that your insertion was successful. You can find the correct primers in the literature and check your designed plasmid to know what size you're amplifying for.

- ii. NOTE: The official detailed protocol for direct injection (and much more useful information about MosSCI) can be found on the MosSCI website: <http://www.wormbuilder.org/test-page/protocol/>

A.3.7 MINIMOS CONFIRMATION AND INVERSE PCR

This protocol was adapted from the Supplementary protocol 2/Inverse PCR individual inserts protocol on www.wormbuilder.org, which was adapted originally from a Mos1 insertion protocol by Boulin & Bessereau in Nature Protocols.

- i. Initial steps post-injection.

- After moving worms to separate plates around 1 hour post-injection, it is important to place the plates at 25°C - this is the optimal temperature for the insertion to actually work!!
 - 1 - 2 days post-injection, add filtered G₄₁₈ at 25 mg/mL, 500 µL per 6 cm plate and allow it to dry under the hood. Place the plates back at 25°C.
 - Plates will take 1+ week to starve out fully - probably longer than 1 week (closer to 2) in reality.
 - Before the plates are fully starved out, you can already start checking for possible successful plates:
 - On the regular scope, check that you see worms surviving the G₄₁₈. You should see worms of multiple ages, not just old adults still crawling around or only a few young/dauer worms. You may see a couple dauers on unsuccessful plates because you can have a few escape the G₄₁₈ and survive.
 - On the fluorescent scope, check that you see some worms with the mCherry fluorescent markers you co-injected with. Only some should have it, as successful insertions will no longer have the co-injection marker.
- If you see a possible success plate, you can pick around 6 worms to separate plates with G₄₁₈ on them and check that they survive and produce progeny that survive.

- When the plates are starved out, heat shock them for 2 - 3 hours at 34°C. Don't stack plates in the incubator.
- 4 - 6 hours (or later) after the heat shock, pick individual worms that don't have the co-injection markers to separate plates (with G₄₁₈ already on them). Continue to grow the worms at 25°C. Check that the progeny survive and look healthy. Growing them on G₄₁₈ will ensure that you are homozygosing your line, as any progeny without the resistance will die before reproducing.
- Optionally, PCR for the miniMos insertion arm markers to confirm that your insertion was successful. If the plate is resistant to G₄₁₈, however, you can be fairly sure that it was a successful insertion and instead first go through the inverse PCR process to determine the location of the insertion. At some point, however, you should make sure you PCR and check that your insertion looks correct.
- NOTE: Once you have worms that are stably growing on G₄₁₈ and non-fluorescent (especially if you've isolated single animals), you can start maintaining them at any temperature. Do keep them on G₄₁₈ until you are fully confident they are homozygous by genotyping for your insertion (PCR).
- NOTE: For all following steps, use aerosol resistant tips at all steps because of contamination, which is an especially big problem when doing two sequential PCR reactions on small amounts of template.

2. Isolate genomic DNA (gDNA).

- Follow gDNA extraction protocol. The genomic DNA isolation kit from Zymo is recommended by the original protocol, though John Calarco's lab can also recommend the Qiagen kit.

3. Digest gDNA with DpnII. (25 μL)

General Notes:

If you get a bad read on the Nanodrop from your gDNA sample (no real peak), just use 10 μL of the gDNA product; otherwise dilute as indicated.

It's important to use the specific buffer associated with DpnII.

The buffer can be thawed at RT (vortex before use), but DpnII should be kept in a blue transport container at all times when outside of the freezer.

- In a PCR tube, mix the following components by vortexing, then spinning down (place PCR tube in larger tube to fit into spinner):

10 μL DNA sample (150 ng - add water to 10 μL)
2.5 μL Restriction buffer DpnII (10x)
1.0 μL Restriction enzyme (DpnII 10 U/ μL)
11.5 μL H₂O (molecular biology grade)

- Place in water bath at 37°C for two hours to overnight to digest.
- Heat inactivate the enzyme after the restriction digest by placing at 80°C for 20 minutes. You can use a PCR machine program for this.

4. Ligate the digested DNA. (25 μL)

General Notes:

The T₄ ligase should be kept in the blue transport container at all times when outside of the freezer. The buffer can be thawed at RT, but because ligase is ATP dependent, it has ATP in it and should therefore be aliquoted so it can go through as few thaw/freeze cycles as possible.

NOTE: Both the T₄ ligase and buffer can be a bit finicky...

- In another PCR tube, gently mix (by pipetting) the following components:

Digested DNA from previous step 2.5 μL
5X (10x) ligation buffer 5 μL (2.5 μL) (Enzymatics ligase buffer)
T₄ ligase 1.0 μL (Enzymatics ligase)
H₂O 16.5 μL OR 19.0 μL

- Leave on the bench at room temperature for 2 hours to overnight. The ligation reactions can then be frozen indefinitely at -20°C before proceeding to the next step. Don't throw it away until you have finished everything!

5. Do first round of inverse PCR. (10 μ L)

General Notes:

NOTE: (from Neil) Phusion is a finicky enzyme.

dNTPs should be aliquoted and stored at -20°C in your personal stock and taken out right before you want to add it.

The Phusion 5X GC buffer can be thawed on ice, but the NEB Phusion Polymerase should be kept in a blue transport box at all times.

- Optimally, make a master mix of PCR ingredients, aliquot it to PCR tubes, and add the “ligation mix” individually to each tube so you don’t have to pipette such small amounts - though the pipette can technically pipette 0.1 μ L. Otherwise, in a PCR tube, mix the following components by pipetting:

2.0 μ L Ligation mix from step 4
1.0 μ L Primer oCF1587 OR oCF1591 (10 μ M)
1.0 μ L Primer oCF1588 OR oCF1592 (10 μ M)
0.2 μ L dNTPs (10 mM)
2.0 μ L Phusion 5x GC buffer
0.1 μ L NEB Phusion Polymerase
3.7 μ L H₂O

- You can start with either set of primers, just make sure you use the corresponding second set when you run the second round of inverse PCR and run them at the right temperature.
- PCR settings:

Initial denaturation: 2 minutes at 98°C
PCR cycles: 30x
Annealing temperature: 64°C OR 62°C
Elongation time: 1 minute

6. Do second round of inverse PCR. (25 μ L)

- Dilute the first round of PCR product 100 fold by transferring 1 μ L of PCR₁ product to a new PCR tube and adding 99 μ L of water. Vortex to mix, then spin down to avoid contamination.

- Mix the following components in a new PCR tube by pipetting, taking the PCR from step 5 from the top of the tube:

1.0 μ L PCR from step 5
 2.5 μ L Primer oCF1589 OR oCF1593 (10 μ M)
 2.5 μ L Primer oCF1590 OR oCF1594 (10 μ M)
 0.5 μ L dNTPs (10 mM)
 5.0 μ L Phusion 5x GC buffer
 0.2 μ L NEB phusion polymerase
 13.0 μ H₂O

- PCR settings are the same as the previous step, using the appropriate temperature for whichever of set of primers you're using.

