# A Microfluidic Platform for Exploring Learning Behavior in *C. elegans*

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## Abstract

Microfluidic technologies are popular for biological research, enabling precise physical and chemical control of the microenvironment surrounding living cells and small organisms. *Caenorhabditis elegans*, a 1 mm long nematode, is capable of olfactory associative learning using the classical conditioning paradigm of pairing an unconditioned stimulus that elicits an innate response, such as food, with a second stimulus, such as an odor, which then elicits a learned behavioral response to this conditioned stimulus alone. Conventional chemotaxis assays on agar petri-plates have been widely used to observe behavioral changes indicative of associative learning; however, reproducibility of these behavioral assays is a major challenge. Here, we describe a microfluidic system that improves the reproducibility of chemotactic behavioral assays by providing better spatiotemporal control of stimuli, gentler worm handling, and more detailed behavioral quantification. Specifically, the microfluidic designs in this study present a uniform conditioning environment followed by a temporally stable linear odor gradient to assess changes to olfactory preference. Stimuli are presented in an enclosed environment to multiple worm populations whose locomotory patterns are analyzed using machine vision. Furthermore, we established an optimized protocol for a positive associative learning paradigm in which animals increase their preference for an odorant, butanone, when previously paired with bacterial food. We reproduced plate-based learning results in wild-type and learning-deficient genetic mutant animals, and demonstrated how developmental stages and chemicals alter the plasticity of olfactory preference.

## I. Introduction

Learning and memory are fundamental components of human and animal behavior, allowing adaptation to changing environments, efficient exploitation of resources, and avoidance of harmful conditions. While it is essential to learn external cues that trigger anticipation of events crucial for survival, it is equally essential to learn to ignore irrelevant cues that have no predictive value. Mechanisms of learning and memory are conserved throughout animal evolution, with many initial insights coming from studies on invertebrates with simpler nervous systems. Early experiments on the marine mollusk *Aplysia* localized behavioral plasticity to biochemical and synaptic changes in specific neurons [1]. Genetic screens in the fruit fly *Drosophila melanogaster* and nematode *Caenorhabditis elegans* (*C. elegans*) provided insights into genes and proteins involved in learning and memory [2-4]. Since invertebrate brains use the same molecules, such as neurotransmitters and neuropeptides, to carry out the same neural functions as in humans [5], invertebrate studies in the field of learning are highly relevant to our understanding of human learning processes as well. For example, most genes involved in learning and memory across multiple organisms are conserved in *C. elegans,* such as: NMDA receptors (*nmr-1* and *nmr-2*), AMPA-type ionotropic glutamate receptor (*glr-1*), MAGI/S-SCAM synaptic folding protein (*magi-1*), and CAMKII (*unc-43*), where *C. elegans* homologs are indicated in parentheses [6-9]. This short list of essential genes touches upon the complexity and conservation of learning behavior across species, and motivates selection of an efficient and experimentally-convenient model organism to study the underlying molecular forces involved in learning.

The nematode *C. elegans* is a compelling model organism for studying genetics, neural activity, and behavior, striking an intricate balance between biological simplicity and behavioral

complexity. The complete wiring of its 302 neurons simplifies the study of neural circuitry and disease pathways in comparison to vertebrate models, yet 60-80% of a *C. elegans'* genome and proteome contain human homologs [10, 11], supporting its use in modeling human diseases. Further advantages to *C. elegans* include the availability of resources and tools to manipulate genes, the ability to quantify locomotory behavior and neural activity via optical imaging through its transparent body, and the ease of cultivating *C. elegans* rapidly from egg to adulthood (3 days at 20 ºC). With these advantages in mind, *C. elegans* continue to be a major model organism for many areas of study in biology.

*C. elegans* detect many stimuli via their amphids, the largest chemosensory organ consisting of 12 sensory neuron pairs with ciliated dendrites [12]. These amphid neurons play a role in locomotory behaviors such as chemotaxis, thermotaxis, and osmotaxis, as well as mechanosensation and other responses such as egg-laying and entry into the dauer developmental pathway [12-15]. Despite its simple neurocircuitry, *C. elegans* are remarkably capable of learning and associating environmental cues that predict food or dangers that should be avoided [6, 16-18]. Prior learning studies in *C. elegans* have probed both nonassociative learning (single-stimulus) and associative learning (paired stimuli) to mechanosensory, thermosensory, and chemosensory cues. Rankin et al. (1990) studied *C. elegans* behavioral plasticity of the 'tap withdrawal response" (TWR), a behavior whereby worms reverse in response to tapping of the petri dish housing the animals. Repeated administration of this nonlocalized mechanical stimulus result in reduced amplitude and frequency of the TWR, a characteristic of nonassociative learning called mechanosensory habituation [19]. *C. elegans* also exhibit associative learning, which was first established when Hedgecock and Russell (1975) found that worms grown with food at a specified temperature

migrate to their temperature of cultivation in a thermotaxis assay [20]. Similarly, chemosensory responses are sensitive to prior experience [21], and chemical cues generally become more attractive when previously paired with favorable conditions like abundant bacterial food, or less attractive when paired with harmful conditions. To date, numerous test odorants (benzaldehyde, diacetyl, butanone, and 1-propanol) have been paired with either favorable (food abundance, food-related odors, NaCl salts) or unfavorable conditions (acidic environment, high osmolarity, high temperature, copper presence, food shortage, overcrowding) for a period of time in order to study how those experiences change future behavioral responses to the test odorants [18, 22-24]. Forward and reverse genetic screens have uncovered several genes required for associative learning. Most studies have demonstrated that learning is stimulus-specific; for example, training to one odor does not affect responses to different odors, even ones detected by the same neurons [25, 26]. Further, learning is often short-term and persists for several hours to one day, and the timecourse of training affects memory retention [22].

Despite great progress made by these studies, there remain numerous additional questions about the nature, mechanism, timing, and location of plasticity within the neural circuit, and how specific training paradigms affect these processes. However, experimental limitations have hindered progress in these areas. Much of the research on *C. elegans* associative learning used agar plate-based chemotaxis assays to quantify changes in behavioral preference, after training paradigms performed in liquid culture or on different agar plates containing training stimuli. The chemotaxis assay requires simultaneous spotting of concentrated odorants on opposite sides of an agar Petri dish to generate a diffusion-mediated spatial gradient, and then counting the fraction of worms that accumulate at each spot [21, 27].

Despite its common use, this technique can have poor trial-to-trial reproducibility due to several factors, including sensitivity to external environmental conditions (i.e. humidity and outside odors), extensive animal handling during washes (between training and behavior testing), instability of odorant concentration over time, and the use of chemical paralysis agents that prevent monitoring behavior dynamics.

To circumvent these issues limiting reproducibility, this study aims to replace both the training phase and the behavioral assay to quantify odor preference with a single microfluidic device that is insensitive to the external environment, can deliver multiple stimuli, and can perform training and behavioral assays without any animal handling or transfer. *C. elegans* behavioral assays based on microfluidic technologies have demonstrated improvements in reproducibility compared with agar plates, due to highly-controlled spatiotemporal delivery of stimuli and a naturally closed system that prevents external environmental influences on the assay outcome [28]. These devices contain microstructured behavioral arenas composed of a transparent, non-toxic, gas permeable, and bio-compatible material polydimethylsiloxane (PDMS) [29], that enable the quantitative recording of locomotory responses to a variety of spatial and temporal chemical patterns, as well as unobstructed optical imaging of neural activity in freely-behaving animals [30].

To demonstrate the suitability and improved performance of microfluidic devices as a learning assay, we selected as proof-of-concept a model of olfactory learning in *C. elegans* that conditions animals to an odorant (butanone) in the presence or absence of bacterial food [18], a paradigm that demonstrates features of both associative and non-associative learning. When *C. elegans* detect bacterial food, they reflexively crawl towards it by detecting food-related odors and they remain in feeding areas via chemical and mechanical cues [31]. This

innate, unlearned reflexive behavior is the unconditioned response (UR) to the unconditioned stimulus (US), food. Butanone is a weakly-attractive volatile organic compound emitted from numerous bacteria such as *Escherichia coli* (*E. coli*) and the nematocidal bacterium, *Bacillus nematocida (B. nematocida)* [32]; thus, it may have some innate attraction as a predictor of nearby food. Worms exhibit chemosensory adaptation to butanone, temporarily blunting their behavioral response after continuous exposure to the chemical alone without food, as it loses its predictive value [18, 23, 25]. Adaptation to butanone is a form of non-associative learning distinct from sensory or motor fatigue (a physical inability to respond) [33, 34], because the response decline can be prevented by prior experience; for example, when both odorant and food reward are presented simultaneously, adaptation is suppressed [18]. Torayama et al. found that exposing animals to butanone and *E. coli* food together not only suppressed adaptation but also enhanced the animals' preference for butanone above the level of naïve fed worms exposed to food without butanone [18]. The enhanced attraction to butanone after prior pairing with food did not carry over to other chemoattractants that are detected by the same neuron that senses butanone, such as benzaldehyde or 2,3-pentanedione.

The butanone enhancement associative learning paradigm in *C. elegans* differs from conventional classical conditioning in mammalian studies, which typically condition a neutral stimulus with multiple spaced training bouts of "forward conditioning," in which the conditioned stimulus is presented before the unconditioned one. The timing between presentation of odor (CS) and food (US), including the number and duration of paired periods, the inter-stimulus interval (ISI) if more than one pairing, and the order in which each stimulus is presented, can impact the effectiveness of paired conditioning. In one study, C. elegans were trained in "backward conditioning" (US precedes CS), "simultaneous conditioning" (both presented

together), and “forward conditioning” (US after CS), and of the three variations, “simultaneous conditioning” proved to be most efficacious for associative memory acquisition and retention [22]. In contrast, mammalian studies generally find that “forward conditioning” leads to fastest learning. The number and duration of training sessions also affects memory formation and retention: massed training consisting of uninterrupted repeated trials tends to form short-term memory, whereas spaced training comprises of repeated trials strengthens long term-memory [35, 36]. Thus, for short-term associative memory studies in *C. elegans*, massed training and simultaneous conditioning are most commonly used [18, 22, 37], and the butanone enhancement paradigm captures the fundamentals of learning, in which the integration of two sensory signals elicit a behavioral modification that is advantageous to the animal’s survival and exploitation of resources

This work presents a novel and reproducible microfluidic learning assay that demonstrates butanone enhancement. Early stages of this work focused on optimizing the protocol, which was then used to test the effects of genetic, developmental, and chemical changes on olfactory associative learning. It is immediately amenable to other learning paradigms, enabling further study of the mechanisms, timing, and location of plasticity within neural circuitry during learning processes.

## 2. Material and Methods

### 2.1 Microfluidic Device Design

The microfluidic device establishes a broad stimulus gradient to determine stimulus preference by recording the preferred location of animals within the gradient. Devices were modified from previous designs [28], and contain two adjacent behavior arenas separated by a

porous barrier that prevents animals from moving between them (Fig. 1A). Each arena contains a worm loading port allowing two different populations of worms to be gently injected into each arena separately via 1 mL syringe. Two liquid stimulus inlets enter mixing channels that establish, depending on flowrate, a symmetric linear or sigmoidal gradient profile from edge to center that is perpendicular to flow. The spatial stimulus pattern may be greatest in the center ("mountain" configuration, Fig. 1B) or lowest in the center ("valley" configuration).

The spatial odor profile was verified before each experiment (Fig 1B) using dye, calculating the relative dye concentration at all positions within the test gradient from video images, correcting for spatial illumination and camera nonlinearities (gamma) as follows. Images were acquired from a single device presenting a 0-10X (0-1 mg/mL) "mountain" gradient of xylene cyanol dye and uniform 0, 1, 2, 5, and 10X dye concentrations across the arenas. Image pixel values were scaled from the 0X dye concentration ($I_0$) via Beer-Lambert Law to obtain an uncorrected concentration, $c = -\log(I / I_0)$ at every pixel. A calibration curve was then generated from the five known dye concentrations, and a second-order polynomial fit corrected for camera gamma at each pixel. These transformations were used to monitor gradient shape and stability over time.

