

## Simultaneous Optogenetic Stimulation of Individual Pharyngeal Neurons and Monitoring of Feeding Behavior in Intact *C. elegans*

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### Abstract

Optogenetic approaches have proven powerful for examining the role of neural circuits in generating behaviors, especially in systems where electrophysiological manipulation is not possible. Here we describe a method for optogenetically manipulating single pharyngeal neurons in intact *C. elegans* while monitoring pharyngeal behavior. This approach provides bidirectional and dynamic control of pharyngeal neural activity simultaneously with a behavioral readout and has allowed us to test hypotheses about the roles of individual pharyngeal neurons in regulating feeding behavior.

**Key words:** Optogenetics, Feeding behavior, Small circuits, Pharynx, In vivo optogenetics, *C. elegans*

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### 1 Introduction

The nematode *C. elegans* is a powerful tool for studying the function of neural circuits, in large part due to its genetic tractability and known synaptic connectivity [1, 2]. Since it is not currently possible to electrophysiologically manipulate the activity of the *C. elegans* nervous system in intact animals [3], the primary method for studying the roles of specific neurons in behavior has been ablation of these neurons in young larvae using a laser beam followed by observation of behavior in adults [4, 5]. However, laser ablation is a permanent, unidirectional manipulation of a neural circuit that does not easily allow the assessment of functional redundancy or developmental compensation [6]. It is therefore suboptimal for understanding dynamic phenomena like neural activity.

Optogenetic techniques, which involve using light-sensitive ion channels to manipulate neural activity and behavior, are ideal for precise temporal control of neural activity in behaving animals, as they afford bidirectional and dynamic manipulation [7]. To date, most optogenetic experiments in *C. elegans* have been performed by illuminating the entire worm after expressing light-sensitive proteins in a desired subset of neurons [8]. However, the cellular specificity of this approach—and therefore its utility for functionally dissecting neural circuits—has been limited by the difficulty of finding a promoter that drives gene expression only in the desired neurons. Genetic intersection approaches have been used [9–12], but even these are not guaranteed to provide the desired overlap. Furthermore, when using promoters with high cellular specificity, a different strain is required to study each subset of neurons, making it impossible to study the functions of multiple neurons in a single worm. To solve this problem, multiple groups have developed different methods for using patterned light to illuminate arbitrary parts of the worm corresponding to neurons of interest. The first of these studies was done using immobilized worms and optically monitoring calcium levels in neurons of interest [13]. More recent work has used locomotion as a behavioral readout of circuit function [14–16].

Here, we describe a method for using spatially restricted optogenetic illumination to investigate the behavioral effects of manipulating individual neurons in the pharynx (feeding organ). The pharyngeal neural circuit is one of the simplest in the worm, containing 20 neurons of 14 classes. However, experiments in which each of the 14 classes of pharyngeal neurons was ablated found only three classes for which ablation caused a clearly identifiable deficit in feeding behavior [17], perhaps due to circuit redundancy. Using an optogenetic approach, we uncovered genetic and neural degeneracy in the circuit for excitation of pharyngeal pumping [18].

We use a digital micromirror device (DMD) to focus light in defined patterns on worms that express light-sensitive excitatory (e.g., blue-light-sensitive ion channel Channelrhodopsin-2 (ChR2)) [19] or inhibitory microbial opsins (e.g., blue-light-powered proton pump (Mac) from *Leptosphaeria maculans*) [20–22]. To stimulate single neurons, we take advantage of the fact that pharynx is internal and use polystyrene beads to immobilize worms [23]. This affords submicron spatial resolution without pharmacological manipulation. We use particle image velocimetry to automatically track movement of pharyngeal muscles during optogenetic manipulations. This approach improves on both the lack of specificity present in typical optogenetic experiments and the lack of sensitivity and intraobserver variability inherent in manual observation [24].

