

Automated Multimodal Stimulation and Simultaneous Neuronal Recording from Multiple Small Organisms

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Abstract

Fluorescent genetically encoded calcium indicators have contributed greatly to our understanding of neural dynamics from the level of individual neurons to entire brain circuits. However, neural responses may vary due to prior experience, internal states, or stochastic factors, thus generating the need for methods that can assess neural function across many individuals at once. Whereas most recording techniques examine a single animal at a time, we describe the use of wide-field microscopy to scale up neuronal recordings to dozens of *Caenorhabditis elegans* or other sub-millimeter-scale organisms at once. Open-source hardware and software allow great flexibility in programming fully automated experiments that control the intensity and timing of various stimulus types, including chemical, optical, mechanical, thermal, and electromagnetic stimuli. In particular, microfluidic flow devices provide precise, repeatable, and quantitative control of chemosensory stimuli with sub-second time resolution. The NeuroTracker semi-automated data analysis pipeline then extracts individual and population-wide neural responses to uncover functional changes in neural excitability and dynamics. This paper presents examples of measuring neuronal adaptation, temporal inhibition, and stimulus crosstalk. These techniques increase the precision and repeatability of stimulation, allow the exploration of population variability, and are generalizable to other dynamic fluorescent signals in small biosystems from cells and organoids to whole organisms and plants.

Introduction

Calcium imaging techniques have allowed the noninvasive recording of *in vivo* neural dynamics in real time using fluorescence microscopy and genetically encoded calcium indicators expressed in target cells^{1,2,3}. These sensors

typically use a green fluorescent protein (GFP), such as the GFP-calmodulin-M13 peptide (GCaMP) family, to increase the fluorescence intensity upon neuronal activation and elevated intracellular calcium levels. Calcium imaging has

been especially powerful in the nematode *C. elegans* for examining how neurons and neural circuits function in living, behaving animals^{4,5,6,7,8,9,10}, as their transparent nature means no surgical process is required for optical access, and cell-specific gene promoters target expression to the cells of interest. These techniques often make use of microfluidic devices, which provide precisely controlled environments to study biological, chemical, and physical phenomena at a small physical scale^{11,12}. Microfluidic devices abound for measuring neural activity, with new designs continually under development, and they are readily fabricated in the research lab. However, many designs trap a single animal at a time, limiting the experimental throughput^{7,9,13}. Neural responses often vary substantially across animals due to differences in prior experience, internal states such as stress or hunger, or stochastic factors such as gene expression levels. These differences establish a need for methods that can simultaneously stimulate and observe many animals and extract information from individuals⁴.

In addition, certain neuromodulatory phenomena become apparent only under specific stimulation conditions, such as temporal inhibition¹⁴, which refers to the brief suppression of responses when stimulation occurs in rapid succession. Electrophysiological systems can drive neural activity across a broad stimulus space for this purpose, modulating, for example, the electrical pulse current, voltage, frequency, waveform, duty cycle, and timing of periodic stimulus trains. Indirect stimulation by naturally detected stimuli or optogenetic systems would benefit from a similar breadth of control mechanisms. Currently, many natural stimuli are presented in a simple "on-off" manner, such as odor presentation and removal, using commercial systems that have been slow to add flexibility. However, inexpensive microcontrollers can now automate the delivery

of several types of stimuli in a manner that is customizable to the researchers' needs. Combined with microfluidics, these systems have achieved the goal of increased experimental throughput and flexibility, allowing neural responses to a variety of precise stimuli to be measured simultaneously in many animals^{4,6}. Multimodal stimulation can be used to further interrogate the neuronal circuitry, such as by monitoring changes in neural excitability when consistently stimulating before, during, and after an orthogonal perturbation such as drug exposure⁴. The benefits of inexpensive, open microscopy systems are clear for advancing scientific research, yet in practice, the need for part sourcing, construction, and performance validation can impede the adoption of these techniques.