## 2.2 Microfluidic Device Fabrication

Monolayer microfluidic gradient devices were fabricated using soft lithography [28] from silicon mold masters produced using conventional photolithographic techniques. Young adult devices were prepared from 70-μm thick photoresist by spincoating SU-8 2035 (Microchem) at 1750 rpm on 4-inch wafers (Silicon Quest). Following softbake (3 min 65°C, 9 min 95°C, 3 min 65°C), the wafer was covered with a 25,000 dpi transparency photomask (Cad/Art Services)

and exposed to collimated 365 nm UV light (23.4 mW/cm$^2$, UV-KUB, Kloe) for 14 sec to crosslink exposed photoresist. Following a post-exposure bake (2 min 65°C, 7 min 95°C, 3 min 65°C), uncrosslinked resist was removed by agitation in SU-8 developer for 7 min. Larval stage 4 (L4) devices were similarly prepared to 50 µm resist thickness by adjusting the following parameters: spincoating at 2200 rpm, softbake (3 min 65°C, 6 min 95°C, 3 min 65°C), UV exposure for 6 s at 93% power, post-exposure bake (3 min 65°C, 6 min 95°C, 3 min 65°C), and development for 5 min. Micropatterned wafers were cleaned with fresh developer, then water, dried, and hardbaked at 150°C for 45 min to anneal the resist. Finally, silicon mold masters were treated with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane (TFOCS, United Chemical Technologies) vapor for 1 hr under vacuum to facilitate PDMS demolding, and cleaned with isopropanol and water washes.

Device casting, coring, and cleaning protocols were adopted from Larsch et al, 2013 [30]. Polydimethylsiloxane (PDMS) silicone polymer was mixed, degassed, and poured over mold masters to 5 mm depth. Inlets were cored with a 1 mm dermal punch (Acuderm) and cleaned with ethanol and water. Glass microscope slides were cut into 4.5 X 5 cm dimensions using a pen-type diamond tip glass cutter and rendered hydrophobic by TFOCS vapor exposure under vacuum for 1 hr. The micropatterned side of the PDMS device was reversibly sealed onto the hydrophobic 4.5 X 5 cm glass slide. Another glass slide with inlet and outlet holes drilled with a diamond-coated bit was placed atop the PDMS device and secured in a custom metal clamp with four screws.

## 2.3 Worm Strains

Standard methods were used for maintaining and manipulating *C. elegans* [38] and experiments were conducted at room temperature between 21-24$^{o}$C. The *C. elegans* Bristol strain, variety N2, was used as the wild-type strain. Worms were isolated at the late L4 stage based on morphology of the developing vulva and cultured at 20$^{o}$C for 24hrs to produce young adults. Experiments on L4 animals were immediately used for training and testing after isolation. The *olrn-1(ut305)* mutant was acquired from the Caenorhabditis Genetics Center (CGC), cultivated, and tested under standard methods.

## 2.4 Odor Stimulus Preparation

Odor dilutions were freshly prepared on the day of an experiment from ≥ 99.0% 2-butanone (11M stock, Sigma-Aldrich) in the specified conditioning medium used: 1X S. Basal buffer [5.85 g NaCl, 1 g $K_2HPO_4$, 6 g $KH_2PO_4$, 1 ml cholesterol (5 mg/ml in ethanol), bring volume with $H_2O$ to 1 liter], Hydroxypropylmethylcellulose (HPMC, Sigma-Aldrich) in S. Basal, or 350mM glycerol in S. Basal. OP50 *E. coli* strain was the bacterial food source for cultivation and during pre-exposure conditions as stated. For pre-exposure, an overnight OP50 liquid culture in LB medium was centrifuged, re-suspended in S. Basal medium, filtered through a 5μm membrane to reduce bacterial aggregates, and measured for optical density at 600 nm using a spectrophotometer. Bacteria suspensions were diluted with S. Basal medium to $OD_{600}$ = 0.50.

## 2.5 Microfluidic Device Setup

Microfluidic devices were degassed in a vacuum desiccator for at least 30 min before flowing 5% Pluronic F-127 surfactant into the entire device through the outlet port in order to prevent bacterial adhesion to microfluidic surfaces. Reservoirs containing the test odor stimulus and S. Basal buffer were positioned on a rack with a height of 30.5 cm from the outflow waste beaker in order to achieve an approximately linear spatial gradient. Each 30mL reservoir of prepared odor stimulus and S. Basal was connected to a 3-way valve, Luer stub needle, 0.02-inch ID (Tygon S-54-HL) microbore tubing, and a metal tube (NE-1027-12; New England Small Tubing) for insertion into the PDMS device [30]. We observed the formation of a symmetric linear dye gradient throughout the device to ensure normal fluid flow before loading animals. Arenas were cleansed by flowing S. Basal before loading animals into the arena. 25 to 30 worms were gently injected through the worm loading ports into each arena via 1mL syringe and ports were later plugged with solid metal tubes (New England Small Tubing). For microfluidic training experiments, the pre-exposure solution was immediately introduced into the arenas through the upstream worm loading port, whereas for microfuge pre-exposure experiments, a 15 to 20 min acclimation period ensured dispersal of animals within the microfluidic arena before initiating a behavioral recording.

## 2.6 Data Analysis

An existing MATLAB software suite, ArenaWormTracker, identifies instantaneous behavioral states from worm tracks, summarizes behavioral state probabilities and animal speed over space and time dimensions [28]. From these data, a new script, CTXsummary (Appendix), tracks relative residence of moving animals and calculates a chemotaxis index (CI)

at every 5 minute interval, as follows. Position information was spatially binned in 0.2-0.3 mm increments along the vertical dimension of the testing arena. Increased residence was observed at the upper and lower arena walls due to geometric effects (Fig. 2F), and these edge effects were omitted by excluding the outer 3 bins from calculation of the chemotaxis index analysis. The remaining bins were then segregated into a middle-region that constitutes 50% of the analyzed arena and two 25% outer regions. Based on these regional demarcations, the Chemotaxis Index (CI) was calculated using the following equation: CI = (# in 50% mid region - # in 25% outer regions) / total worms in arena. Thus, a positive CI indicates that animals prefer the center stimulus, and a negative CI indicates that animals prefer the outer edges. All experimental groups consist of at least n=3 arenas with each arena containing 20-30 worms. The average and SEM are presented in each figure. *$p \leq 0.05$ indicate statistically significant differences by unpaired student t-test and one-way ANOVAs as mentioned in text.

## 3. Results

### 3.1 Characterization of the gradient device

A gradient microfluidic design was modified from a previous design employing diffusive mixing [28] and adopted for the butanone enhancement assay. Because gradient shape depends on fluid flowrate, we first characterized the gradient profile throughout each arena using xylene cyanol dye to visualize the symmetric variation in dye concentration along the vertical dimension of a microfluidic arena (Fig. 1A,B). Xylene cyanol (538.6 g/mol) is a larger molecule than most odorants, including our test odorant, butanone (72.11 g/mol), and therefore has a lower diffusivity. Since faster diffusion results in more linear stimulus profiles and slower diffusion leads to sigmoidal profiles, the true odor profile will be more linear than

dye observations. From top to bottom vertical dimension, the dye gradient profile is rounded in the center ~4mm of the 16mm wide arena, and linear in the adjacent 4 mm bands above and below (Fig. 1B). Moreover, gradient shape remained constant over at least 2 hours, longer than a typical assay. Notably, our microfluidic gradient shape differed from the approximately exponential gradient shape presumed to form from an odorant spot on an agar plate, but due to odorant diffusion and evaporation in agar plates, odorant concentration on agar plates were difficult to quantify and variable over time.

### 3.2 Reproducibility of a chemotaxis behavioral assay within a microfluidic platform

During training, previous studies have pre-exposed animals to a combination of odorants and *E. coli* bacteria immersed in liquid medium [23, 37] or soaked in an agar plate [9, 18]. We initially pre-exposed worms in liquid medium and then tested for behavioral changes in a microfluidic setting. We hypothesized that the stable delivery of a butanone gradient within a microfluidic system would improve the reproducibility of behavioral results. As a starting point, we chose pre-exposure ($10^{-4}$ dilution, 1.1 mM) and test ($10^{-6}$ dilution, 11 μM) butanone concentrations based on previously used plate-based assays and the typical 1000x lower concentration required to elicit similar responses in a microfluidic versus agar plate system [21, 28]. We subjected animals to S. Basal only (naïve), food only (US), butanone only (CS), and butanone plus food (CS+US) conditioning within liquid S. Basal medium for 1 hr in 2 mL microfuge tubes (Fig 1C). After conditioning, animals were washed in S. Basal medium, transferred to the microfluidic device, and given 15-20 minutes to acclimate to their new surroundings before presenting a butanone “mountain” gradient, ranging from zero at the outer edges to butanone $10^{-6}$ (11 μM) at the center, for 1 hr. Worms demonstrating strong attraction

towards a stimulus were represented by a highly positive CI close to one (Fig 2D) and we observed only minimal odor adaptation or response decline (Fig 2G). However, beyond 30 min of testing, a slight response decline was observed, suggesting that behavioral plasticity from a 1 hr pre-exposure was short-lived. Therefore, all chemotaxis indices were calculated from the first 30 min of the test recording. For control experiments, we replaced test odor with S. Basal buffer and observed minimal spatial bias and a CI near zero (0.09, Fig. 2E-G), indicating no preference.

We compared experimental groups with control groups having similar motivational states such as fed worms with or without butanone to look at behavioral plasticity, and likewise, we compared starved worms presented with or without butanone to look at an adaptation effect. During the microfluidic testing, we observed a statistically insignificant increase of attraction in worms tube-conditioned to butanone and food (CS+US) compared to worms tube-conditioned to food (US) alone ($p = 0.26$, $n = 3$ Fig 3A). Further, a statistically insignificant adaptation effect was observed in worms conditioned to butanone (CS) alone versus naïve animals. However, compared to worms conditioned to butanone (CS) alone (-0.12 ± 0.18), animals pre-exposed to CS+US (0.56 ± 0.14) manifested significantly higher chemoattraction towards butanone. This difference demonstrates that food addition modulated the subsequent response to butanone pre-exposure, either by suppressing butanone adaptation, or increasing butanone attraction, or both (Fig 3A). Extra worm handling when transferring animals from the conditioning tube to microfluidic environment may have increased the variability within experimental groups, necessitating a further increase sample size to observe the individual enhanced butanone and adaptation effects.

To eliminate assay variability caused by numerous worm-handling steps, handling time, and environmental differences between microfuge tube pre-exposure and microfluidic assay, we next sought to perform pre-exposure and testing within the same microfluidic device. We predicted that minimizing the number of user-subject interruptions within the assay would reduce within-group variability to better measure associative learning. Similar to worms that underwent tube pre-exposure, we pre-exposed worms with the same conditions: S. Basal only (naïve), butanone $10^{-4}$ (CS) only, food (US) only, and butanone $10^{-4}$ + food (CS+US) in a microfluidic device for 1 hr and tested immediately with a butanone $10^{-6}$ gradient in the same device (Fig 3B). Under these conditions, we observed a significant butanone enhancement in fed worms presented with butanone (0.53 ± 0.05) compared to worms fed without butanone (0.31 ± 0.05). The net positive difference between CI's from fed worms pre-exposed with or without butanone signifies an enhancement that was attributed to the combined effect of food-related suppressed adaptation and paired association effect where the animal had learned to increase the value of butanone as an attractive stimulus. In addition, we also noticed a significant adaptation effect, in which animals conditioned with butanone only (0.02 ± 0.06) exhibited a drastic response decline towards a butanone test odorant compared to starved naïve worms (0.22 ± 0.06) (Fig 3B). Overall, worms pre-exposed and tested within a microfluidic device provided more consistent results than animals pre-exposed in microfuge tube followed by microfluidic testing, and this procedure was also experimentally more convenient than tube-to-device transfer.