7. Run the PCR products on a 1% agarose gel, excise clear bands from gel, and gel purify.

- Follow the gel excision and purification protocol (see Appendix B).
- Only excise one band from each reaction - though if you don't know what you're doing or looking for, just go ahead and excise multiple.
- Do not excise bands that are not clearly distinct or bands where there is a smear.
- Only excise bands that are larger than 100 bp.
- Follow the sequencing protocol to send the gel purified product in with oCF1590 OR oCF1593.

8. Determine insertion site.

- Once you get the sequence read back, you can determine the insertion site.
- Search the sequence read for the following sequence: "ACATTTTCATACTTGTA-CACCTGA." Allow for two mismatches to accommodate poor sequence calls. This is the end of the Mos1 transposon. The next two nucleotides should be a "TA", where the Mos1 transposon inserted. The rest of the read is the genomic DNA insertion site.

- Go to Wormbase and blast search.
Change “Query Type” to Nucleotide.
Change “E-value Threshold” to $1E-4$.
Unclick “Filter”.
- Identify the correct match to your insertion site. Typically it will be the best match but make sure the query match starts at position “1”. Otherwise the read is probably finding part of the *unc-119* rescue gene or the transgene you put in. Some insertions cannot be mapped to unique locations because of repetitive regions in the genome or too short reads.

9. No bands?

- Redo the PCR reactions with oligos that anneal at the other end of the transposon. Start with the ligated DNA from step 3. This corresponds to repeating steps 5 - 7 with the other set of primers.
- Still no bands? Repeat protocol with another restriction enzyme, for example HpaII.

A.3.8 FREEZING WORM STRAINS

1. Make freezing solution if needed (1L):

5.85 g NaCl
6.8 g KH_2PO_4
250 mL 100% glycerol
5.6 mL 1 N NaOH
ddH₂O to 1L

Mix all ingredients in an autoclaveable bottle, autoclave 15 - 30 minutes, then add 3 mL sterile 0.1M $MgSO_4$ post-autoclave.

2. Prepare freezer tubes:

- (a) Take a plate that has little to no food and lots of L1s (but not dauers). Add 2 mL of M9W to the plate, swirl it around, and transfer it to a 15 mL conical. Fill the conical to 15 mL with M9W.
- (b) Spin the conical at 2500 rpm for 3 minutes.
- (c) Remove the supernatant carefully, making sure not to disturb the loose worm pellet (you can pipette it out, or use vacuum suction if you have that set up).
- (d) Add 1.5 mL of the freezing solution, then add enough M9W to bring the total volume to 3 mL.
- (e) Aliquot 1 mL of this mixture into each of 3 cryotubes. Cryotubes should be labeled with strain name, initials, and date of freezing on the front.
- (f) Place the tubes in Styrofoam box in the -80°C freezer.
- (g) Make sure to track the strains you are freezing in your personal notes (Excel, etc.).

3. Verifying the freeze:

- (a) Wait at least 5 days to check your frozen worm strain!
- (b) Thaw the aliquot at room temperature, then pipette 250 - 500 μL onto a seeded NGM plate. Let it dry under the hood.
- (c) Check for worms on the plate in 2 - 3 days (or earlier - it's just easier to see later). Make sure to also check for coselectable markers if it's a strain with an extrachromosomal array or for anything else special about the strain - temperature restrictions on growth, males, etc.
- (d) **DO NOT DISCARD THE PLATES YOU'RE PROPAGATING UNTIL YOU HAVE VERIFIED THE FREEZE!!**

4. General notes:

- (a) You can make more than 3 tubes, you'll just have to have space to store them in the -80°C freezer. If it's a strain you're working with a lot, it could be good to make more.

- (b) If it's a low penetrance chromosomal array, you need to use more than 1 plate if you're making more than 3 tubes. You'll also need to check your plate for the marker before you freeze to make sure you'll have some survivors carrying the strain.
- (c) Try to always have some worms frozen down when you either get or make a new strain - in case you have lines that die out because they're starved too much or there are contamination issues. Also, frozen strains are closer to the original because they haven't acquired as many mutations as the lines you are currently propagating.
- (d) When you thaw a strain, set aside a plate to re-freeze it as soon as possible.

A.4 qPCR

GENERAL NOTES

For my experimental timeline, you want to start a qPCR experiment around 55 - 60 hours after plating, which gives weird timing for plating and egg prep, unfortunately.

A.4.1 EGG PREP - SYNCHRONIZATION VIA BLEACHING

1. Make a 20% alkaline hypochlorite solution (15 mL) - make fresh every time:

9.75 mL water
3.75 mL 1M NaOH
1.5 mL concentrated bleach

2. If you're working with a chunked plate, tap the chunk onto the lid of the plate.
3. Pipette 2 mL of M9W onto the plate and gently swirl it to dislodge the worms.

4. Tilt the plate and use a plastic bulb pipette to transfer the M9W from the plate to a 15 mL conical. If you're worried about the worms that might stick to the sides of the pipette, you can do multiple plates.
5. Fill the conical to 15 mL with M9W and centrifuge it at 2500 rpm for 3 minutes.
6. Aspirate as much of the M9W as possible without disturbing the worm pellet.
7. Add 15 mL of the 20% alkaline hypochlorite solution to the tube. Mix by gently inverting the tube for 5 minutes and no more!! Put a drop on a glass slide after 5 minutes to check that the adults have lysed open, but don't leave the bleach too long, or you will damage the eggs.
8. After the adults have lysed, centrifuge the tube at 2500 rpm for 2 minutes.
9. Aspirate as much of the bleach solution as possible without disturbing the worm pellet, and quickly add 15 mL of M9W to dilute out the bleach.
10. Centrifuge again at 2500 rpm for 3 minutes.
11. Aspirate out the supernatant, being careful not to disturb the worm pellet, and repeat this rinse at least 1 more time (rinse = pour M9W, centrifuge, aspirate). There should be no bleach smell left after you have done the rinse steps.
12. Add 14 mL of M9W and agitate by hand to resuspend the pellet.
13. Optional: Split this into two 15 mL tubes with 7 mL each.
14. Label the tubes with the date and time and place them on the nutator (mini-mixer).
15. The worms will take 8 - 12 hours to hatch. Since there is no food, they'll arrest at L1. After hatching, pipette 100 μ L or more onto a seeded plate and put it under the hood to dry. You can check how successful your egg prep was by seeing how many worms you get on the plate. For a strain with an extrachromosomal array, you may want to do multiple plates if the array isn't super penetrant.
16. It takes 48 hours from plating for the worms to reach young adult (WT worms growing at 25°C - mutants and different growing temperatures may vary in time to reach young adult).
17. Egg preps can be used up to a week after making them. The older the egg prep, the less synchronized the population will be because the time it takes to get out of the L1 arrest will fluctuate more.

A.4.2 TIME POINT SAMPLE PREPARATION FOR 50 WORMS.