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## 2 Materials

1. Optical table (at least 1.5 m × 1.2 m surface).
2. 473 nm laser (e.g., Shanghai Laser and Optics Century model BL473T3-150).
3. Leica DMI3000B inverted microscope and associated filter cubes.
4. Leica Plan Apo 63× oil immersion objective lens with N.A. = 1.4.
5. Photometrics DV2 multichannel imaging system.
6. Cooled CCD camera with software capable of 30 frames per second (e.g., Andor iXon 885 and Andor SOLIS software).
7. 1024 by 768 pixel digital micromirror device (DMD) with Discovery 4100 Explorer software (Discovery 4100 DLP, Texas Instruments/Digital Light Innovations).
8. Power meter (e.g., Coherent FieldMate).
9. MATLAB software.
10. Aluminum mounting box for DMD control board (approx. 15 cm × 20 cm).
11. 6.24 mm focal length aspheric lens (e.g., Thorlabs C110TME-A).
12. 75 mm focal length planoconvex lens (e.g., Thorlabs LA1608-A).
13. 100 mm focal length, 2-in. diameter achromatic lens (e.g., Thorlabs AC508-100-A).
14. 2 200 mm focal length, 2-in. diameter achromatic lenses (e.g., Thorlabs AC508-200-A).
15. ~7 mirrors (e.g., Thorlabs PF20-03-P01 and PF10-03-P01).
16. Two irises (e.g., Thorlabs ID15).
17. Longpass dichroic filter (e.g., Thorlabs DMLP490R).
18. Hardware for mounting each optic.
  - (a) Post holders (e.g., Thorlabs UPH3).
  - (b) Posts (e.g., Thorlabs TR3).
  - (c) Lens and mirror mounts (e.g., Thorlabs LMR1, LMR2, KM100, KM200).
  - (d) Post clamps (e.g., Thorlabs RA45).
  - (e) 1/4"-20 cap screws (e.g., Thorlabs HW-KIT2).
  - (f) 8-32 cap screws (e.g., Thorlabs HW-KIT3).
19. Sodium fluorescein.

20. NGM buffer (NGM from [25] but without agar, cholesterol, or peptone).
21. Agarose.
22. Serotonin HCl.
23. Slides and coverslips.
24. 0.5 mm thick plastic shim stock (Arduus Yellow 0.020 Inches).
25. 250 mL beaker.
26. 50 mL centrifuge tube.
27. Disposable spatulas (e.g., VWR International 80081-188).
28. 2.5 % (v/v) suspension of 50 nm diameter polystyrene beads (Polysciences 08691-10).
29. Transgenic nematode strains expressing opsins in neurons of interest.

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### 3 Methods

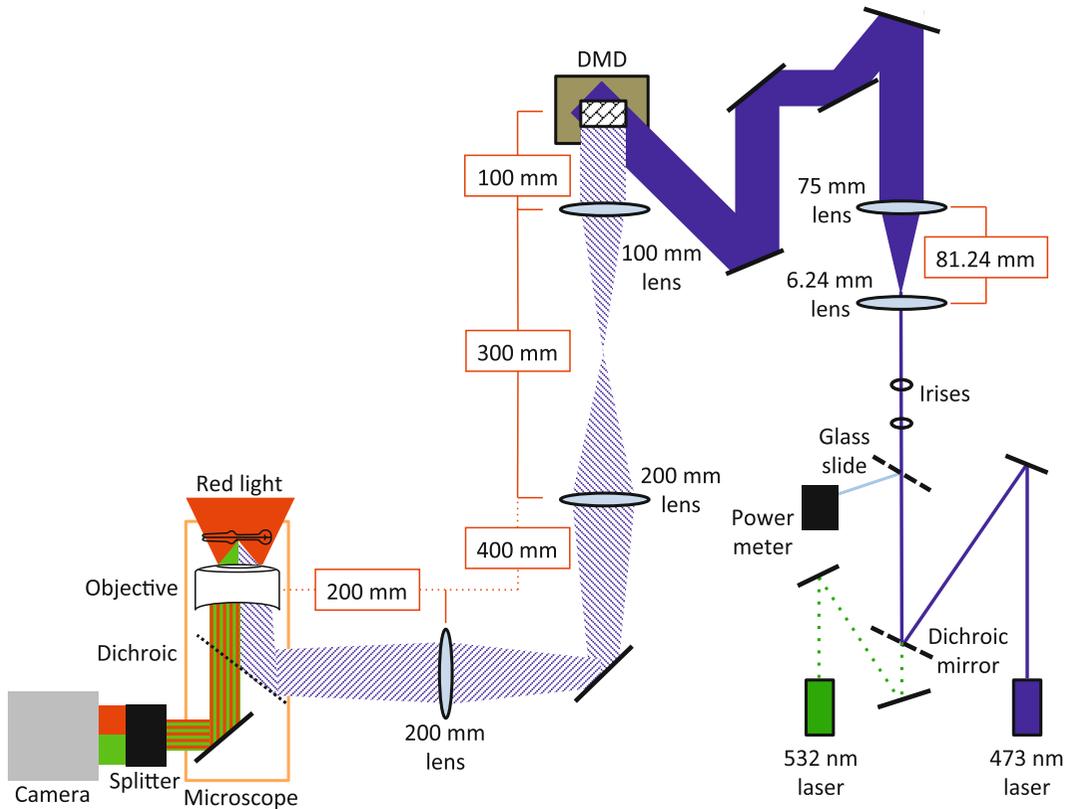
The methods will be described in five sections: (a) Building the rig, (b) Constructing the worm strains, (c) Immobilizing the worms, (d) Performing the experiments, and (e) Analyzing the data.