### 3.3 Optimization of test and pre-exposure conditions for butanone chemotaxis assay in a microfluidic device

Next, we sought to optimize test and pre-exposure butanone dilutions that elicited the largest associative learning response, which was determined by the largest chemotaxis difference between CS+US and US only experimental groups. Even though *C. elegans* are capable of detecting a wide range of odorant concentrations, there is an upper and lower limit for odorant desirability that is crucial for activating processes responsible for attraction; thus, presenting odor concentrations that were too high may activate neural processes for avoidance behaviors. We aimed to optimize butanone test concentrations ranging from $10^{-7}$ to $10^{-5}$ dilutions while maintaining the same CS butanone $10^{-4}$ dilution for pre-exposure conditions. To detect the largest increase in butanone attraction due to associative learning, we compared worms conditioned in CS+US and US only groups that were tested at the same butanone concentration. Based on our results, butanone $10^{-6}$ dilution faired as the optimal test concentration for observing butanone enhancement with a mean difference of 0.22 ± 0.08 or a 42% CI increase in worms presented with butanone and food compared to worms pre-exposed with food only (Fig 4A). At butanone test concentrations of $10^{-5}$ or above, the CI declined because animals showed a wider band of residence in the center, avoiding the inner edges of the outer 25% regions less strongly; this suggests saturation of the behavioral response at these concentrations.

We next aimed to optimize the butanone pre-exposure dilution in order to elicit a maximum butanone enhancement effect. We pre-exposed animals to food paired with butanone dilutions ranging from $10^{-6}$ to $10^{-3}$ for 1 hr, and tested them with a butanone $10^{-6}$ test gradient in each case. Compared to the food only (US) control group, no significant differences

were observed in CI for the CS+US condition across all butanone dilutions (Fig 4B). Strikingly, the only significance noted was the diminished butanone preference in worms pre-exposed to butanone $10^{-3}$ (0.22 ± 0.03) when compared to the other three dilutions, suggesting that pre-exposure conditions should not exceed the $10^{-3}$ butanone level, which may saturate sensory responses or activate additional aversive neurons. Although we did not observe significant enhancement of butanone response in fed animals at any pre-exposure concentration, this is attributed to a loss of statistical power due to comparing multiple experimental groups against one food-only control. Based on these data, we chose butanone $10^{-4}$ (1.1 mM) as the pre-exposure CS concentration in the microfluidic device followed by a gradient of butanone $10^{-6}$ dilution (11 μM) to test behavioral responses, for the remainder of our study.

### 3.4 Validation of microfluidic-based learning assays with genetic mutants

The mutant olfactory learning-1 *olrn-1(ut305)* was identified in a forward genetic screen for defects in butanone enhancement and benzaldehyde adaptation [18], yet the mechanism of its activity remains unclear. To see whether our microfluidic chemotaxis assay could reproduce associative learning abnormalities in mutant animals, we tested *olrn-1* mutants using the assay conditions optimized for wild-type animals. Associative learning, observed as the butanone enhancement effect (a positive increase of chemotaxis index in the CS+US conditioned group compared to the US only group), was observed in wild-type animals, but no significant butanone enhancement was observed in *olrn-1* mutants pre-exposed to food and butanone when compared to *olrn-1* mutants conditioned to food alone. Observations of no explicit butanone enhancement in *olrn-1* animals may not be attributed to defects in butanone detection, because naïve fed *olrn-1* animals exhibit normal attraction towards butanone.

Although wild-type animals exhibited significant adaptation when conditioned to butanone alone, a similar but insignificant trend was observed between *olrn-1* worms pre-exposed to butanone alone (0.09 ± 0.11) and *olrn-1* starved naïve animals (0.41 ± 0.15) (Fig 5).

### 3.5 A customized microfluidic gradient device that enables associative learning studies on various developmental stages of *C. elegans*

During maturation, *C. elegans* pass through four larval stages (L1-L4). While the nervous system is largely developed by larval stage 1 (L1), neural circuit activity and responses to external stimuli may differ during development. Associative learning experiments in *C. elegans* have examined young adult animals, but little is known about age-dependent changes in learning. Therefore, we investigated learning in L4 worms by modifying the chemotaxis assay we established for young adults (YA). Due to the smaller size of L4 animals versus young adult animals (0.6 mm L4 versus 1 mm YA in length), we reconfigured the micropost dimensions in the microfluidic arena by scaling down all dimensions by ~60%. *C. elegans* thrash in a whip-like motion when submerged in liquid medium, but micropatterned posts with optimized dimensions appropriate for a specific aged worm enable them to traverse through the arena in a sinusoidal crawling motion similar to movements observed in worms crawling on agar plates. Keeping the microchannels that conduct diffusive mixing of the test odorant unchanged, we only modified the microposts within the arena for L4 worms, reducing the post radius from 100 μm to 60 μm, post spacing from 100 μm to 60 μm, and microfluidic channel height from 70 μm to 50 μm, respectively (Fig 6A-C). This new L4-stage microfluidic device was chosen from several reduced-dimension devices and validated for smooth crawling locomotion of L4 animals in this design. For example, a 40 μm tall device with the same post

radius and spacing was appropriate for young L4 animals, but restricted movement in older L4 animals.

We hypothesized that the response profile for associative learning may change with age. Therefore, we tested butanone responses of L4 animals in the modified gradient device, employing the same protocol used for young adults. Our results demonstrate that both starved naïve and butanone only pre-exposed L4 groups behave similarly as young adults. However, the butanone plus food conditioning group exhibited a CI slightly below zero (-0.02 ± 0.06), which was comparable to worms that have adapted to butanone in the CS conditioned group (0.01 ± 0.06) (Fig 6D)**.** The inability of food to increase the chemotaxis in L4 worms after butanone exposure suggests a strong age-dependent difference in the interaction between these two cues. The lack of a feeding effect may arise from an inability to increase preference due to pairing, an inability to integrate the food and odor signals, an inability to suppresses butanone adaptation, or any combination of these. To distinguish among these possibilities, it will be important to compare these responses with naïve fed animals (an experiment not yet performed). If naïve fed L4 animals showed similarly weak positive attraction as naive starved L4 animals, the suggestion would be that younger animals are unable to exhibit associative learning and unable to suppress butanone adaptation. Finally, if instead naïve fed L4 animals showed no response to butanone, this result would be consistent with either the inability to suppress adaptation but intact associative learning, or normal suppression of adaptation but defects in associative learning; in either case, their effects exactly cancel. In summary, our results indicate that responses to food and odor pairings may change with age and genotype, similar to other studies that have examined how behavioral plasticity such as salt chemotaxis

and olfactory associative learning have been impeded by aging mutations such as *age-1* and *daf-2* [39, 40]. Further studies will be required to determine the nature of these defects.

### 3.6 Chemical and physical alterations to training conditions perturb associative learning

Our results indicated that microfluidic assays could quantify behavioral effects due to short-term associative memory, thus motivating the extension of the method towards long-term associative studies. Unlike agar plates, feeding nematodes in a PDMS microfluidic device for long periods can be challenging due to the bacterial aggregation and settling at very slow flow rates, which may lead to device clogging or a non-uniform flow of food throughout the microfluidic arenas. We attempted two solutions: (1) replacing the bacterial food with exogenous application of an internal food signal, serotonin, and (2) modifying the conditioning buffer to prevent bacterial aggregation and settling.

Serotonin (5HT) is a monoamine neurotransmitter that is involved in nematode pharyngeal pumping during the presence of bacterial food, and exogenous serotonin can induce feeding motions [41]. However, 5 mM serotonin dissolved in a conditioning agar plate did not induce butanone enhancement but merely suppressed butanone adaptation [11]. We asked whether serotonin could mimic food, and assigned it as our unconditioned stimulus *in lieu* of food, in a pilot study where animals were pre-exposed to serotonin in a microfuge tube and tested against butanone in our microfluidic behavioral assay. A prior study where electrophysiological recordings were taken from *C. elegans* exposed to 10 mM 5-HT while trapped in a microfluidic channel demonstrated serotonin-induced pharyngeal pumping [41]. Since our assay was also in a microfluidic format, we decided to initially condition animals with the same 10 mM serotonin concentration. Unfortunately, we noticed that animals pre-exposed

to 10 mM 5-HT alone for 30 minutes manifested transient paralysis (data not shown), so we lowered the concentration to 5 mM 5-HT. Movement was unaffected when worms were pre-exposed to 5 mM 5-HT for 1 hr but they did not elicit normal chemoattraction towards butanone compared to worms conditioned to food alone in a microfuge tube and tested in the microfluidic device (CI). Instead, animals pre-exposed to 5 mM 5-HT showed no preference for the test butanone with a CI close to zero (0.00 ± 0.13). Surprisingly, worms pre-exposed with 5 mM serotonin in the presence of butanone $10^{-4}$ exhibited a chemotaxis index (0.44 ± 0.03) significantly greater than animals pre-exposed to 5-HT alone (0.00 ± 0.13), indicating that serotonin works in conjunction with the butanone chemosensory pathway to increase preference for butanone (Fig 7A). Thus, our data suggests that serotonin pre-exposure does alter learning behaviors, but it does not exactly mimic bacterial food in the case of 5-HT alone versus food alone. While pairing serotonin with butanone does significantly increase attraction to butanone, it is unclear whether this represents associative learning, as it remains to be seen whether animals are attracted to serotonin itself. It is also possible, though perhaps unlikely, that serotonin causes adaption to butanone, and butanone pre-exposure can suppress serotonin-induced adaptation. Future studies employing a serotonin gradient on wild-type animals within the microfluidic arena will garner evidence that may support a larger push for serotonin's role in contributing to associative learning if we observed attractive bias as a function of serotonin concentration gradient, whereby worms move towards an increasing concentration of serotonin and away from decreasing concentrations.

As an alternative strategy to implement extended bacterial feeding for long-term associative studies, we then tried to physically deter bacterial sedimentation and aggregation by using an inert solution with similar density as *E. coli* bacteria. Hydroxypropylmethylcellulose

(HPMC) polymer has been used as a densifier and viscosifier with no reported behavioral effects. 5% (w/v) HPMC has a similar density to *E. coli* culture and effectively prevents settling over >1 day (data not shown). Animals exhibited normal sinusoidal movement within the HPMC-containing conditioning solution, despite an increase in viscosity. However, we noted interesting differences between animals conditioned in S. Basal medium versus those conditioned in HPMC when subjected to the same microfluidic pre-exposure and test conditions as previously used on wild-type animals. Naïve N2 worms pre-exposed to HPMC displayed higher baseline levels (0.52 ± 0.06) of butanone attraction than naïve N2 worms (0.22 ± 0.06) conditioned in S. Basal medium. Surprisingly, worms conditioned with food (US) only and butanone (CS) only within the HPMC solution also showed stronger attraction to butanone than their S. Basal pre-exposure counterparts (Fig 7B). Pre-exposure with HPMC heightened butanone attraction across all groups, regardless of food or butanone pre-exposure, making it impossible to assess learning behaviors. We concluded that 5% HPMC modulated or masked behavioral responses on its own, and therefore cannot be further used to circumvent bacterial clumping for long-term associative studies. Other densification agents, such as Ficoll or polyvinylpyrrolidone (PVP) may be suited for long-term associative learning studies, so long as the adaptation and associative learning phenotypes are tested and preserved. Another possibility is the use of mucin polymers normally found in human digestive tracts as it has been shown to reduce bacterial surface adhesion by 20-fold over a 1 hr period without harming bacterial viability [42]. While HPMC has proven to be an implausible solution for long-term bacterial feeding in a microfluidic device, future experimentation on alternative agents will help screen for physical deterrents of bacterial clumping and settling.