1. Label 1.5 mL microcentrifuge tubes for all samples.
2. At the microscope, add 30 μL of TRI-reagent to the lid of a tube using filter tips.
3. Pick 50 - 100 worms and place them in the lid in the TRI-reagent under a microscope, making sure that you can count all worms when added.
 - Pick 10 worms at a time so you're sure all 10 worms make it into the tube.
 - It's important that you have the exact same number of worms for each time point, as the qPCR can be sensitive to that.
 - Between each picking, wipe the tip of your pick off with a Kim wipe before flaming it to try to get rid of all the TRI-reagent.
 - Note that TRI-reagent is a nasty chemical, so the Kim wipes should go into chemical waste along with the tips and anything else used.
4. Add 170 μL of TRI-reagent to the tube, close the lid (making sure not to spill what's in the lid), and carefully shake the tube.
5. Centrifuge at 50 g for 30 seconds.
6. These tubes can be stored in the -80°C freezer for up to 1 month - thaw at RT when ready to use.
7. After taking a time point, clean the benchtop and bottle when you finish using them. Everything that comes into contact with the TRI-reagent should go in hazardous waste, including your gloves, all tubes, and the paper towel you wipe down the bench with.
8. NOTE: It takes about 30 minutes to pick one time point (2 repeats), so you want to stagger the time points for different experimental conditions by 30 minutes so you can pick all worms. Repeats will be 5 minutes apart for 50 worms (or more depending on how fast you are at picking).

A.4.3 RNA EXTRACTION

General notes

- Open a new RNase free water whenever you feel like you should. Water is a major source of contamination, so don't be afraid to just open a new one. Date after opening.
- BCP (used in this step) is also toxic and needs to be disposed of as hazardous waste.
- All of the chemicals you use in these steps should have their own separate hazardous waste containers in the RNA hood.
- Never put more than 12 tubes in the 4°C centrifuge at a time, and when you are first learning qPCR, you should do no more than 1 - 4 tubes at a time.
- Anytime you're working with something with TRI-reagent in it, use filter tips.

A.4.4 DAY 1 STEPS (1 - 2 HOURS)

1. Clean the hood before starting:

- 30 minutes of UV sterilization.
- Wipe everything down first with 70% EtOH, then with RNase away (+ Kim wipes). This includes everything inside the hood that you'll use: bottles, pipettes, benchtop surface - make sure these are thoroughly cleaned. Also things outside the hood: hood handle, cart handle, drawer handle, door handle, your gloves, etc.
- Use new items every time you start an RNA experiment - new tips (don't autoclave, just take tip boxes that are still wrapped in the plastic, open in the hood), a new bag of 1.5 mL tubes, a new bottle of water, and a new box of Kim wipes.
- Change gloves frequently - change after you clean the hood. Also at least re-clean your gloves with RNase away every time you go back into the hood to work after working outside of the hood.

- Also clean the centrifuge in the 4°C room with RNase away, and make sure to keep it closed at all times that you're not loading/removing samples.
2. Get ice in an external ice bucket and dump it into the RNA hood ice bucket, making sure to wipe down the RNA hood ice bucket first. Place the RNase free water in it.
 3. Centrifuge a 1.5 mL phase lock gel tube (heavy) for 1 minute at 10,000 g in the room temperature centrifuge.
 4. Working in the RNA hood, add 200 μL of sample (in TRI-reagent), 75 μL of RNase free water, and 6 μL of glycogen.
 5. Add 0.1 mL of BCP (1-bromo-3-chloropropane) per 1 mL of TRI-reagent - i.e. 20 μL for 200 μL TRI-reagent. Cover the samples tightly and shake vigorously for 15 seconds.
 6. Store the mixture at room temperature for 2 - 15 minutes (Lucy recommends 10 minutes). After this incubation, you should see 3 layers: a white-ish gel on bottom, a red middle, and a transparent top.
 7. Spin the tubes at 12,000 g (12000 rpm) for 15 minutes in the 4°C centrifuge (in the 4°C cold room). After centrifugation, you should see that the bottom and middle layers have switched positions.
 8. In the RNA hood, move the transparent phase to a new (non gel lock) 1.5 mL tube, taking care to not touch the gel and contaminate your sample. Cap and freeze the remaining sample at -80° .
 9. Add 0.5 mL IsOH (isopropanol) for every 1 mL of TRI-reagent (i.e. 100 μL for 200 μL TRI-reagent) and mix by gently inverting. Store the tubes at room temperature for 5 - 10 minutes, then move them carefully to a box for storage in the -20°C freezer for >20 hours.

A.4.5 DAY 2 STEPS (3 - 4 HOURS)

1. Repeat the hood cleaning steps.

2. Get ice as before and place the 75% EtOH and RNase free water in it.
3. Carefully remove the tubes from -20°C . The RNA has precipitated, so you don't want to disturb them. They will still be liquid (because of the IsOH), so you don't need to thaw them.
4. Spin the tubes at 12,000g for 8 minutes at 4°C . Be careful placing the tubes into the centrifuge and removing them from the centrifuge as not to disturb pellet. Use a 1.5 mL tube rack from the RNA hood to move the tubes to and from the 4°C room, just be sure to wipe down the rack (but not the tubes) every time you put it back in the RNA hood.
5. While centrifuging, preheat the heat block to 58°C and label a 50 mL tube for IsOH waste. Place it in the RNA hood after having cleaned it.
6. After centrifuging, you should be able to see a TINY white spot attached to the bottom of the tube; it's very difficult to see, and you can't always see it.
7. Remove the supernatant to a waste container with a 200 μL tip, only removing down to the line at the bottom of the tube. Use multiple tips per tube if needed.
8. Add ice-cold 75% EtOH. Use at least 1 mL EtOH for every 1 mL TRI-reagent used, e.g. 200 μL EtOH for 200 μL TRI-reagent, making sure not to stick the pipette tip very far into the tube. Gently shake (not ridiculously gentle, but not vigorous) the tube a few times after adding. The white dot (RNA pellet) should detach from the tube wall, and you should be able to see it floating in EtOH. Make sure you can see the pellet in every tube - if you can't see it, you may not have a sample... Also make sure the sample settles to the bottom of the tube and doesn't get stuck on the side - you can also lose a sample this way.
9. Centrifuge the tubes at 4°C at 7500 g (9000 rpm) for 5 minutes. You should be able to see a tiny white dot at the very tip of the tube, preferably at the very base, but perhaps slightly up the wall.
10. Remove the EtOH. Start with a 200 μL tip, then move to a 20 μL tip, both set to max volume. Make sure you remove all the EtOH, including droplets on the side of the tube. Use as many tips as needed (Lucy went through 2 - 3 200 μL tips and 4 - 5 20 μL tips per tube). BE VERY CAREFUL! This is Lucy's least favorite step because it's so finicky.