#### 3.1 Building the Rig

In the optical setup, a laser beam is first expanded in diameter by about ten times by a telescope consisting of two lenses (Fig. 1). This laser beam is routed via mirrors to the DMD, which restricts the beam to a set of pixels selected by computer. Next, the image on the DMD is relayed to an intermediate image plane of the microscope with 2× magnification using lenses with focal lengths 100 and 200 mm. Finally the image is relayed again to the worm via a relay lens system composed of a 200 mm focal length lens and the objective lens. The steps for constructing this setup are described below, and a schematic is depicted in Fig. 1.

*Before starting work, obtain the appropriate laser protection eyewear, light curtains, and other safety equipment. Consult your laser safety protection officer to determine what is needed.*

1. Place the microscope and laser on an optical table and secure them so that they will not inadvertently move in relationship to the rest of the table (*see Note 1*).
2. Mount each mirror or lens in an appropriate mount, and attach each mount to a post and post holder. Fasten post holders to the table in an out of the way location so that they are secure while you are performing the other steps.
3. Mount the DMD by attaching the DMD to a KM200 mirror mount and attaching the control board to the mounting case using screws and board standoffs. Ensure that the DMD power cable can be plugged in to the control board after mounting.



**Fig. 1** Setup for single neuron stimulation of pharyngeal neurons. Light leaves the blue laser and is reflected off a mirror (*black line*) and dichroic beam splitter. A small percentage of the light is then reflected to a power meter to monitor laser output. The light then passes through two irises before being expanded through a telescope composed of two lenses. The beam is then, through a series of mirrors, reflected onto the DMD (*brown box*) such that it covers the entire grid of mirrors. This beam then passes through a series of lenses along the light path. It then enters the microscope through an auxiliary port, where it is reflected by a dichroic on a custom filter cube into the back of the objective. The stage of the microscope is illuminated with *red* light. This *red* light and the *green* light emitted by fluorescent proteins then pass through a DV2 beam splitter, which separates the red and green channels, which are then recorded by a camera

4. Place two irises in the path of the laser, and adjust them and the laser so that the laser beam is perfectly horizontal and passes directly through the center of the irises.
5. Place the 6.24 mm focal length lens and the 75 mm focal length lens so they are 81.24 mm apart (the sum of the focal lengths), beyond the irises. The lenses should be oriented according to their design specifications, with the sides designed for short conjugate distance facing each other.
6. Align these lenses so the beam passes through the center of the lenses and is collimated (i.e., neither converging nor diverging) after exiting the 75 mm focal length lens (*see Note 2*).
7. Install a filter cube and dichroic filter that reflects light from the desired port through the back of the objective. For GFP