On a wider scope, short-term associative learning not only involves the use of positive reinforcement, but also negative reinforcements such as repellents. Hukema et al. demonstrated *C. elegans* avoidance from a 25 mM NaCl attractant after fed animals were simultaneously pre-exposed to glycerol and NaCl for 30 minutes [37]. Therefore we opted to use glycerol as our negative unconditioned stimulus to observe whether it interferes with butanone attraction within our microfluidic chemotaxis assay. Since we witnessed osmotic shrinkage and transient paralysis in animals pre-exposed with 500 mM glycerol within the microfluidic environment, we lowered the glycerol concentration that was previously used on agar plates from 500 mM to 350 mM. Although we still noticed temporary paralysis from 350 mM, the time delay between pre-exposure and testing reduced to 30 minutes compared to 1 hr if using 500 mM glycerol. Our results showed that naïve worms conditioned in glycerol alone (0.37 ± 0.10) elicited normal butanone chemotaxis; however, animals conditioned to butanone alone (0.19 ± 0.10) within glycerol medium unexpectedly sustained a positive preference for butanone compared to naïve worms pre-exposed to glycerol alone. Thus, butanone adaptation appeared impaired by glycerol, although this result was not significant. Since *C. elegans* are highly sensitive to osmolarity changes, it is possible that switching from a glycerol-containing pre-exposure medium to S. Basal during test conditions may have relieved some osmotic stress when animals settled in the latter environment; therefore, inducing a sustained butanone attraction even after a 1 hr pre-exposure to the odorant itself. Meanwhile, worms conditioned with butanone and food together (CS+US) in glycerol medium showed a reduced attraction towards butanone $10^{-6}$ test odorant (0.12 ± 0.12) (Fig 7B). A comparison between the CS+US and US only groups is required to distinguish whether glycerol affects associative learning, but data for the US only group remain to be determined, and the only conclusion from this data set

is that glycerol impedes butanone enhancement in an unknown manner. In principle, glycerol could modify the chemosensory and gustatory signals, or their integration, that reinforce associative learning in a negative fashion. In summary, our results demonstrate that presenting a negative cue such as glycerol interferes with associative learning, but whether glycerol prevents food's ability to inhibit butanone adaptation, associative learning, or both remains to be determined.

## 4. Discussion

### 4.1 A microfluidic system that offers a reliable and reproducible behavioral chemotaxis assay for butanone enhancement observations

We established an associative learning assay on a new microfluidic platform that addressed some of the issues arising from a plate-based assay. Unlike the open environment agar-plate assay, PDMS-based gradient devices enable direct control of stimulus delivery at precise concentrations in an enclosed environment for freely moving animals to roam up and down an odorant gradient. Animal position can then be recorded and quantified as a chemotaxis index (CI) over time, without paralyzing the animals as was previously done on agar plate assays to simplify quantification. Similar to chemotaxis plate assays, attraction behavior within a microfluidic device is defined by a worm's movement up a test odorant gradient and its residence at higher stimulus concentrations. We adapted the assay that tests for a butanone enhancement phenotype and modified butanone concentrations and timing to employ this assay in a microfluidic setting. We aimed to reproduce behavioral results from chemotaxis agar plates in this new microfluidic platform, first pre-exposing animals in a microfuge tube as previously reported, and then performing pre-exposure and chemotaxis

behavior tests in the same device to minimize animal handling. We diluted pre-exposure and test butanone concentrations by 1000-fold based on previous studies that compared saturating responses in plate-based chemotaxis versus microfluidic assays [28]. Our optimization studies confirmed that these initial concentrations were good estimates, because this protocol enabled us to observe similar behavioral trends as Torayama et al. when animals were subjected to a butanone test gradient within the microfluidic arena [18]. By keeping the conditioning odor concentration high, we observed strong butanone adaptation effects, which could be suppressed by pre-exposure with food. Associative learning, defined as the positive chemotaxis index (CI) difference between fed worms that were pre-exposed with or without the conditioned stimulus (CS), butanone $10^{-4}$ (1.1 μM) was more noticeable in the protocol that pre-exposed and tested worms within the same microfluidic device. While butanone is a natural weak attractant stimulus, emitted by several bacterial strains, animals exposed to butanone in the presence of food learned to build a stronger association between the CS, butanone and the US, food. Thus, associative learning has a value-altering effect in that butanone now becomes a more valuable stimulus or a better predictor of food reward in trained animals. Overall, the microfluidic learning assay reproduced agar-plate-based findings when pre-conditioning and odor preference testing in the same device, demonstrating the feasibility of running a sensitive behavior assay in this type of micro-environment.

In general, plate-based chemotaxis assays show daily fluctuations as well as differences among research labs and researchers. Comparisons of chemotaxis results are therefore usually made on data collected on the same day, and/or with large numbers of repeats. Here, at least three different experimenters, including two graduate students and an undergraduate trainee, ran the same wild-type assay on different days and the resulting

chemotaxis indices varied no greater than among repeats by the same researcher on the same day. This is at least suggestive that microfluidic assays are reproducible. We suspect that similar behavioral results are due to (1) reduced user intervention and worm handling, which could vary among researchers (2) more uniformity in the test odor gradient, and the closed microfluidic environment that insulates from the effect of external environmental factors, such as temperature and humidity that may fluctuate over time. In all, the benefits of a microfluidic-based chemotaxis learning assay make it an attractive alternative to behavioral assays done on agar plates.

**4.2 Learning behavior changes in mutants**

One substantial advantage of *C. elegans* as a model system is the ability to uncover molecular mechanisms via genetic screens for mutant behavior. To test whether the microfluidic learning assay could identify genetic mutants, we tested *olrn-1* animals previously discovered from a forward genetic screen on an agar-plate-based assay. Despite a normal butanone preference in its naïve state, *olrn-1* showed the most severe associative learning defect. We observed a reduced but insignificant butanone attraction in *olrn-1* animals pre-exposed with butanone alone, and similarly we observed increased but insignificant butanone attraction in *olrn-1* mutants conditioned to butanone plus food compared with the fed only *olrn-1* mutants. Compared to wild-type animals, the *olrn-1* mutants showed decreased butanone enhancement, similar to plate-based assays of *olrn-1* animals that underwent CS+US conditioning. These results suggest that defects were visible in the microfluidic assays, but that greater response variability by mutants with smaller condition effects may require additional experiment repeats. Despite this potential limitation for initial genetic screens, the microfluidic

system may offer substantial benefits for further analysis of learning mechanisms, including the ability to observe neural and behavioral changes continuously before, during, and after training.

The biology underlying *olrn-1* behavioral plasticity defects remains unclear; however there is evidence pointing to the necessity of having an $AWC^{ON}$ neuron for normal butanone enhancement. DNA constructs containing an *str-2* promoter to drive wild-type *olrn-1* expression in AWC neurons failed to rescue the *olrn-1* mutant phenotype because it lacked an $AWC^{ON}$ neuron. Interestingly, experiments using the *slo-1(ky389gf)* gain-of-function mutant with *slo-1* encoding for a voltage- and calcium-activated (BK) $K^+$ channel, was combined with *olrn-1(ut305)* to make a *slo-1(ky389gf); olrn-1(ut305)* double mutant that gave rise to a mixed population of animals expressing $2AWC^{ON}$, $2AWC^{OFF}$, and $1AWC^{ON}/1AWC^{OFF}$. Torayama et al. showed that animals with at least one $AWC^{ON}$ neuron from this mixed population of the same genotype manifested normal butanone enhancement, suggesting that $AWC^{ON}$ cell-fate was a determinant of whether an animal was capable of butanone associative learning. Similar to other mutants with $2AWC^{OFF}$ such as *daf-11(m84)*, the *olrn-1* mutation is involved in hampering the left-right AWC cell-fate symmetry and therefore indirectly impedes associative learning (Fig 8). AWC and other chemosensory neurons play a role in food sensation, where this information largely contributes to the process of making a paired odor-food association for butanone enhancement. Altogether, defects in experience dependent associative learning in *olrn-1* mutants were not attributed to reduced butanone sensation because naïve *olrn-1* animals chemotaxed toward butanone, but instead, due to the lack of a functional $AWC^{ON}$ neuron or $AWC^{ON}$-like properties.

Current findings from butanone enhancement agar-based assays on mutants have paved an avenue for further exploration into the AWC ON/OFF neurons and its possible

downstream interneuron targets. A closer look at changes in neural properties such as activity patterning between neurons in *olrn-1* mutants and wild-type young adults is necessary to further understand the biology underlying associative learning. Neural imaging of AWC ON/OFF neurons and interneurons before, during, and after butanone plus food conditioning will enable us to answer how past experiences or neural activity in *C. elegans* alter learning behavior. The *olrn-1* mutation may perturb signaling to AWC's downstream targets, prevent food sensation, or inhibit the integration of sensory information. In support of localizing learning deficiencies to AWC target interneurons, evidence from Ha et al. suggested that experience-dependent plasticity occurred downstream of sensory neurons [43]. *C. elegans* never exposed to the pathogenic bacterium, *Pseudomonas aeruginosa* (PA14), often prefer the smell of pathogens compared to benign bacteria such as *E. coli* OP50; however this preference to PA14 odors was greatly reduced after worms were pre-exposed to a fresh lawn of PA14 for 4-6 hrs. Moreover, intracellular calcium responses in $AWC^{ON}$ neurons did not correlate with the animals' olfactory preference away from PA14; implying that experience-dependent associative learning occurred downstream of the AWC sensory neuron. Furthermore, a downsized microfluidic device to closely capture neural activity from a single neuron can be a powerful tool to not only address the mechanistic unknowns within an *olrn-1* mutant at a cellular and network level, but to also address similar questions in other transgenic mutants for future studies.

### 4.3 Learning behavior changes during development

There have been numerous studies on associative learning in young adults and aged *C. elegans* [23, 35, 44], but none focused on larval stage 4 (L4). Results from aged worm

populations suggest that long-term olfactory memory is the first cognitive decline in *C. elegans*, followed by motility, neuronal structure defects, and then synaptic function [45, 46]. Mutations that alter a worm's lifespan have also been heavily studied for its effects on associative learning during adulthood [46]. Considering that changes in lifespan have a systemic effect on neural development, it is important to understand the biology underlying effective learning and memory retention during developmental stages. In order to examine younger worms in an associative learning paradigm, we first reduced the micropatterned post dimensions of the gradient device to a scale that accommodates smaller L4 worms: specifically, a 40% reduction in post radius and spacing, and 30% reduction in post height, enabled L4 worms to exert normal sinusoidal locomotion throughout the arena.

The nervous system is largely complete by the end of L1, but developmental changes persist into the L4/adult molt stage. We employed the optimized conditions previously used for young adult wild-type worms on L4 animals and observed normal butanone adaptation but a complete lack of response to butanone when pre-exposed with butanone and food. Future experiments will help clarify if this is due to a lack of associative learning, or inability to suppress butanone adaptation, or both. Interestingly, the effect of age and development was observed only in the fed state; starved naïve L4 worms and those pre-exposed to butanone alone displayed similar behavioral responses as their young adult counterparts. In contrast, L4 worms conditioned to butanone paired with food showed a significantly lower chemotaxis index than young adults that underwent the same pre-exposure. Even though the connectome of an L4 worm's nervous system is complete, changes in butanone preference may be due to neural activity patterns that differ from young adults. Furthermore, whether changes in butanone

preference are gradual during the progression from L4 to adulthood, or occur step-wise immediately after the L4/adult molt, will be interesting to observe.

### 4.4 Chemical and physical perturbations alter learning behavior

Flow-through of the *E. coli* bacterial food source for 1-2 hrs poses no experimental issues, however, longer periods may result in bacteria settling to the bottom of a reservoir and lead to inconsistent flow of bacteria concentration over time or device clogging. Before extending the applications of our short-term associative learning studies to long-term, these challenges must be resolved by either substituting the food source with a substance lacking adhesive properties or a chemical agent that prevents bacterial aggregation and settling. We first attempted to replace the bacterial food source with serotonin because it had been shown to trigger pharyngeal pumping in *C. elegans* to the same extent as if the animal detected food [41]. Exogenous addition of serotonin (5HT) alone suppressed butanone attraction in wild-type animals, demonstrating immediately that it does not exactly mimic bacterial food. This reduction could be concentration dependent, and worms conditioned to lower 5HT concentrations, below 5 mM, may exhibit positive butanone chemotaxis similar to bacterial fed animals. It is possible that the attraction to butanone is suppressed by a residual aversion to serotonin, and whether animals show an aversion to serotonin alone remains to be seen by testing responses to a serotonin gradient in the same type of device.