11. Open the tubes to dry after you have removed the EtOH from ALL tubes. Leave them open 2 - 10 minutes to dry under the hood, 10 minutes MAX because if you dry too long, the sample will separate from the tube and you will lose it. If you don't dry long enough, the sample will be contaminated with EtOH. You should be able to see a small pellet still.
12. Resuspend the pellet in 20 - 50 μL of nuclease free water (Lucy used 40 μL). If the samples already appear to be drying out, you can add water before you're done removing EtOH from all of the samples to make sure they don't dry out too much. Many (but not all) patches will become clear as they dry - if it goes clear very fast, it means it's already v. dry and you should add the water sooner. Give the tube 1 - 2 minutes with the lid open to dry, then add water before going back to the rest of the tubes.
13. NOTE: You can make small aliquots of the water (in 1.5 mL tubes) to avoid contamination that you can use for this step and then take to the Nanodrop with you.
14. Incubate the tubes 10 minutes at 58°C in the heat block water bath. This incubation is technically optional, but Lucy recommends doing it every time (including the pipetting in the next step) because RNA isn't very soluble in water.
15. After 10 minutes, move the tubes to the RNA hood on ice. Remove the tubes from ice one at a time and gently pipette around 15 times each to mix. Leave them on ice for 20 minutes (starting from after you finish pipetting all tubes). You should get new ice for this step if the ice used previously is melting.
16. Optional: Clean RNA with RNeasy kit (you only need to do this if you're sequencing - you don't need to do it for qPCR).
17. Nanodrop the samples to determine concentration. Bring with you: samples on ice (with lid on), water aliquot on ice, RNase away bottle, pipette, tip box (unopened), and flash drive to save data.
18. To use the nanodrop: Clean everything with RNase away first, then with water from the aliquot you bring. Initialize the device, then select RNA-50, not DNA-50. Blank with water, then measure samples as usual - pipetting each sample a few times before sampling. If you change "overlay control" to "accumulate until clear", you will get all the plots on same graph. Make sure to add a sample ID each time if you do this (230 nm). Wipe with a kimwipe between samples but no RNase away.

19. NOTE: You're hoping for something greater than 5 ng/ μL .

A.4.6 cDNA SYNTHESIS (2 - 3 HOURS)

General Notes:

- This step removes the DNA from your sample so you have pure RNA.
- DNase I is stored in a -20°C freezer, as is the cDNA synthesis kit.
- You can also find a protocol for the DNase I rxn from NEB online. It uses 10 μg RNA sample + buffer to fill to 100 μL in a PCR size tube. If you don't get enough RNA from the extraction, you can scale this back for the amount you get.

Protocol :

1. Repeat the hood cleaning step if you're doing this another day, though it can easily be done after the RNA extraction steps in one (long) day.
2. Use the protocol from NEB's website - "a typical DNase I reaction" - but for example, we had to scale down to 43 μL from 100 μL on 5/21/16.
3. Get new ice if needed, cleaning the bucket properly before placing it in the hood. Also clean the RNase free shuttle you'll keep the DNase I in.
4. Work in PCR tube strips - label these first before starting anything.
5. Transfer the RNA sample (dumping the original 1.5 mL tube into the proper hazardous waste container).
6. Add DNase I (opening tubes one at a time to add).
7. Mix by pipetting the tubes one at a time - not too rough or you can degrade the DNase I.
8. DNase I PCR program in Lucy's folder (see NEB protocol).

9. The second step is to add something to stop the reaction (see NEB protocol). You really should add less than 1 μL for less than 100 μL sample, but 1 μL is the minimum we can add, so do that if you have limited sample.

A.4.7 qPCR REACTION (2 - 3 HOURS SET-UP, 2 - 3 HOUR RUN)

General Notes:

- The qPCR kit (KAPA SYBR FAST qPCR Kit) is stored in the -20°C freezer. It uses Master Mix (2X) Universal (in the kit).
- All you need is the master mix, your qPCR primers, your samples, and water.
- You'll always have at least 2 genes (# genes = # sets of primers), aka your gene(s) of interest and a housekeeping gene (usually *snb-1*) to make sure everything ran well.
- You'll use qPCR 96-well plates and special tape for these steps.
- Since this is just DNA, you can work on your benchtop now instead of the RNA hood.

Protocol:

1. Dilute the primers to the proper concentration if new. It's technically best to do this in Tris or something else, but Lucy just uses water.
2. Get ice to keep the samples and master mix (MM) on.
3. Mix one set of MM for each primer set. Keep these on ice in 1.5 mL tubes and note that the MM is sensitive to light, so you should cover the ice buckets with foil if needed and save mixing until the last step.
4. In PCR tube strips, mix (1 sample X 1 MM) per tube - 48 μL per tube.
5. Split each tube into 2 wells of the qPCR plate - 20 μL per well for 2 technical repeats.
6. Note that the MM is very bubbly and you don't want any bubbles in the wells, so be very sensitive when mixing and transferring between tubes and plate.

7. To run the qPCR program, turn on the computer (front) and qPCR machine (back left).
Open “realplex” on the computer and log in with the password “qbiolab”. Choose File → Open Assay → Lucy’s folder → KAPA SYBR assay.
8. Set up the names of the wells using the blue tube icon for “Unknown”. Label as you see fit; Lucy doesn’t usually do too much detail because she also keeps her own separate record of the set-up in her documentation, but it’s always good to label well.

A.4.8 ANALYZE QPCR DATA

B

Miscellaneous Protocols

B.1 RNAi

This protocol was compiled from the protocols of Eunjun (Lucy) Lee, Andrzej Nowojewski, Kathie Watkins, and the Ahringer Lab.

DAY 0:

1. Streak out your RNAi clone onto an LB + Carb 15 cm plate. Make sure you streak from either a -80°C freezer stock or a single colony from a previous streaking. Grow overnight at 37°C , and the plate can be stored for up to 2 weeks in the 4°C fridge with parafilm around the edge.
2. Make Carbenicillin and IPTG stocks if needed. You can make multiple aliquots of the stocks and freeze them for future use. Both chemicals dissolve in water.
 - (a) Carbenicillin, 50 mg/mL
0.05 g + 1 mL OR 0.1 g + 2mL OR 0.15 g + 3 mL OR 0.2 g + 4 mL OR 0.25 g + 5 mL
 - (b) IPTG, 1M
0.24 g + 1mL OR 0.48 g + 2mL OR 0.72 g + 3mL OR 0.95 g + 4mL OR 1.19 g + 5 mL
3. Once the stocks have been mixed (by vortex), both solutions need to be filter sterilized with $0.2\ \mu\text{m}$ syringe filters, then aliquoted into 1 mL tubes and stored at -20°C .

DAY 1:

1. Make standard NGM agar plates with Carbenicillin and IPTG. Because the plates will only be good for 1 - 2 weeks, you don't want to make too many. Mix the pre-autoclave ingredients and 1 L water, then autoclave for 30 minutes. After the solution cools to around 50°C , add the post-autoclave ingredients and pour your plates (6 cm). Cover the plates with aluminum foil after you're done pouring to protect them from light.
 - (a) Pre-autoclave:

3 g NaCl
20 g Bacto Agar
2.5 g Bacto Peptone
 - (b) Post-autoclave:

25 mL KP buffer
1 mL Cholesterol
1 mL 1M CaCl₂
1 mL 1M MgSO₄
1 mL 1M IPTG (final concentration 25 µg/mL)
500 µL 50mg/mL Cb (final concentration 1mM)

2. Pick a single colony of your RNAi clone and grow it in culture for 8 - 12 hours. Note that before plating your bacteria, you will want to concentrate it 10x, so use 3 mL of LB for each plate that you want to be able to make. Grow your cultures in Falcon tubes, 1 tube per plate.