- optics, use a longpass dichroic filter that reflects wavelengths shorter than 500 nm and transmits wavelengths longer than 500 nm (*see Note 3*).
8. Place one of the 200 mm lenses 200 mm away from the back of the objective, along the light path, with the curved side facing the microscope (*see Note 4*).
  9. Place the other 200 mm lens 400 mm beyond the first 200 mm lens, along the light path. The curved side should face away from the microscope (*see Note 5*).
  10. Place the 100 mm lens 300 mm beyond the furthest 200 mm lens. The curved side should face the 200 mm lens.
  11. Place the DMD 100 mm beyond the 100 mm lens. Screw down loosely, as the position will likely need to be adjusted later (*see Note 6*).
  12. Plug in the DMD and use the Discovery 4100 Explorer software to set it so that all the mirrors are in the ON position.
  13. Use mirrors as necessary to project the laser beam onto the DMD (*see Note 7*).
  14. Adjust the angle of the DMD so that with the mirrors in the ON position, the laser beam is reflected along the optical path of the microscope, through the center of the 100 and 200 mm lenses (*see Note 8*).
  15. Place a glass slide at an angle in the light path shortly after the beam leaves the laser by taping the slide to an optical post (*see Note 9*).
  16. Set up the power meter so the light reflected off the glass slide hits the center of the sensor.
  17. Place a red filter in the transillumination light path to enable behavioral observation during the optogenetics experiments without off-target stimulation effects.
  18. To allow independent analysis of the green (GFP/targeting) and red (brightfield/behavior) signals, attach the DV2 and camera to the imaging port of the microscope.
  19. Align the DV2 according to the instructions that come with the device (*see Note 10*).
  20. Register the DMD coordinates to the field of view of the camera. Create a series of images that contain a square moving in a matrix across the DMD (*see Note 11*).
  21. Prepare a slide with a thin layer of 1 M sodium fluorescein in water or glycerol between two shims under a coverslip and place it on the microscope stage.
  22. Pass the series of images to the DMD (**step 20**) and record where (if at all) a fluorescent spot appears on the camera.

23. Compute the correspondence matrix of the coordinates passed to the DMD and coordinates where fluorescence was detected.
24. Use this matrix to define an image transformation between the DMD and the camera.

### 3.2 Creating Worm Strains

1. In general, the same strains that are used for whole worm optogenetics can be used for this method (*see Note 12*).
2. These worms should be grown in the dark following standard procedures. Where needed, add 2  $\mu\text{L}$  of 100 mM all-*trans* retinal (ATR) in ethanol to the bacterial suspension immediately before seeding (*see Note 13*).
3. Laser ablations, if desired, can be performed as previously described [4, 5].