On the other hand, worms conditioned with either 10 mM or 5 mM 5HT paired with butanone showed higher butanone preferences than worms pre-exposed to 5HT alone. This sudden change in butanone response may be attributed to two possibilities: 1) the addition of butanone in the paired conditioning may have nullified the unknown downstream serotonergic

pathways involved in suppressing butanone preferences suggesting that the effector of behavioral changes might be localized to upstream chemosensory neurons; or 2) butanone may have strengthened synaptic signaling within a serotonergic dependent circuit required for associative learning. Further neural imaging experiments on the AWC chemosensory neuron may be a good start to distinguish between the two hypotheses. Any indicators of neural activity changes in the AWC neuron during butanone and 5HT paired conditioning may point towards the first possibility; otherwise, future neural imaging experiments to look at AWC's downstream synaptic targets may be needed to examine the second possibility. We cannot infer from our current data that the increase in butanone preference after paired conditioning resembles associative learning because serotonin alone elicited an indifferent response towards butanone that produced a close to zero baseline when compared to naïve fed worms. However, we conclude that even though serotonin itself cannot completely mimic the effects of bacterial food, it does modulate behavior through mechanisms that remain to be determined. This result is not unprecedented, as serotonin is involved in both aversive and attractive olfactory learning in *C. elegans* [47]. In one example, a key component for aversive olfactory learning is the upregulation of serotonin in ADF chemosensory neurons upon exposure to pathogenic bacteria [35], while increased serotonin levels in NSM neurons facilitate attractive olfactory learning [47]. Previous studies have also demonstrated that serotonin may modulate the sensitivity of ASH or ASE neurons, which mediate osmotic avoidance and attraction, respectively [37]. Altogether, serotonin appears to be involved in numerous neural circuits for learning. Therefore, studying the neural activity patterns before, during, and after conditioning with serotonin plus butanone may help us understand the neural networks involved in associative learning.

Alternative feeding methods were explored, including sonicating bacteria until they rupture and release their biological contents into the medium where we could then separate cell components such as its membrane and organelles that are likely to sediment into a cell pellet during centrifugation, leaving behind supernatant containing attractive chemicals reminiscent of *E. coli*. However, as filter feeders, particulates may be important for nutrients to reach the *C. elegans* gut. Thus, we next explored methods to prevent clumping and settling that left the bacterial cell intact. OP50 *E. coli* bacteria sedimented, aggregated, and clumped after eight hours of continuous flow through a microfluidic device (data not shown). Hydroxypropylmethylcellulose (HPMC) is a viscoelastic polymer that matches the density of bacteria at a 5% w/v concentration, and its common use as a thickening agent in food additives, an ophthalmic lubricant, and an excipient that facilitates drug delivery, absorption, and solubility, suggests it is generally inert [48, 49]. While animal movement appeared normal in this solution, 5% HPMC unexpectedly heightened the animals' preference for butanone in all pre-exposure groups regardless of food (US) or butanone odor (CS) conditions. To rule out the possibility that HPMC may deter locomotion causing an artificial outlook that animals appear attracted to the middle region of highest butanone dilution, we allowed the worms to roam evenly across each arena before testing to remove any geometric bias. Despite normal locomotion, animals may have disliked the 5% HPMC such that behavior during the butanone test gradient solely reflected the removal of HPMC, rather than the effect of butanone and/or food pre-conditioning. Overall, HPMC obstructed the assessment of changes in learning behavior in *C. elegans*, making it an unsuitable solution for associative learning studies. Other densifiers such as Ficoll or polyvinylpyrrolidone (PVP) may be used to create isopycnic solutions, but any additive will need to be tested for any effects on the learning assay.

Another variation of associative learning in *C. elegans* is aversive learning, wherein a negative reinforcement is presented simultaneously with an attractive conditioned stimulus so that the paired association drives the animal to avoid a cue that was formerly attractive. Glycerol is a high osmolality solution that is typically used as a repellent for *C. elegans* because it elicits the most $Ca^{2+}$ influx in polymodal aversive ASH neurons [50], which signals avoidance through GLR1/GLR-2 channels and NMDA ionotropic glutamate receptors (NMRs) [51]. We hypothesized that by using 350 mM glycerol as our negative stimulus we would induce changes to butanone attraction after pairing this noxious stimulus with butanone alone. Starved naïve worms showed normal chemoattraction towards butanone even in the presence of glycerol, but animals conditioned with butanone only showed an insignificant adaptation effect compared to naïve, and butanone did not take on a negative association. One possibility is that animals pre-exposed to glycerol exhibit transient paralysis due to the osmotic shock and required a 30-minute recovery period before full locomotion returned. The time delay before testing may have diminished short-term memory retention, therefore leading to more response variability and reduced attraction phenotype, especially for worms pre-exposed to both food and butanone. Another possibility is that osmotic shock the animals experienced during pre-exposure with butanone in glycerol solution created a highly unfavorable environment such that once the medium changed to S. Basal during testing, animals inherently expressed a stronger butanone attraction because it is now presented with a more desirable S. Basal environment. Meanwhile, low butanone attraction in worms pre-exposed to both food and butanone within glycerol medium suggests that noxious cues from glycerol diminished the animal's olfactory preference to butanone despite the presence of food. Despite the incomplete data set for the food only group in glycerol solution, it would be worth investigating the net

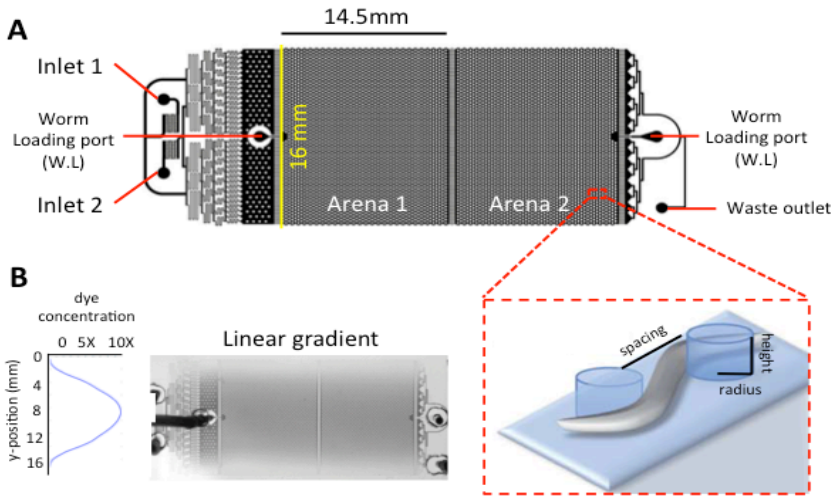
change in CI when comparing the food only group with worms pre-exposed to both food and butanone in the presence of glycerol in order to determine how strongly glycerol negatively impacts learning behavior when both a CS and positive US are present. Although glycerol was not a potent repellent to induce strong avoidance responses, it would be interesting to explore other chemical repellents, such as 1-octanol, quinine, $Cu^{2+}$, $Cd^{2+}$, and garlic extract that do not increase osmolarity and may be effective negative reinforcers without requiring a recovery period after pre-exposure.

## 5. Conclusion

Learning is a mechanism that enables an organism to adapt to changing environments and improve its chances of survival. *C. elegans* dwell in moist soil, which harbors a vast array of chemical stimuli that may or may not be a prelude to injury. Here we have shown that *C. elegans* learn to enhance their response towards the attractant butanone when it is paired with a nutritional reward such as food, which has been widely used in traditional agar plate behavioral assays. Agar plate-based chemotaxis assays have been the conventional method to determine changes to odor preference during learning and memory, in part due to their ease of setup, ubiquity, and inexpensiveness. However, results from these assays are often variable due to numerous confounding factors such as humidity, odorant evaporation, animal handling, and subtle variations in protocol across users. The microfluidic behavioral testing system presented in this study exhibits great potential in resolving many inherent issues that laboratories face when running a plate-based chemotaxis assay. Although plate-based assays are still convenient and sufficient for addressing certain hypotheses on mechanosensory or thermosensory learning, there are limitations on what aspects of learning can be studied in a plate-based assay. For example, identifying a specific neuron and correlating its activity to a

behavioral output may require experimental conditions that are beyond the means of what an agar plate can provide, such as the sudden addition or removal of the test stimulus. In this case, a small-scale microfluidic device is more suitable for implementing this type of testing than an agar plate because its transparent environment enables high-magnification fluorescent imaging of individual neurons and its enclosed environment ensures precise spatiotemporal delivery of stimuli that is necessary for correlating rapid stimulus-invoked behaviors with neural activity of identified neurons [30].

Utilizing the microfluidic device, we investigated experience-dependent behavioral plasticity which can be impeded by genetic mutations as seen with *olrn-1* mutants, by age as indicated by L4 worms, by neurochemicals such as serotonin, and by other molecules such as HPMC and glycerol. With an established microfluidic-based chemotaxis assay protocol, we can apply this method to test other transgenic *C. elegans* mutants modeling human diseases that hamper cognitive function. Future experiments studying odor-invoked behavioral responses and neural activity of transgenic mutant strains will help us advance our understanding about the molecular and synaptic complexities that underpin learning in *C. elegans*. Findings from such invertebrate models can be translated to higher model organisms for future therapeutic strategies to treat human diseases associated with learning and memory impairment.


A
14.5mm
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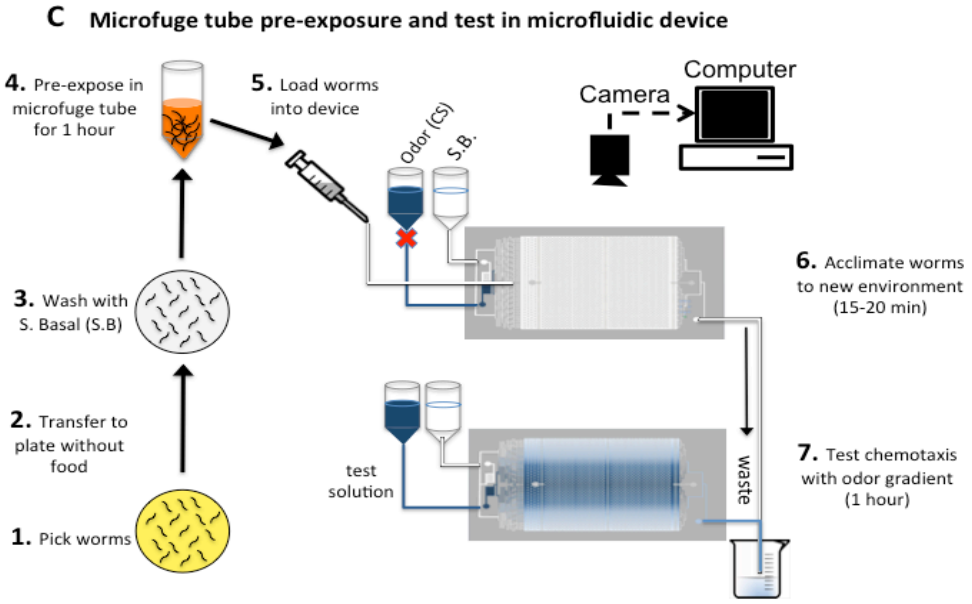

C Microfuge tube pre-exposure and test in microfluidic device
4. Pre-expose in microfuge tube for 1 hour
5. Load worms into device
Odor (CS)
S.B.
Camera
Computer
6. Acclimate worms to new environment (15-20 min)
3. Wash with S. Basal (S.B)
2. Transfer to plate without food
1. Pick worms
test solution
waste
7. Test chemotaxis with odor gradient (1 hour)