1 plate: 3mL LB + 3 µL Cb₅₀ (final concentration 50 µg/mL Cb)
2 plates: 6mL LB + 6 µL Cb₅₀
3 plates: 9mL LB + 9 µL Cb₅₀
4 plates: 12mL LB + 12 µL Cb₅₀
5 plates: 15mL LB + 15 µL Cb₅₀
6 plates: 18mL LB + 18 µL Cb₅₀

DAYS 2 - 4:

1. Centrifuge the overnight cultures at 4000 rpm for 10 minutes. Aspirate the supernatant.
2. Resuspend them to make a 10X concentrate. Per Falcon tube, add and vortex to mix:

300 µL LB
0.6 µL Cb₅₀ (final concentration 100 µg/mL Cb)
3 µL 1M IPTG (final concentration 10mM)

NOTE: The higher concentration of 100 - 200 µg/mL Cb in this culture helps to prevent plasmid loss.

3. Without exposing the plates to UV first, uncover them (take away the aluminum foil), and spread 200 - 300 µL of the concentrate on each 6 cm RNAi plate. Pipette the concentrate on

and then either tilt/swirl the plate to spread or use a spreader. It's easier to use a spreader if you're only dealing with one RNAi clone; it's easier to just tilt/swirl if you're using multiple and worried about accidental contamination of your plates by accidentally using the wrong spreader.

4. Keep the plates under the hood with the blower on for 30 - 60 minutes to let them dry.
5. Wrap your plates in aluminum foil and let them grow at 25°C for 2 days. Any plates you didn't seed should be wrapped in aluminum foil and stored in the 4°C room for use no more than 2 weeks later.

DAYS 5+:

The plates are ready to use. You can either put synchronized L1s (from a bleach prep) or semi-synchronized embryos on the plates and grow them to the desired stage at the desired temperature.

SPECIFICS FOR ROL-6 RNAI PROTOCOL:

DAY 1:

1. Make 3.5 cm NGM/no antibiotic plates if you don't have any stocked up - follow the same protocol as regular NGM plates (see below), just don't add ampicillin. Having these ready is the limiting factor in being able to do your RNAi, so do this first. Only make 500 mL of NGM because that makes a lot of plates. 3.5 cm plates need 3 - 5 mL of NGM, depending on the type of plate. These plates can be stored at 4°C for several months and used later.
2. In the evening, streak out your RNAi clone onto an LB + Cb or LB + Amp 15 cm plate. Cb is more stable than Amp.
3. Grow at 37°C overnight.
4. This plate can be stored at 4°C with parafilm around the edge for up to 2 weeks if you will need to use it again.

DAY 2:

1. In the morning, mix 6 mL LB and 4 μ L Amp in a Falcon tube and inoculate it with a single colony from your streak plate. This makes a 6 mL culture that can make 6 RNAi plates.
2. Grow the culture 8 - 12 hours at 37°C (on the shaker), not over 12, ideally 8 - 10 hours. Do this in the morning so you don't have to come back super late to make the plates.
3. Spin down the bacteria at 1100 RCF (not the normal units on the centrifuge - make sure to change) for 10 minutes.
4. Resuspend each pellet (if you make multiple tubes) in a 600 μ L IPTG/Amp mixture.
 - Pour the LB out after spinning it down; the pellet will be solid enough that you can just decant it.
 - IPTG/Amp mix (makes 600 μ L), mix in a 1.5 mL tube:
 - 240 μ L 1M IPTG (can be thawed at room temp)
 - 18 μ L 100 mg/mL Amp (same as used before)
 - 342 μ L H₂O
 - Add 600 μ L of this mix directly to the Falcon tube with the pellet and vortex on a low speed to resuspend it.
5. Pipette 100 μ L of the resuspended bacteria onto a 3.5 μ cm NGM/no antibiotic plate and swirl the plate around to spread the bacteria and cover the whole plate.
6. Put the plates (with their lids cracked) near a Bunsen burner that's turned on and leave there for 5 minutes or so until the bacteria dries. Don't put under the hood!
7. Cover the plates in foil, leaving them right side up, and incubate them for 48 hours at room temperature (can just leave on your bench).

DAY 5

1. 48 hours later, the plates can either be used immediately or stored at 4°C for up to 2 weeks.
2. To use the plates:

- Do a bleach prep on your strain of interest (see separate protocol). Note, you can do this ahead of time and keep the L1 larvae on the shaker for up to 1 week.
- Put a drop (20 μ L) of your synchronized L1 larvae on an NGM plate and count the number of worms so you'll see how many you get per drop. Also for this strain, check that you see enough worms with the red marker. You'll want 25 - 30 worms per RNAi plate, so dilute if needed.
- Put 1 - 2 drops of the synchronized L1 larvae on an RNAi plate. It's probably best to do multiple plates to make sure you'll have enough worms.
- Grow at 25°C to induce the highest RNAi response (use an incubator).
- Check plates for nonrollers 2 - 3 days later or at the developmental stage of interest. For this strain, also make sure to select for mCherry.

NOTE: To propagate the rol-6 line before the RNAi (and after), you must make sure you're selecting for only the worms that show red fluorescence.

B.2 MAKING TAE BUFFER

- TAE buffer comes in 50x, but we want it in 1x for use.
- Measure 20 mL of 50x TAE buffer into a graduated cylinder. It is best to keep a separate graduated cylinder just for this so it won't get contaminated.
- Fill it the rest of the way up to 1000 mL (980 mL added) with sink water. Put parafilm over the top and shake it to mix.
- This can be stored in a glass bottle indefinitely, and the bottle doesn't need to be washed between uses, unless it starts looking grungy.

B.3 MAKING 1% AND 2% AGAR

- Make 1% up in a 500 mL bottle; make 2% up in smaller amounts because it is used less (and only make if you need it).
- Put your initials and the date on the bottle you are using.
- Mix TAE (or water) and agarose in appropriate amounts, and swirl the bottle/flask you are making it in. Microwave on full power (with the cap off, if it's a bottle) for about 2 minutes. Stop anytime within those 2 minutes that you see it starting to boil, swirl it, and then put it back in for more time. It should be clear when all the agarose is dissolved.
- When it's clear, put it in the 65°C water bath to store it.
- The percentage is in weight (grams) to volume (mL), i.e. 3 g agarose and 300 mL 1x TAE buffer or water will give you 1% agar while 1 g agarose and 50 mL 1x TAE buffer or water will give you 2% agar.

B.4 THAWING WORM STRAINS

- Find where the strain you are thawing is stored and make a note that you are removing the strain with the date and your name. Also note if you are removing the last tube of that strain.
- Take the tube out of the -80°C freezer and thaw it at room temperature.
- When it's fully thawed, pipette about half of it (150 μL) onto a seeded OP50/NGM plate and put the plate under a hood in the media prep room with the lid not fully on. Let it dry for 10 - 20 minutes.
- When it's fully dried, close the lid and store as usual. It's best to keep it at room temperature, and you should have worms within two days.
- You can also use the second half of the thawed tube to make a second plate. Otherwise, throw it out (it doesn't get refrozen).