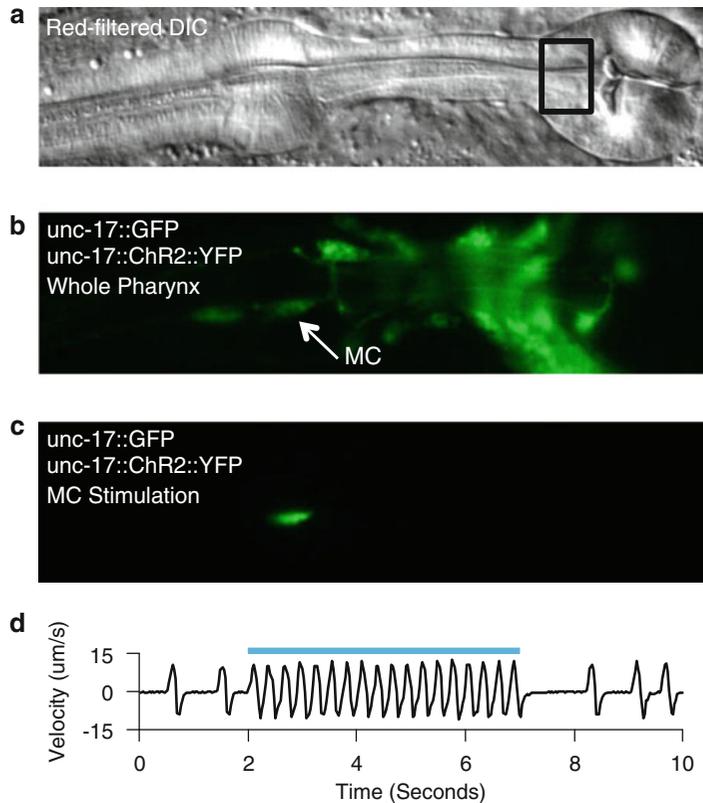
### 3.3 Preparing Agarose Pads

1. Prepare six slides by placing 0.5 mm thick shims, approximately 1 cm by 2 cm, on each end of each slide.
  2. Prepare agarose pads by mixing 0.20 g agarose and 4 mg 5-HT into 2 mL of NGM (10 % agarose, 10 mM 5-HT) in a 50 mL centrifuge tube (*see Note 14*).
  3. Swirl the mixture gently so that the agarose is evenly dispersed in the liquid rather than a large clump, but avoid getting too much of the mixture on the sides of the tube.
  4. Rest the lid on top of the centrifuge tube, but do not screw it on.
  5. Place the centrifuge tube in a 250 mL beaker.
  6. Fill the beaker to ~50 mL with tap water, just above the level of liquid in the centrifuge tube.
  7. Microwave the beaker and centrifuge tube for 50 s at high power, at which point the agarose mixture should be clear, and may have some bubbles.
  8. Using a disposable plastic spatula, place a drop of agarose mixture about 1 cm in diameter onto the middle of a slide prepared with shims and immediately cover this slide with a second slide (*see Note 15*).
  9. Make slides until all the agarose has been used (*see Note 16*).
  10. Allow slides to set for at least 1 min before adding worms.
- Note: **Steps 11–16** should be performed quickly for optimal immobilization.
11. Load 1.5  $\mu\text{L}$  of a 2.5 % (v/v) suspension of 50 nm diameter polystyrene beads into a 20  $\mu\text{L}$  pipettor [23] (*see Note 17*).
  12. Remove top slide from one of the pads and place the slide with the pad onto a plate lid on the microscope stage.

13. Use bacteria to stick up to ten worms onto the bottom of a worm pick, but do not put them on the pad yet.
14. Expel the 1.5  $\mu\text{L}$  of beads onto the pad.
15. Gently, and as quickly as possible, place the pick into the beads and allow the worms to transfer into the beads on the pad (*see Note 18*).
16. Quickly place a coverslip on top of the pad to immobilize the worms.
17. Wait 5–10 min before performing experiments to allow the worms to habituate.

### **3.4 Performing the Experiments**

1. Set the frame rate of the camera and the laser power (*see Note 19*).
2. Place a slide on the microscope stage and find a worm at 10 $\times$  on the microscope under brightfield illumination (*see Note 20*).
3. Switch to 63 $\times$  and move the pharynx of the worm into the center of the field of view under brightfield illumination (*see Note 21*).
4. Manually take a z-stack of the pharyngeal neurons by illuminating the entire field using the DMD, and focusing through the relevant regions. This illumination period should be less than 1 s.
5. If desired, use MATLAB to convert the images into JPEG files. MATLAB scripts for this and all other steps are available upon request (*see Note 22*).
6. Identify the images in the z-stack that most clearly show the neurons of interest.
7. Use the `roipoly` function in MATLAB to select the regions of these images you would like to use to stimulate each neuron (*see Note 23* and Fig. 2b, c).
8. Use MATLAB to create an image mask that contains the value 1 for the region you outlined in the previous step and the value 0 elsewhere, and transform this file into DMD coordinates using the transformation computed in step A23 (*see Note 24*).
9. Create a Discovery 4100 script that tells the DMD which images to display and at which times they should be displayed (*see Note 25*).
10. Acquire a baseline recording of behavior without any illumination (*see Note 26*).
11. Run each script while recording from both the red and green channels (*see Note 27*).



**Fig. 2** Stimulation of single pharyngeal neurons. (a) DIC image of the pharynx. *Black* box denotes region used for velocity calculations. (b) Wide field GFP fluorescence image of the same field of view as in (a). The *arrow* points to an MC soma. (c) GFP fluorescence image of the same field of view as in (a) and (b), during selective illumination of an MC soma. (d) Velocity from PIV algorithm during ChR2-mediated stimulation of the MC neurons. Each peak in the velocity represents a pump. The *blue* bar represents timing of laser illumination. Modified from Trojanowski et al. [18] with permission from American Physiological Society

### 3.5 Analyzing the Data

The following steps should be repeated for each experiment:

1. If desired, convert the images into JPEG files (*see Note 22*).
2. Use the `roipoly` function in MATLAB to draw a ROI around the brightest part of the neuron that was stimulated during the experiment (*see Note 28*).
3. Use MATLAB to track the pixel intensity of this region over the course of experiment.
4. Use MATLAB to identify the times at which the intensity of this region sharply increased or decreased. These are the times at which the stimulus was turned on and off, respectively.