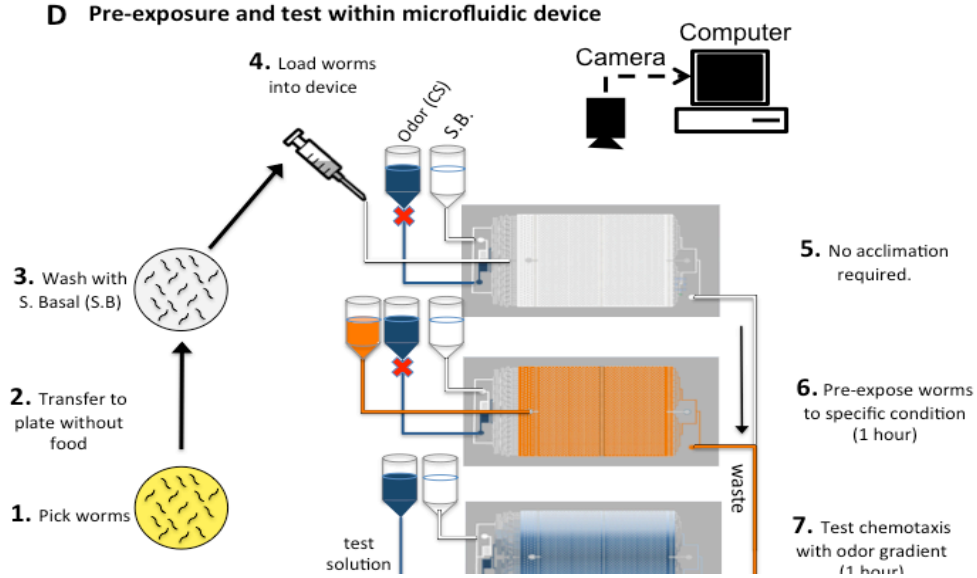

D Pre-exposure and test within microfluidic device
4. Load worms into device
Odor (CS)
S.B.
Camera
Computer
5. No acclimation required.
3. Wash with S. Basal (S.B)
2. Transfer to plate without food
1. Pick worms
6. Pre-expose worms to specific condition (1 hour)
waste
test solution
7. Test chemotaxis with odor gradient

**FIGURE 1:** Relative odorant distribution in a microfluidic gradient device. A) Diagram of a 2-Arena microfluidic device containing two inlets to allow: 1) S. Basal (S.B) (inlet 1) and Dye (inlet 2) in-flow, two worm loading (W.L) ports, and one waste outlet. Enlarged illustration of a worm situated between two micro posts within an arena (red). B) Quantification of a dye concentration used to visually represent the odor gradient distribution shows a linear curve for the gradient stripe that runs across both arenas shown on the right. C) Schematic of experimental workflow when animals were pre-exposed in microfuge tube: *1)* Young adult worms were manually picked from a plate seeded with OP50 *E. coli,* then *2)* transferred to a new plate without food and *3)* washed once with S.B. *4)* Pre-exposures of various conditions were done in microfuge tubes for 1 hr. Thereafter, worms were immediately transferred from S.B wash to a 1mL syringe for device loading*. 5)* Worms from the syringe were carefully flowed through the worm loading port for both arenas and *6)* allowed to acclimate to their new microfluidic environment for 15 to 20 minutes under constant gravitational flow of S.B while the odor reservoir was shut off. 7*)* Valves for both S.B and odor solutions were turned on to allow an odor gradient flow across the two microfluidic arenas. All liquid waste exits through the waste outlet. D) Schematic of experimental workflow when animals were pre-exposed and tested in one microfluidic device: *Steps 1-3* were carried out in the same manner as shown in *C. 4)* Worms from the syringe were carefully flowed through the worm loading port for both arenas. *5)* Once worms were loaded, no acclimation period was required. *6)* Valves controlling S.B and odor flow were closed, and a third reservoir containing experimental conditioning solution connected to the worm loading port for a 1 hr worm pre-exposure. *7)* The third pre-exposure reservoir was then removed, and valves for both S.B and odor solutions were turned on to allow an odor gradient flow across the two microfluidic arenas. All liquid waste exits through the waste outlet.

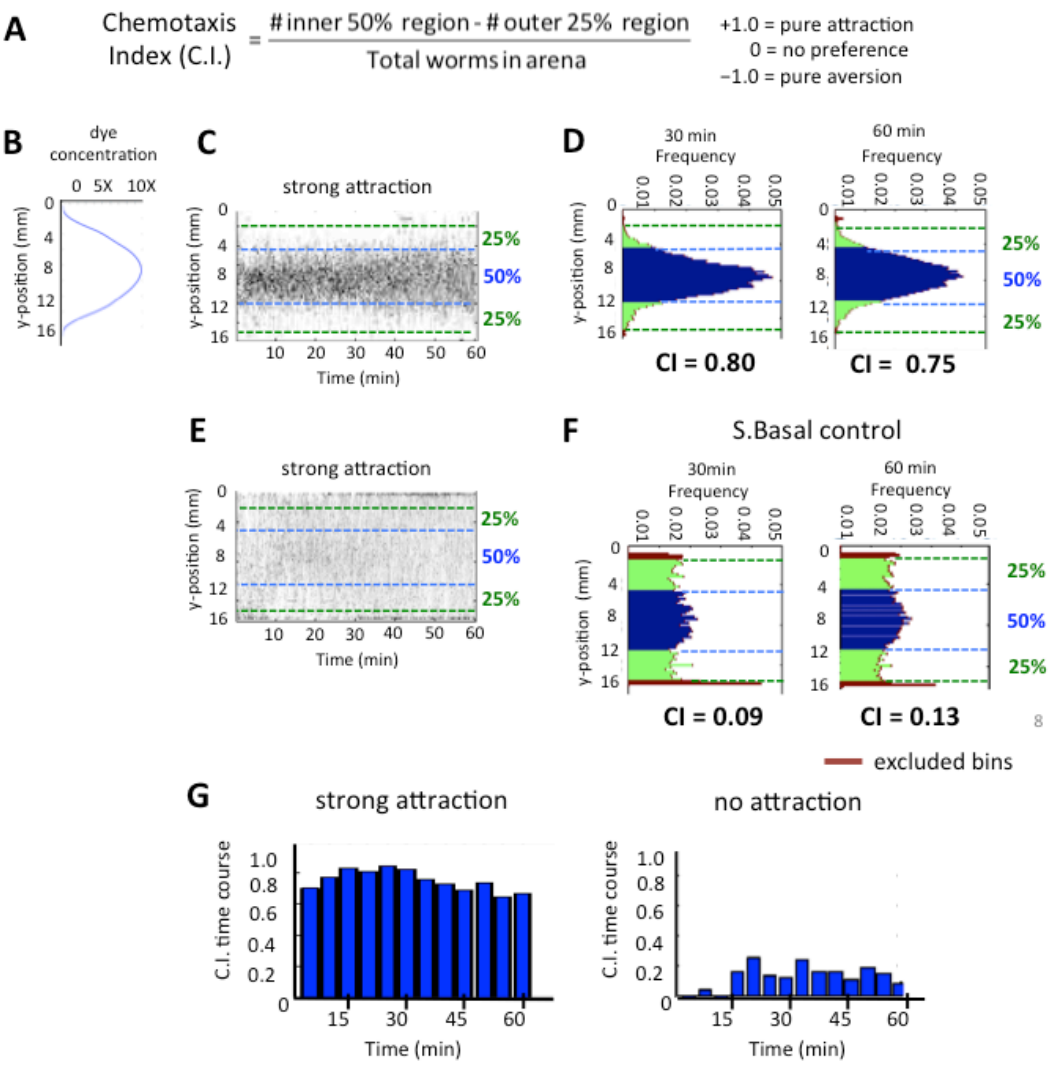

A
Chemotaxis Index (C.I.) = # inner 50% region - # outer 25% region / Total worms in arena
+1.0 = pure attraction
0 = no preference
−1.0 = pure aversion
B
dye concentration
0 5X 10X
y-position (mm)
C
strong attraction
25%
50%
25%
Time (min)
D
30 min Frequency
60 min Frequency
CI = 0.80
CI = 0.75
E
strong attraction
F
S.Basal control
30min Frequency
60 min Frequency
CI = 0.09
CI = 0.13
excluded bins
G
strong attraction
no attraction
C.I. time course
Time (min)

**FIGURE 2:** Worm distribution within a microfluidic device was quantified as the chemotaxis index over a one-hour period. A) Chemotaxis index (CI) was calculated by subtracting the number of worms residing in the outer 25% of the edges from those in the inner 50% mid-region, divided by total worms tested in the arena. B) Quantification of a dye concentration used to visually represent the test odorant gradient distribution shows a linear curve for the gradient stripe that runs across both arenas. C) Relative proportion of animals residing at every bin of the vertical y-position in a microfluidic arena, in this case it represents a strong attraction. D) When animals exhibit strong chemoattraction, they move within the highest concentration gradient as depicted by 30 minute CI = 0.80 and 60 minute CI = 0.75. E) Relative proportion of animals residing at every vertical y-position (mm) in a microfluidic arena containing only S.B, in this case it represents no attraction. F) When animals exhibit no attraction, they move randomly across the arena as indicated by a 30 minute CI = 0.09 and 60 minute CI = 0.13. The outer 25% edges *(green)* and the inner 50% mid-region *(blue)* are incorporated into the CI calculation. Peripheral regions containing higher frequency of animal residence are omitted *(red).* G) Changes in CI were observed over 1 hr in 5 minute bins for worms showing strong attraction (*left*) and no attraction (*right*).

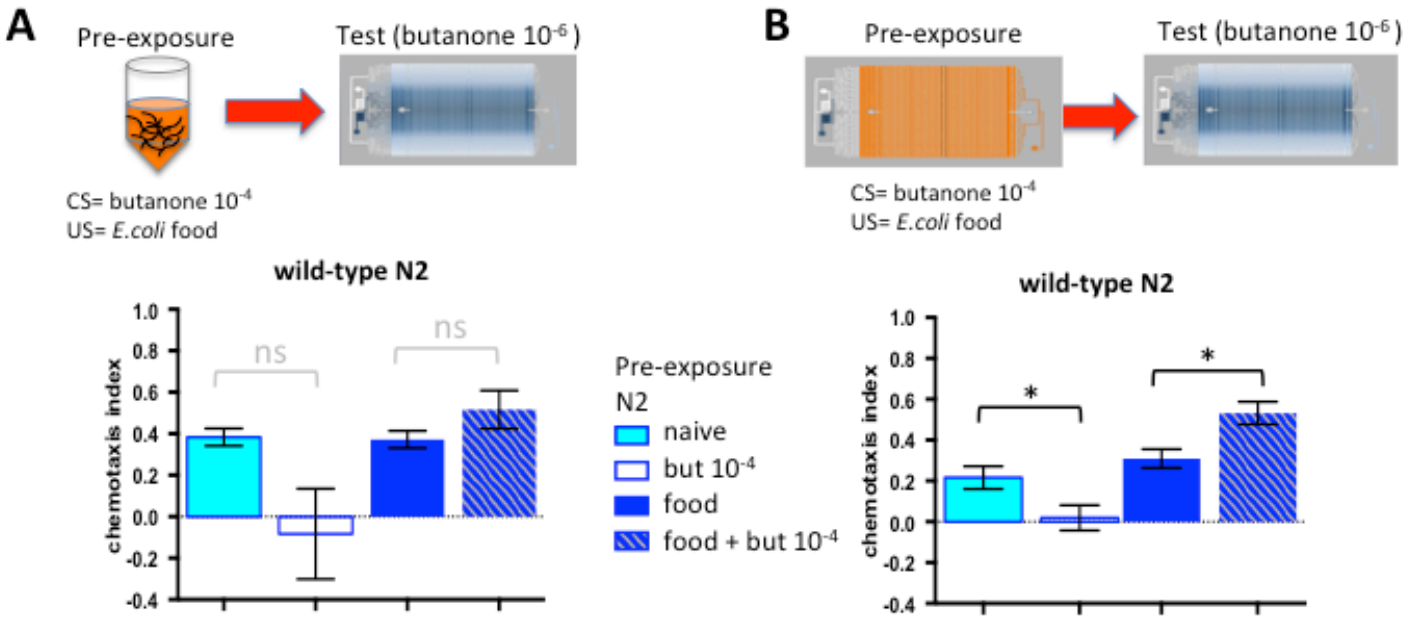



**FIGURE 3:** Wild-type N2 animals pre-exposed to butanone plus food for 1 hr in a microfluidic device exhibits noticeable butanone enhancement when tested in the same device. A) Pre-exposure conditions were first held in microfuge tubes (*top*), and then all experimental groups were tested for chemotaxis towards butanone $10^{-6}$ for 1 hr. This 30 minute chemotaxis index showed that butanone ($10^{-4}$ dilution) plus food was not significantly higher than the food only pre-exposure group. However, the presence of food repressed butanone adaptation and sustained attraction. B) Pre-exposure conditions were held in microfluidic devices followed by a chemotaxis test within the same device already in use (*top*). This 30 minute chemotaxis index shows significant butanone enhancement when butanone is added in the presence of food (0.53 ± 0.05) compared to food alone (0.31 ± 0.05). Worms pre-exposed to butanone $10^{-4}$ alone (0.02 ± 0.06) manifested butanone adaptation compared to starved naïve worms (0.22 ± 0.06), *p ≤ 0.05. 1 hr pre-exposure conditions include: naïve, food only, butanone $10^{-4}$ only, butanone $10^{-4}$ plus food. Error bars indicate SEM. Microfuge tube pre-exposure experiments, n = 3-4. Pre-exposure and test in microfluidic device, n = 6-8.