B.5 MAKING 70% ETHANOL

- Higher proof ethanol is stored in the flammables cabinet!
- To calculate how much ethanol and water to add:

$$((.95) * (\text{amount you need to add})) / (\text{total amount you want}) = 0.7$$

- Solve for the amount you need to add - that's the amount of 95% ethanol to add (you can either pipette directly from the container or use a graduated cylinder to measure it, or just pour and guesstimate the amount - accuracy is not super important). Subtract that from the total amount of 70% ethanol you want to have, and that's the amount of distilled water to add.

B.6 PCR WITH GENOMIC DNA

- Because the DNA has already been extracted from the worms, only the second PCR cycle of a normal SWPCR needs to be run.
- To make the master mix for this cycle, mix 1 μL genomic DNA, 1 μL forward primer, 1 μL reverse primer, 25 μL 2X one taq, and 22 μL water, for 50 μL total. Note that you always want the volume of 2X one taq to be half of the total volume you are making. You can change the amount of water you add to the master mix to make this the right ratio.
- Everything else in the protocol is the same - PCR this on the appropriate cycle and run 10 - 20 μL on a gel; you should get very strong bands. This can be used to make sure primers you made and ordered are working properly if you are having trouble seeing results from regular SWPCR.

B.7 MAKING NaOH + BLEACH (20% ALKALINE HYPOCHLORITE SOLUTION)

- This should be made fresh each time you need to use it. This recipe makes 15 mL, so it can be made in a 15 mL tube:

8.25 mL ddH₂O (measure 8 mL with glass pipette, 250 μ L with normal pipette)
3.75 mL 1M NaOH (measure 3 mL with glass pipette, 70 μ L with normal pipette)
3.0 mL bleach (measure 3 mL with glass pipette)

- Use autoclaved MilliQ water and non-germicidal bleach (and not the super concentrated bleach). The order of adding ingredients is not important.
- This is used in the procedure for synchronizing worms via bleaching, but also to decontaminate worm strains.

B.8 MAKING A NEW STOCK OF BACTERIA FOR INOCULATION

- This protocol is via Kathie Watkins.
- Find the stock of OP₅₀₋₁ in the -80°C freezer. The “-1” is important because it means that the strain has streptomycin resistance, and streptomycin is usually used in all standard NGM plates.
- Let the stock thaw partially (but not all the way, this won’t take long). Then, taking a streaking tool (handle with wire loop on the end), streak some of the bacteria on a new, unseeded NGM + Strep plate. Put this in the 37°C incubator overnight.
- Take the plate out in the morning and place it in the 4°C fridge until 3 PM or so. At 3 PM, use an autoclaved toothpick (and gloved hands!) to pick a single colony from the plate and drop the toothpick into a bottle of LB broth + Strep. Make sure to flame the bottle after opening and before closing it to keep it clean. Close the cap, but not all the way (want it to be a little loose) and place it on the bottom shelf of the 37°C incubator (it doesn’t need to shake).

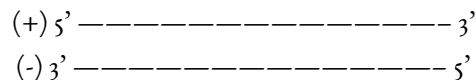
- The next morning, the bottle is ready for use. It can be taken out and placed in the 4°C fridge.

B.9 MAKING A NEW THERMOCOUPLE TIP

- Before constructing the tip, make sure to resolder the tip of the thermocouple wire if it is not a new one and check that it is working. If there is no soldered tip, the thermometer will show that the thermocouple is not working. To solder it, keeping it connected to the thermometer/power supply, put the thermocouple tip above a very hot fire source (blue fire) for a second or two, then remove the tip quickly. If the tip is soldered, the thermometer will show temperature. After you verify the thermocouple is working, you can build the tip.
- Cut a piece of size 5 tubing (the same size we use to load worms) about 5 cm long and string it onto the thermocouple.
- Take a syringe tip off (the metal portion) and use a flame to get any plastic residues off. Break off about a 1 cm piece of the syringe tip.
- Take a pipette tip (medium size). First trim the tip so that a syringe tip (metal piece) fits in it, then trim the other end so that the total length is about 1.5 cm.
- String the pipette tip onto the thermocouple next, then the syringe tip.
- Lastly, cut a piece of size 1 tubing about 5 mm long and string that onto the thermocouple as well.
- Thread the syringe tip into the pipette tip, then the smaller tubing into the syringe tip, and bring this to the end of the thermocouple. Move the bigger tubing down as well. Drip super glue into the pipette tip, making sure there is no air, and let it set.
- Note that it is important that the tip of the thermocouple wire (the soldered bit) sticks out of the smallest tubing just a little bit, and that the smallest tubing sticks out of the syringe tip a little bit as well. The smallest tubing only needs to go into the syringe tip several mm, not the entire length.

B.10 PICKING AND ORDERING PRIMERS

- Go to wormbase.org to find the sequence of the DNA in the region in which you want to pick primers; it should be listed under molecular details. Select the region in which you are trying to pick primers and copy/paste it to Primer3 (can also specify a right and left primer if you have something picked out already, such as ending the primer with the one base that has been mutated in a strain). Primer3 will give you a variety of possible primers to choose from.
- After picking a couple primers from the list given by Primer3, use two other websites - Primer BLAST and NetPrimer (which requires a login with username and password) to check the primers. Check to make sure the sequence on WormBase was correct (sometimes it can be wrong - if it's right for the given gene, the primer-picking software should recognize it as that gene) and to determine which primer is the best. You want to maximize G/C content and minimize hairpins/self complementarity/etc, all of which the programs will check for.
- A note about the order of the bases for primers – double stranded DNA:



The strand/sequence listed on WormBase is the positive strand; this is the sequence that the mRNA will have. The primer runs from the 5' end to the 3' end. This is the forward primer.

- The negative strand, also known as the antiparallel or complementary strand, runs the opposite direction. This is the sequence that the mRNA will be copied from (thus the mRNA will be reverse complementary to it). The primer again runs from the 5' to the 3' end. This is the reverse primer. When programs calculate the best primer for you, the program may return the primer as a sequence from the positive strand; you want to order the reverse complement of this sequence for the reverse primer. There is a reverse complement website that will do this switch for you, or you can check “reverse complement” when you enter the sequence on the ordering website.
- To order primers, use the Operon website. To add items to the shopping cart, go to Shopping Cart → Add Oligos → input a name for your primer, the sequence (with the option to check reverse complement), and check that the settings are salt-free and 10 nM.

B.11 COLONY FORMING UNITS TEST (CFU COUNTING)

- Take your bacterial suspension and make dilutions in 1.5 mL tubes:

1 mL suspension no LB
 10^1 100 μ L suspension 900 μ L LB
 10^2 10 μ L suspension 990 μ L LB
 10^3 1 μ L suspension 999 μ L LB

You can go all the way up to 10^6 if necessary, depending on how concentrated your suspension is to begin with.

- Take an LB plate with no antibiotics and pipette 10 μ L of each dilution in a row across the top of the plate. Tilt the plate to let these drops streak out, taking care to not let them run into one another. Alternatively, you can just plate 100 μ L of the suspension spread out on a plate.
- Place the plate at 37°C overnight and count the colonies in the morning.
- The CFU/mL can then be calculated as: (# colonies)/(0.01 mL* dilution) where 0.01 mL is for the 10 μ L you plated, and the dilution is 1, 10^1 , 10^2 , etc...