5. Use MATLAB to select the rectangular region of the terminal bulb just anterior to the grinder (*see* **Note 29** and Fig. 2a).
6. Using the freely available package PIVlab, a Time-Resolved Digital Particle Image Velocimetry Tool for MATLAB, track the velocity of this region over time (*see* **Note 30** and Fig. 2d).
7. Use this trace of velocity over time to identify the times at which a pump occurred by drawing a threshold and recording the times at which a positive-going threshold crossing occurs (*see* **Note 31**).
8. Determine the number of pumps that occur during each interval in which the stimulus is on or off (*see* **Note 32**).
9. Divide the number of pumps in a stimulus interval by the length of that stimulus interval to find the average pump rate (*see* **Note 33**).

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## 4 Notes

1. Our setup uses a Leica DMI3000B inverted microscope with a Leica Plan Apo 63 $\times$  oil immersion objective lens with N.A. = 1.4. Any microscope with a port that allows direct access to the back of the objective should suffice. An inverted microscope may be easier to set up due to the lower height of the fluorescence illumination path, but an upright microscope could be used as well.
2. The distance between the 6.24 mm lens and the 75 mm lens must be  $\sim 81.24$  mm, but the distances from 75 mm lens to the irises, between the irises, and between the irises and the laser, can be arbitrary. Our rig is partially designed to work with a 532 nm laser as well as a 473 nm laser, so we use a system of mirrors and a dichroic beam splitter to allow us to use these two lasers through the same optical path. If you only wish to use one laser, you can aim it directly through the irises, though we find it useful to have the beam reflect off at least one mirror before entering the irises, because it is easier to precisely adjust the tilt angle of the mirror than that of the laser.
3. For the Leica DMI3000B, the fluorescent light port in the rear of the microscope contained optics of unknown parameters, so we used a side auxiliary port and custom filter cube (Nuhsbaum Inc.) that allowed direct access to the back of the objective.
4. You should adjust this position so that a sharp back-scattered image through the objective forms 200 mm away beyond the lens. It is difficult to measure the 200 mm distance from the back of the objective to this lens precisely, so you may need to adjust this lens empirically later. If this lens needs to be adjusted

after all the other lenses are set up, it may be easier to adjust the position of the microscope rather than move all the optics.

5. Due to spatial constraints, we put a mirror between the 200 mm lenses. This is fine so long as the total distance along the light path between the lenses is 400 mm.
6. The 100 mm distance here should be adjusted empirically so that a crisp back-scattered image from the microscope appears on the DMD and is centered on the grid of mirrors, covering it completely.
7. The laser beam should just barely cover the DMD. If this is not the case, then you likely need to adjust the two smaller lenses so that the beam is not changing in size after it exits the 75 mm lens.
8. To do this, the light must be incident onto the DMD from the lower right, if you are facing the DMD. We find that is easiest to use multiple mirrors to reflect the laser beam at the correct angle, as this provides multiple degrees of freedom for adjustment.
9. This will allow a large amount of the light to pass through but will reflect a small fraction without disrupting the direction of the laser beam. You can measure the light power reflected and the light power at the objective to determine the percentage of light that is reflected, and use this relationship to determine the light power at the objective at any time.
10. Alternatively, a beam splitter could be built in-house with similar optics to those in the DV2.
11. In order to target a specific region of the microscope stage, you must first identify the DMD coordinates that correspond to this region. This process will need to be repeated if any of the optics are inadvertently bumped, so make sure that all of the optics are tightly fastened to the table before beginning this step.
12. It is essential that the opsin of interest is tagged with an appropriate fluorescent protein (we used blue light to excite ChR2 and Mac, so we used opsins tagged with YFP or GFP). However, we found that for some transgenes the ChR2::YFP was not bright enough to resolve neuron processes (e.g., *zxxIs6[unc-17p::ChR2(H134R)::YFP+lin-15(+)]*), likely because the ChR2 is membrane bound. After confirming expression in the relevant cell bodies, we crossed strains containing dim transgenes into a strain that expressed cytoplasmic GFP under the same promoter (we used *vsIs48[unc-17::GFP]*). We found this particularly useful for looking at off-target effects during ablation experiments, where we wanted to determine the effect of stimulating processes near a cell body after killing the cell body.