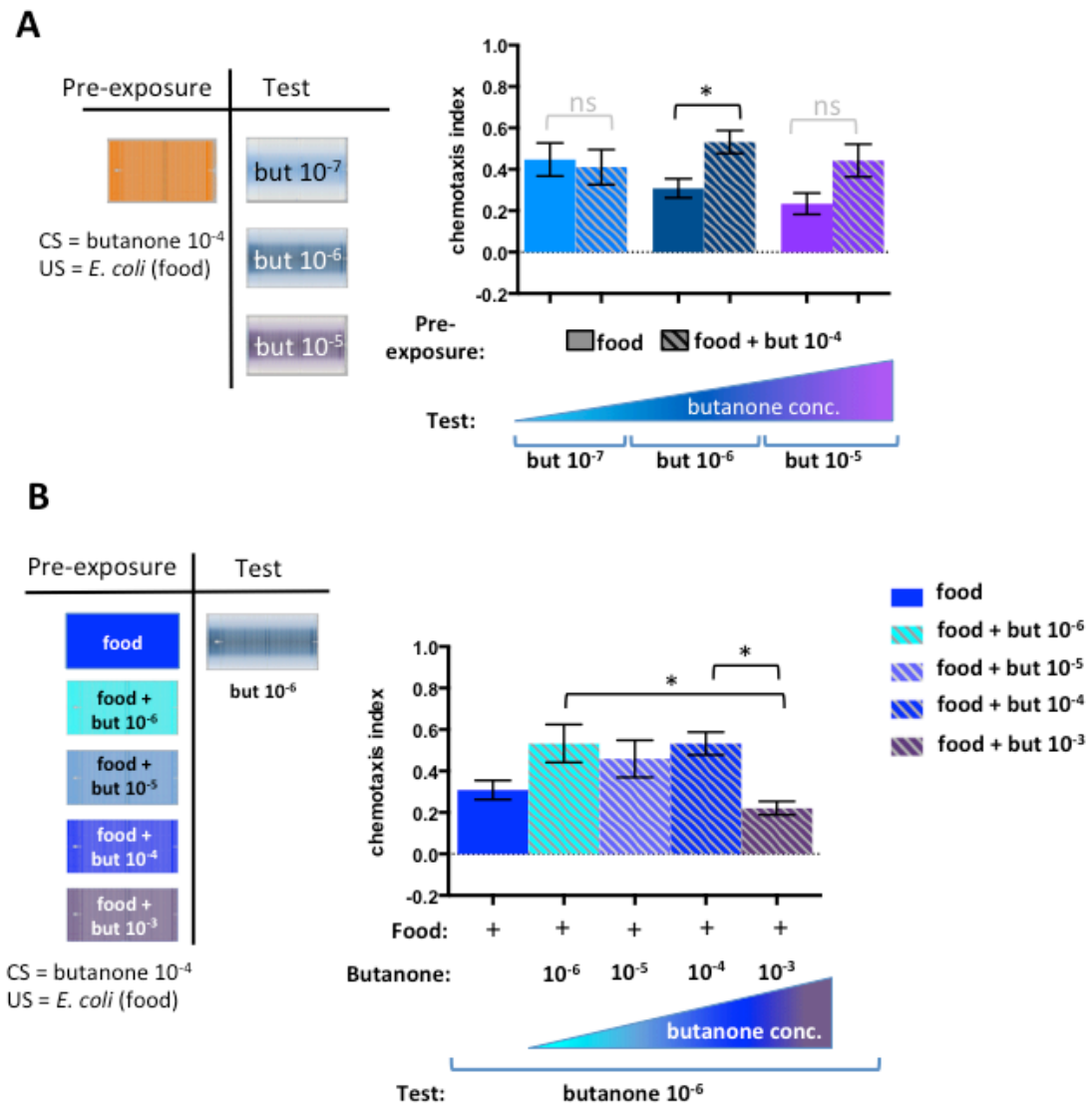



**FIGURE 4:** Optimization of test and pre-exposure conditions for a microfluidic-based chemotaxis assay to test for butanone enhancement phenotypes. A) 1 hr test conditions examined butanone dilution ranges: $10^{-7}$ to $10^{-5}$, while 1 hr pre-exposure conditions remained the same as listed: (food only *(filled bars)*, food plus butanone $10^{-4}$ *(diagonal striped bars*). Test concentration of butanone $10^{-6}$ (*p ≤ 0.05) offered the most robust butanone enhancement of all three dilutions when compared to their respective food only pre-exposure control groups using unpaired t-test. B) Following establishment of a test butanone concentration (butanone $10^{-6}$), a variety of pre-exposure conditions with butanone concentrations ranging from $10^{-6}$ to $10^{-3}$ were examined to observe which of the four dilutions induced the most robust butanone enhancement phenotype. No significance was observed across all pre-exposure groups when compared to one food only group using one-way ANOVA. Upper limit for pre-exposure conditions should not exceed butanone $10^{-3}$ because there was a significant drop in CI (0.22 ± 0.03) compared to butanone $10^{-6}$ and $10^{-4}$ dilutions. Error bars indicate SEM, *p ≤ 0.05, n = 6-8.

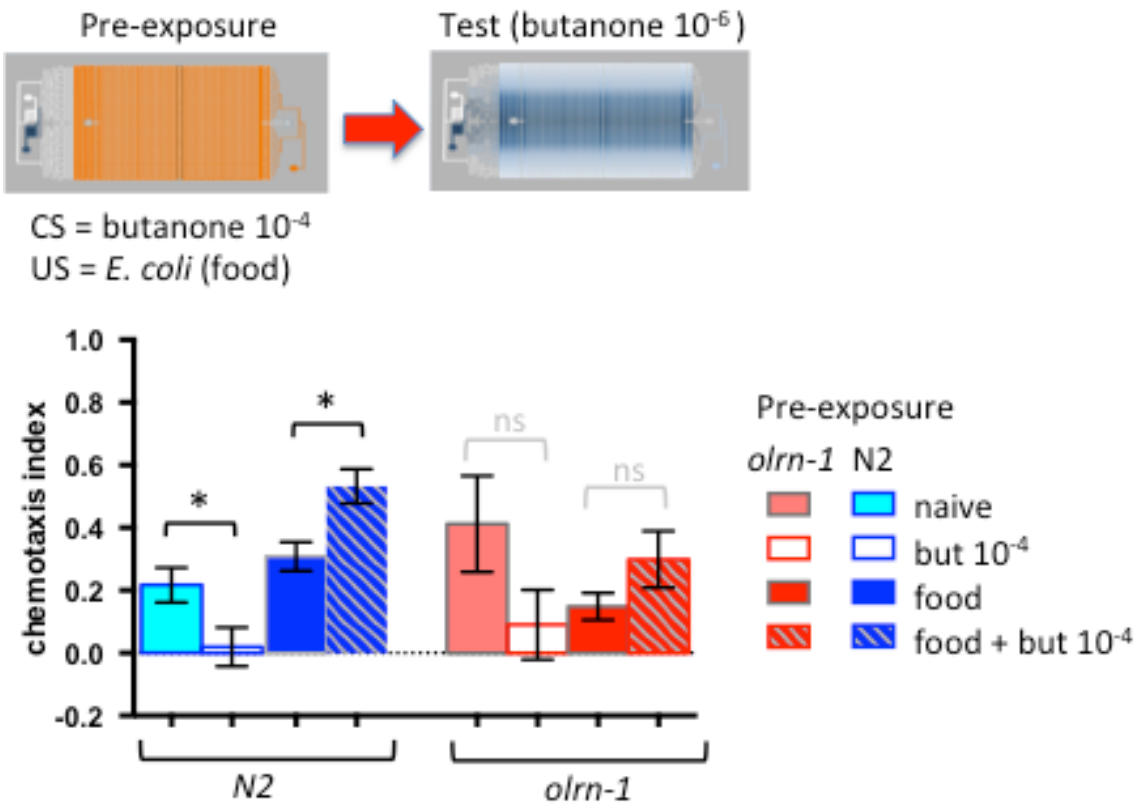
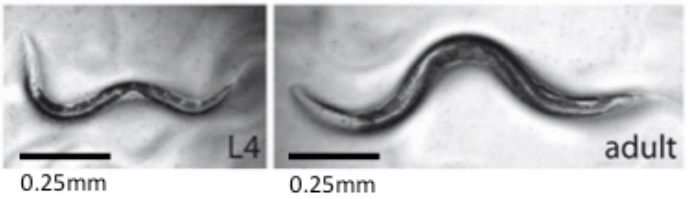



**FIGURE 5:** Applying the optimized microfluidic chemotaxis assay conditions to test *olrn-1* mutants. Using the same wild-type N2 *(blue)* data from Fig 3B for comparison, we noticed similarities and differences between the two genetic strains. Normal butanone preference in naïve *olrn-1* mutants was observed (0.41 ± 0.15), but no significance was observed for butanone adaptation or associative learning in *olrn-1* young adults *(red)*. Appropriate pair-wise comparisons between naïve and butanone for adaptation, and butanone plus food and food only for associative learning showed no significance as indicated in gray (ns). Error bars indicate SEM, *p ≤ 0.05, n = 6-8.

**A**

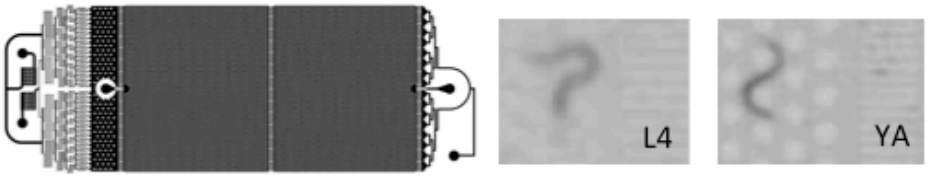



**B**

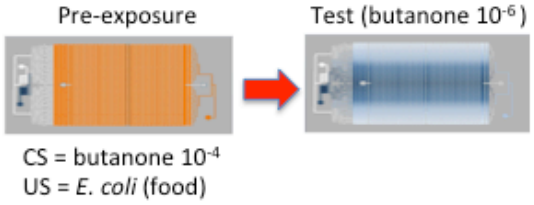
Micropost dimensions for larval stage 4 (L4) *C. elegans*

| Device | Post Radius (μm) | Spacing (μm) | Height (μm) |
|---|---|---|---|
| Young Adult | 100 | 100 | 70 |
| Larva 4 | 60 | 60 | 50 |

**C**




**D**



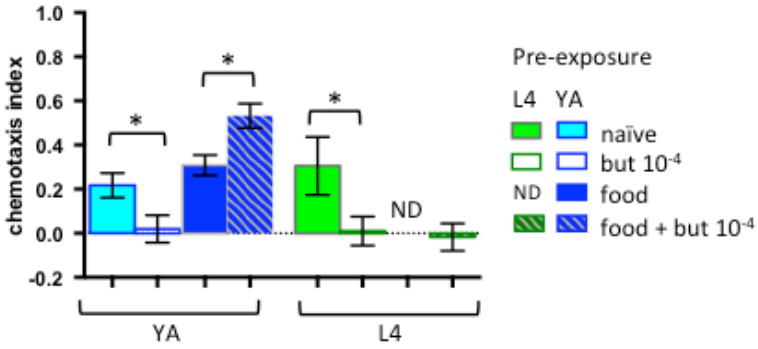



**Figure 6:** Sustained butanone attraction was absent in larval stage 4 (L4) worms when pre-exposed to butanone plus food. A) Morphological and size differences between L4 and young adult (YA) worms are displayed for comparison. B) Table listing micropatterned post dimensions that enable normal sinusoidal movements of young adult or L4 worms within the microfluidic arena. C) Diagram of modified device for L4 worms and an image of L4 worm within the microfluidic arena juxtaposed to a YA worm (*right*). D) L4 worms still exhibit innate attraction to butanone in the naïve state (0.31 ± 0.13) and showed normal butanone adaptation (0.01 ± 0.06, *p ≤ 0.05) after pre-exposure to it for an hour in the absence of food. Worms pre-exposed to butanone + food showed almost no preference for butanone (-0.02 ± 0.06). Authorized permission to use preliminary data for L4 worms from a graduate rotation student, Jeremy Shui (2014). L4 pre-exposure to food only group was not determined (N.D). Error bars indicate SEM, *p ≤ 0.05, n = 3-4.