B.12 MAKING 1M NaOH

- Note that the molecular weight of NaOH is 39.997 g/mol.
- To make 1M NaOH:

10 g NaOH
250 mL milliQ water

- DO NOT AUTOCLAVE THIS. Because it's a strong base, and you make it with milliQ water, it should already be sterile.

B.13 MAKING BACTERIAL STOCK CONCENTRATIONS

- Stock concentrations are always in mg/mL, whereas working concentrations are in ng/ μ L, which is the same as μ g/mL.
- Higher stock concentrations may not fully dissolve, or may precipitate at -20°C , so you'll have to adjust your stock concentration accordingly.
- Store all stocks at -20°C .
- Any antibiotic LB plates should just be made with fresh antibiotic stock, not from the -20°C stock because of the amount needed.
- All stocks of antibiotics - both for plates and for stock - need to be filter sterilized with a $0.22\ \mu\text{m}$ filter before usage! The powder antibiotic stocks are not sterile!

KANAMYCIN To make:

1 mL stock: add 0.01 g Kanamycin to 1 mL water. Filter sterilize.

5 mL stock: add 0.05 g Kanamycin to 5 mL water. Filter sterilize.

Aliquot into 1 mL stocks for the -20°C .

Working concentration: 50 ng/ μ L (50 μ g/mL)

Stock concentration: 10 mg/mL

Add: 5 μ L/mL

STREPTOMYCIN To make:

1 mL stock: add 0.06 g Streptomycin to 1 mL water. Filter sterilize.

5 mL stock: add 0.3 g Streptomycin to 5 mL water. Filter sterilize.

Aliquot into 1 mL stocks for the -20°C .

Working concentration: 180 ng/ μ L (180 μ g/mL)

Stock concentration: 60 mg/mL

Add: 3 μ L/mL

CARBENICILLIN To make:

1 mL stock: add 0.05 g Carbenicillin to 1 mL water. Filter sterilize.

5 mL stock: add 0.25 g Carbenicillin to 5 mL water. Filter sterilize.

Aliquot into 1 mL stocks for the -20°C .

Working concentration: $100\text{ ng}/\mu\text{L}$ ($100\text{ }\mu\text{g}/\text{mL}$)

Stock concentration: $50\text{ mg}/\text{mL}$

Add: $2\text{ }\mu\text{L}/\text{mL}$ to solution

B.14 MAKING 40% (BY VOLUME) GLYCEROL STOCK

- Mix 20 mL glycerol and 30 mL water (use autoclaved, sterile water) in a 50 mL conical by shaking.
- Store at RT.

B.15 MEDIA PREP

GENERAL NOTES

- Label your glassware with the name of the media, your initials, and the date. Folding over one end of the tape before sticking it on makes removal easier after autoclaving.
- If you use a spatula to measure chemicals, to properly clean it, first rinse it with water from the sink and rub it clean. Next, rinse it with ddH₂O, taking care not to touch it. Then rinse with ethanol, again not touching, and air dry it.
- To make a 1 M solution of something, find the molecular weight on the container (or look it up online). The molecular weight is the number of grams to add to 1 L of water for it to be a 1 M solution. To make 100 mL of a solution that is 1 M, therefore, divide the molecular

weight by 10 and add that many grams. For example, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ has a MW of 246.48 g, so you want to add 24.648 g to 100 mL of ddH₂O.

- It's very important to check the exact chemical formula - you want the same number of waters as listed under the ingredients, and some chemicals are sold in multiple formats.
- Kyung Suk's recommended method of measuring is to use a graduated cylinder to accurately measure 1 L of ddH₂O into a bottle. Mark where it is with a Sharpie, then pour some of the water back into the graduated cylinder - back down to 800 mL or so. Add your ingredients, dissolve them, and then pour in ddH₂O to get to your Sharpie line.
- Note that it is important to dissolve ingredients in an amount of water less than your total solution volume before topping off the water because some of the chemicals have water in them and will increase the volume of your solution when added.
- When autoclaving, use a liquid autoclave cycle. Place glassware into a plastic tub with a small amount of water in the bottom and use hot gloves to place it in the autoclave. Use Program 10 (30 minutes, liquid) if there is a lot of agar; use Program 12 (20 minutes, liquid) if there isn't a lot of agar. It should take about an hour to complete the cycle (but book the autoclave for at least 1.5 hours).

M9

INGREDIENTS (FOR 200 ML):

3 g KH_2PO_4 (potassium phosphate, monobasic)

6 g Na_2HPO_4 (sodium phosphate, dibasic)

5 g NaCl (sodium chloride)

1 mL sterile 1 M MgSO_4

METHODS:

1. Put 800 mL of MilliQ water and a stir bar in a 1000 mL beaker.
2. Add the first 3 ingredients pre-autoclave and let it mix, then pour it into a graduated cylinder (more accurate measurements) and fill to 1000 mL. Cover the top with parafilm and mix it up. Pour it into bottles (it's usually stored in 500 mL bottles).
3. NOTE: The graduated cylinder does not have to be autoclaved before use since the M9W will be autoclaved, but rinse it and make sure to wash/autoclave it after use.
4. Autoclave the bottles.
5. When it has cooled down completely, add the 1 mL 1 M MgSO₄. Unless the M9 is needed urgently, it is usually easiest to wait and add this the following day, giving the media overnight to cool.

1 M MgSO₄

INGREDIENTS (FOR 100 mL):

24.648 g MgSO₄ ⋄ 7H₂O

100 mL ddH₂O

METHOD:

1. Start with 80 mL of water. Add the chemical and mix, then add water to get to 100 mL.
2. Sterilize by autoclaving (liquid), then store at room temperature.

B.15.1 LB BROTH

INGREDIENTS (FOR 1 L):

25 g LB Broth

1000 mL ddH₂O

METHODS:

1. Add 800 mL MilliQ water and a stir bar to a beaker.
2. Mix 25 g of the LB broth in. Again, pour into a graduated cylinder and fill to 1000 mL, covering the top with parafilm to mix. LB broth also gets stored in 500 mL bottles.
3. Autoclave using the bottles
4. Let the bottles cool off, then place them in storage on your bench.

NEMATODE GROWTH MEDIUM PLATES

INGREDIENTS (FOR 200 mL):

3 g NaCl

17 g Bacto Agar

2.5 g Bacto Peptone

0.2 g Streptomycin (powder form)

1 mL Cholesterol

1 mL 1 M CaCl₂

1 mL 1 M MgSO₄

25 mL KP buffer

METHODS:

1. Add 1000 mL of MilliQ water and a stir bar to a flask.
2. Add the first 4 ingredients pre-autoclave (sodium chloride, bacto agar, bacto peptone, and streptomycin) and let it mix. Cover the container with aluminum foil and it is ready to be autoclaved.
3. Autoclave using the liquid autoclave cycle, 30 minutes.
4. Post-autoclave, cool the bottle down to about 50°C on a hot plate with the stirrer on (using a temperature strip to measure), then add the last four ingredients listed above. If you need to wait longer than it will take to cool to 50°C (an hour or two), you can keep the hot plate on to prevent it from cooling too much (and the agar solidifying).
5. Pour it in 10 mL increments into 6 cm plates (see below).