13. We stored ATR-seeded plates at 4 °C for up to 1 week before use.
14. We make the agarose mixture fresh for each experiment. 5-HT is necessary for inducing a basal pumping rate during immobilization. Other drugs can be added at this step as desired, though we found that adding too many ionic salts (more than 10 mM) caused the polystyrene beads to clump and made immobilization difficult.
15. We have found that the flexibility of the plastic spatulas makes it easier to get all the agarose out of the centrifuge tube, compared to stainless steel spatulas, and their low thermal mass may prevent premature cooling of the agarose.
16. We are usually able to make 6 slides from 2 mL of agarose solution.
17. We store 100  $\mu$ L aliquots of polystyrene beads at 4 °C between uses.
18. When the pads contain 5-HT, the beads clump quickly after they are added to the pad, which reduces the quality of immobilization. Thus, we try to have the beads on the pad for as little time as possible before we add the cover slip.
19. We use an exposure time of 30 ms, which produces a frame rate of 32.7 Hz. We set the laser power so that the irradiance of the laser at the objective is approximately 37 mW/mm<sup>2</sup>, well above the saturation irradiance of ChR2 [26]. These settings can be adjusted to increase the visibility of the fluorescent signal, if necessary.
20. Because of the way the DV2 splits the camera field of view, it is important that the head of the worm is close to aligned with the long direction of each channel's field of view (within  $\sim 30^\circ$ ). If the head is not aligned in this manner, rotate the slide or select a different worm on the slide. Do not rotate the camera, or you will have to re-register the DMD and camera images.
21. It is important that the grinder is visible in the camera's field of view, since its motion will later be used to quantify feeding rate. It is also important that the locations of the neurons of interest are visible in the camera. For pharyngeal neurons, this should not be an issue as long as the worm is aligned as described in **Note 20**.
22. The Andor iXon 885 and Andor SOLIS software produce images as multipage TIFF files. We find it easier to work with JPEG files than multipage TIFF files, but this is a lossy compression and may increase noise.
23. This should be a region slightly larger than the cell body—about 2–3  $\mu$ m in diameter—to allow constant stimulation as the neural cell bodies move during the pump. It is important that the neuron of interest remains in this region during the

entire experiment, because the fluorescent signal from this neuron will be used to determine the times of stimulation *post hoc*. Counterintuitively, we found it difficult to immobilize some paralyzed mutants, in which case we found it necessary to use a substantially larger stimulus region to ensure the neuron of interest was stimulated during the entire experiment.

24. The DMD mirrors can be set in either the ON or OFF position, so we pass an image that contains 1 s (representing ON) in locations corresponding to the area of the stage we wish to illuminate and 0 s (representing OFF) elsewhere.
25. The DMD works by reading in images that represent the pattern it should display and displaying this pattern, then pausing for a defined period, then reading in the next file, and so on. For our experiments, we begin with an all-off image for 5 s, followed by 5 s of illumination of the neuron of interest, followed by an all-off image for 5 s, repeating up to ten times and ending with an all-off image.
26. We record the baseline for 30 s.
27. We wait 2–3 min between running each script, i.e., between each neuron we stimulate. We found that the behavior was most robust when experiments were performed within 90 min of immobilization.
28. Here we find it better to use a ROI smaller than that used for the experiment, because it is easier to detect changes in brightness when just looking at the brightest parts of the neuron.
29. We found that using a rectangle that extends from the anterior edge of the terminal bulb to the grinder, and is just slightly wider than the grinder, provides the best signal.
30. Tracking the velocity of this region over time will show a series of spikes, each representing a pump: a positive change in velocity represents the movement of the grinder towards the posterior and a negative change in velocity represents anterior movement.
31. We found that using a threshold of half of the maximum velocity provided high sensitivity and specificity, though a wide range of threshold values will give the same result on a good recording.
32. We did this for each stimulus interval by finding the number of pumps for which the value (pump time-stimulus time) was greater than 0 but less than the time between when the stimulus turned on and when it turned off.
33. The pumping rate during the stimulus-off windows is sometimes lower than the initial baseline, likely due to post-excitatory inhibition, so we typically do not use these values for looking at the effect of neuron stimulation. Rather, we use the pumping rate that we get from step D10 as the baseline.

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