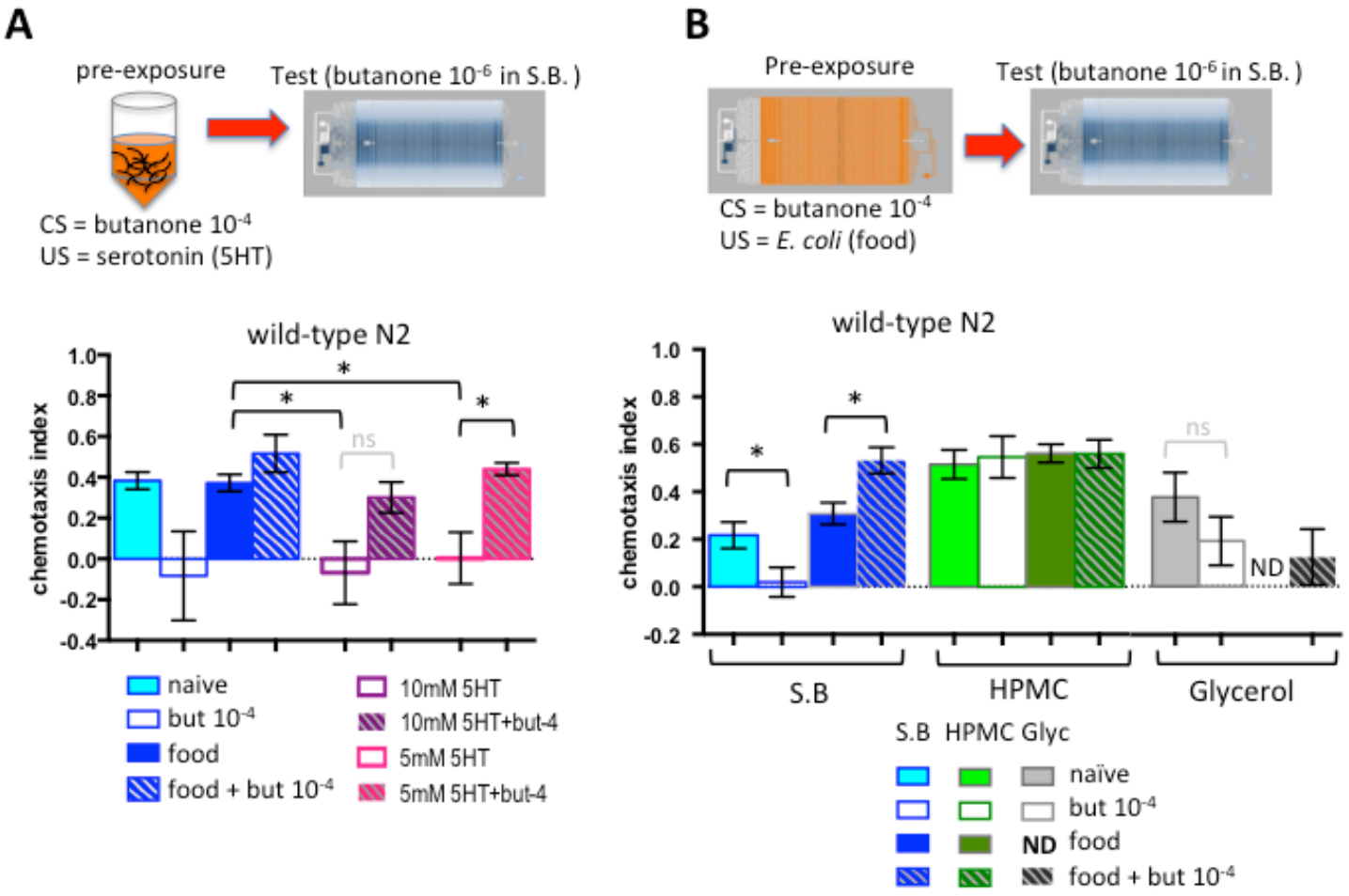



**Figure 7:** Chemical and physical perturbations to learning behavior in a microfluidic-based chemotaxis assay A) Schematic for 1 hr pre-exposure conditions within a microfuge tube, followed by a 1 hr chemotaxis test with butanone $10^{-6}$ *(top)*. The previous 30 minute chemotaxis analysis of tube-conditioned N2 young adults is shown in blue. Both 10 mM and 5 mM 5HT pre-exposed worms were significantly different from food only groups, indicating that serotonin itself does not mimic food (*p ≤ 0.05). Worms pre-exposed to either 10 mM (purple bars) or 5 mM (pink bars) exogenous serotonin exhibit either suppressed adaptation to butanone $10^{-6}$ or enhanced butanone attraction when tube-conditioned and tested within a microfluidic device. B) N2 worms underwent pre-exposure conditions in S. Basal (S.B, *blue,* previous data from Fig 3A), 5% Hydroxypropylmethylcellulose (HPMC, *green*), and glycerol (Glyc, *grey*). While testing with butanone $10^{-6}$ occurred in S.B within a microfluidic device (top) for all pre-exposed groups compared Pre-exposure conditions in HPMC showed no significant differences with each other, thus deeming it a non-neutral medium and unsuitable as a bacterial suspension for future associative learning studies. The presence of glycerol prevented positive butanone attraction when N2 worms were pre-exposed to butanone plus food (0.12 ± 0.12) for 1 hr, and glycerol also hampered butanone adaptation in worms conditioned to butanone alone (0.19 ± 0.10). Food only experimental group was not determined (N.D) for glycerol conditioning. Error bars indicate SEM. *p ≤ 0.05, n = 6

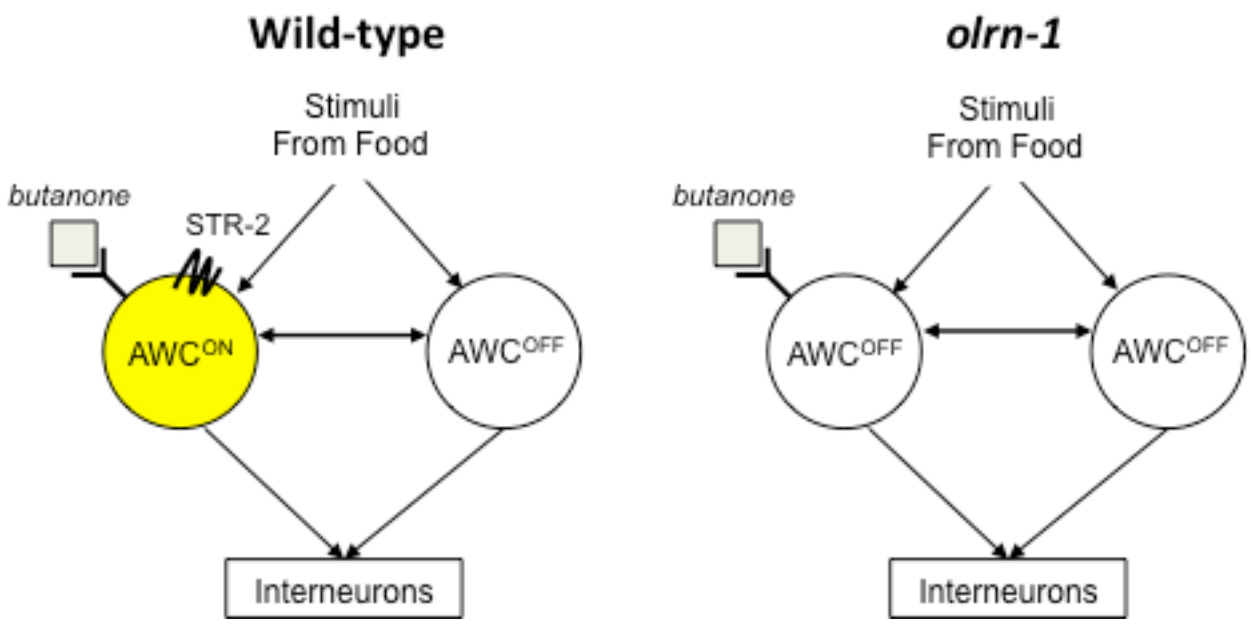



**Figure 8:** Olrn-1 loss-of-function mutation impedes AWC ON/OFF cell-fate**,** consequently preventing normal butanone enhancement. Schematic of synaptic relationships between the left-right AWC neurons and their interneuron targets show for wild-type (*left*) and olrn-1 mutants (*right*). A known difference between the two strains is the presence of an $AWC^{ON}$ neuron as indicated by its STR-2 expression. It remains to be determined which type of butanone receptors are expressed in both AWC ON/OFF neurons and the impact of cell-fate on cell-cell interaction between the left-right chemosensory neurons and between chemosensory and interneurons.

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# Appendix

## CTXsummary

```
% Definitions and settings
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figh = 30;
set(0,'DefaultAxesFontSize',8);

%load experiment file names
load('Z:\Karen Tran\Experiments\Gradient Assay\GradientFileList.mat');

saveFolder = uigetdir;

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```

```
for g = 1:size(groups,1)

    conditionname = groups{g,1};

    allexplist = groups{g,2};

    numexpts = length(allexplist);
    numpages = ceil(numexpts / rows);

    for p = 1:numpages
        indexlist = (p-1)*rows+(1:rows);
        indexlist = indexlist(find(indexlist <= numexpts));
        explist = allexplist(indexlist);

        figure(figh); clf;

        for i = 1:length(explist);
            expnum = explist(i);

            f = GradientFileNames{expnum};
            shortname = f(max(strfind(f,'\')):(strfind(f,'_density')-1));

            load(f);
            % TimeY = Track.TimeY; % use all data
            TimeY = msum(struct2mat(3,Beh,[1 3 6],{'TimeY'}),3);  % removed pause
state (2)

            rownum = i;

            % heatmap
            subplot(rows,5,(rownum-1)*5+[1:2]);
            [tbins,ybins]=size(TimeY);
            %imagesc((1:tbins)/2,1:ybins,Track.TimeY');
            image((1:tbins)/2,1:ybins,index2rgb(TimeY',flipud(gray)));
            %colormap(flipud(gray)); colorbar;
            if i==rows xlabel('Time (min)'); end
            ylabel('y-bin');
            title(shortname,'Interpreter','none','FontSize',8);

            % timecourse CTX
            interval = 10;
            ctx = [];
            for t = 1:floor(tbins/interval)
                temp = CTX(TimeY, (t-1)*interval+(1:interval));
                ctx = [ctx, temp];
            end
            subplot(rows,5,(rownum-1)*5+[3]);
            bar((1:length(ctx))*interval/2,ctx,'b');
            ylabel('CTX timecourse');
            xlabel('Time (min)');
            xlim([1 125]);

            % 30 min CTX
            subplot(rows,5,(rownum-1)*5+[4]);
            CTX(TimeY,1:61);
            ylabel('30 min CTX');
```

```
            colormap(jet);
            xlim([1 80]);
            if rownum == 1
                title(sprintf('%s_page%d %d/%d exp#%d',conditionname, p, i+(p-
1)*rows, numexpts, expnum),'Interpreter','none');
            end

            % 60 min CTX
            subplot(rows,5,(rownum-1)*5+[5]);
            CTX(TimeY,1:121);
            ylabel('60 min CTX');
            xlim([1 80]);

        end % experiment plot loop

        orient(figh,'landscape');
        saveas(figh,fullfile(saveFolder,sprintf('%s_page%d.pdf',conditionname,
p)),'pdf');

    end % page loop

end  % group loop
```