CHOLESTEROL, 5 MG/ML

INGREDIENTS:

250 mg (.25 g) cholesterol

50 mL 100% ethanol

METHODS:

1. Measure the cholesterol on a scale in the media prep room. Pipette the ethanol from a stock bottle.

2. Mix them in a 50 mL tube and place on a shaker for a couple hours (or overnight) to dissolve the cholesterol - it will take some time.
3. Technically you should store this below room temperature, but it's okay to store it at room temperature with parafilm around the lid to prevent evaporation.

1 M CaCl₂

INGREDIENTS (FOR 100 mL):

11.099 g CaCl₂ (no water!)

100 mL ddH₂O

METHOD:

1. Start with 80 mL of water. Add the chemical and mix, then add water to get to 100 mL.
2. Sterilize by autoclaving (liquid), then store at room temperature.

1 M MgSO₄ See above.

POURING PLATES:

1. After the agar solution has been started in the autoclave, come back and stack your plates - you should get about 100 plates for every liter of agar that you make. Stacks of 5 work well for gripping and counting.
2. When the flasks get removed from the autoclave, let the solution cool down to 45°C - 50°C; use temperature-reading tape located in the media prep room to track the temperature. While the agar is cooling, keep the flask on a hot plate (set to 60°C or so) with a stir bar going. If the agar gets below 37°C, it will start solidifying and be unusable, so be careful.

3. Add the four post-autoclave liquid ingredients, and let them stir in. Once cooled and everything is added, you're ready to pour.
4. Move the flask under the fume hood (with regular fluorescent light and the exhaust running). Use the large pipette to get about 55mL of the agar. Put 10 mL in each petri dish, moving through a stack as you go from bottom to top. (Top button of pipetter sucks up liquid, bottom releases.) Get rid of any bubbles that form, as they will not pop on their own.
5. Let the plates solidify overnight.

INOCULATING PLATES:

1. Before inoculating, spread the plates out to a layer one plate thick and leave the germicidal light on for 30 - 60 minutes to kill any contamination.
2. Inoculation is also done under the hood. You need the plates (restacked in stacks of 5), the alcohol burner, a jar of alcohol, a petri dish with a small quantity of bacteria, and the bacteria spreader.
3. Pipette some of your overnight culture of bacteria into an empty petri dish.
4. Sterilize the end of your bacteria spreader (dip in alcohol, then burn off), then dip the bottom side of the triangle in the bacteria. Make a rectangle of the bacteria on the surface of the agar by dragging the dipped side across it. Try not to spread to the edges of the plate or get bacteria on the walls. Again, work through one stack at a time, bottom to top.
5. Repeat for all plates. When you finish, leave them in stacks for a bit, then flip them upside down and move them into a Tupperware container. Place them in an incubator overnight for the bacteria to grow; remove them the next morning. They can also grow at room temperature, it will just take longer. After growing, they can either be placed in the 15°C fridge to use or stored at 4°C.

S-MEDIUM

INGREDIENTS (FOR 200 mL):

195 mL S-Basal

2 mL potassium citrate

2 mL trace metals

600 uL CaCl_2

600 uL MgSO_4

METHODS:

1. Mix all ingredients in a clean, autoclaved bottle, making sure to properly flame bottles, etc. as they are opened to keep everything sterile. Shake to mix. Use regular pipettes or glass pipettes to measure ingredients, depending on the volume.
2. Note, to test that the S-basal is good, you can mix some with OP50, add worms, and keep it on a shaker for 8 hours or so, then spin it down and remove the worms and the eggs they produced and see if they develop normally. You don't need to do this every time, just when you make new ingredients if you want to test them.

S-BASAL

INGREDIENTS (FOR 1 L):

5.84 g NaCl

1 g K_2HPO_4

6 g KH_2PO_4

METHOD:

1. Dissolve all 3 ingredients in 800 mL ddH₂O, then add ddH₂O to bring up to 1 L.
2. Sterilize by autoclaving (typical liquid autoclave).
3. After autoclaving, add 1/1000 the volume of 5 mg/mL cholesterol in ethanol 100% (for 1 L, this is 1 mL to be added). You can warm the bottle overnight at 37°C to help dissolve the cholesterol.
4. Store at room temperature.

1 M POTASSIUM CITRATE, pH 6.0

INGREDIENTS (FOR 200 mL):

42.02 g citric acid, monohydrate

200 mL ddH₂O

34 g solid KOH (will be adjusted)

METHOD:

1. Add the citric acid to about 160 mL of water and mix in a bottle.
2. To measure the pH, you need an ice bucket, pH paper, and buffer solutions at pH 4 and 7. First, dilute the buffers (about 0.5 - 1 mL in a 15 mL tube, then top off to 15 mL with ddH₂O). You can just pour, this doesn't need to be accurate, it's just to get rid of the color of the solution). Pipette 20 μ L of the buffer solution on each colored spot on the pH paper, making sure not to touch the tip of the pipette to the paper, and compare the colors to the given pH color chart on the packaging.
3. Add close to the asked for amount of KOH to your solution and mix. Note that this reaction will generate a lot of heat, and pH measurements vary depending on the temperature, so use an ice bucket to cool the solution to room temperature before making pH measurements.

- Drop 20 μL of the solution onto a pH strip and decide what the current pH is. Make a chart tracking how much KOH you have added and what you think the pH is, then add KOH in 0.5 - 1 g increments to the solution until you get to 6.0 pH. Note that it's easy to overshoot, so when you think you're getting close, add smaller amounts.
- When you've gotten to 6.0, add water to bring up the volume to 200 mL
- Sterilize by autoclaving (liquid) and store at room temperature.
- Note that KOH collects moisture from the air very easily, so always close the lid of the container immediately.

TRACE METALS 100X

INGREDIENTS (FOR 500 mL):

500 mL ddH₂O
0.93 g disodium EDTA
0.345 g FeSO₄ \diamond 7H₂O
0.1 g MnCl₂ \diamond 4H₂O
0.145 g ZnSO₄ \diamond 7H₂O
0.0125 g CuSO₄ \diamond 5H₂O

METHOD:

- Measure all chemicals on the high-precision balance. Be especially careful with the copper sulfate since it is such a small amount. Add them to 500 mL of ddH₂O in a bottle. Because it is such a small amount of all of the chemicals, you don't have to worry about the additional water gain. Shake to mix.

2. Sterilize by filtration with a $0.22\ \mu\text{m}$ filter. To do this, rinse a 30 - 50 mL syringe with ddH₂O, then filter the solution in 30 - 50 mL quantities with the filter attached.
3. After filtering into storage bottles, wrap the bottles with aluminum foil to protect the solution from light. Store at room temperature.
4. It is recommended to make 500 mL as described above to make your measurements more accurate since they are so small, even though you don't use very much of the solution in medium. You can store it in 250 mL bottles (or smaller) after filtering.

1 M CaCl₂ See above.

1 M MgSO₄ See above.

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