This protocol aims to alleviate some of these technical challenges. Whereas previous protocols have focused on microfluidic device use and basic stimulation^{9,15,17}, we describe here the construction and use of a flexible, automated, multimodal stimulus delivery system for neural imaging in *C. elegans* or other small organisms utilizing previously described microfluidic devices⁴. The open-source system is programmed *via* simple text files to define the experiments, and the NeuroTracker data analysis program semi-automatically extracts the neural activity data from the microscope videos. We demonstrate this system with examples of assessing temporal inhibition, disinhibition, and stimulus crosstalk using the chemosensory neuron AWA, which depolarizes in response to different food odors or in response to light when expressing optogenetic light-sensitive ion channels^{5,6}.

Protocol

1. Neural imaging equipment

NOTE: See Lawler and Albrecht¹⁵ for detailed instructions on building the imaging and stimulation system, which controls the microscope illumination timing, image acquisition, and stimulus delivery (**Figure 1**). An inexpensive Arduino Nano stimulus controller actuates the fluidic valves through digital signals to a valve controller and controls the optogenetic illumination through analog voltage signals to an LED controller. Other stimuli, such as vibration motors and thermal heaters, can be controlled using digital or analog signals. The stimulus controller synchronizes the stimulation and image recording *via* camera signals, as specified by the open-source Micro-Manager microscope control software (μ Manager)¹⁶. See the **Table of Materials** for details related to all the materials, reagents, equipment, and organisms used in this protocol.

1. Set up the neural imaging equipment, including an epifluorescence microscope with GFP optics, an sCMOS camera with a digital exposure output signal, and a stimulus controller¹⁵.
2. Connect the stimulus controller to the desired systems *via* digital or analog signals. For the examples presented below, the following systems are used:
 1. Use a valve controller for chemical stimulation. Connect the valve controller to fluidic solenoid or pinch valves (**Figure 1**)¹⁵.
 2. Use a 615 nm red LED and controller for optogenetic stimulation, mounted above the microscope stage.

3. Set up the computer with the microscope control software, create a Configuration Preset with equipment settings, and ensure proper operation of the stimulus control¹⁵.

2. Microfluidic device fabrication

NOTE: See Lagoy et al.¹⁷ for detailed information about obtaining or fabricating the master molds and the production, use, and cleaning of the microfluidic devices. These steps are summarized below.

1. Obtain or fabricate a master mold using the microfluidic design file provided (albrechtlab.github.io/microfluidics)¹⁷. For young adult *C. elegans*, ensure that the channel height is 55-70 μ m.
2. Combine the PDMS base and curing agent at a 10:1 ratio by weight and mix thoroughly with transfer pipettes.
3. Degas for 30-60 min in a vacuum desiccator until the bubbles disappear.
4. Place the master mold into a large (150 mm diameter) Petri dish, and pour the degassed PDMS up to a depth of 4-5 mm (~100 g). Inspect and remove any dust or bubbles with a transfer pipette.
5. Bake at 65 °C on a level oven shelf for 3 h to overnight.
6. Once cured, use a scalpel to cut the PDMS from the mold and a straight razor blade to separate the devices.
7. Punch inlet and outlet holes using a 1 mm dermal punch, and clean them with dH₂O, ethanol, and again with dH₂O. Dry the device in an airstream (see **Figure 2A**).
8. Clean both sides of the PDMS device with adhesive tape, removing any dust or debris.

- Prepare the glass slides to complete the microfluidic device as described¹⁷. Drill inlet holes in the top slide using a diamond bit, and render the bottom slide hydrophobic by exposure to TFOCS vapors or by applying water-repellant glass treatment (**Figure 2B**).
- Assemble the glass-PDMS device sandwich into a clamp (**Figure 2C**).

3. Animal preparation

- Obtain or create animals with genetically encoded calcium indicators expressed in the neurons of interest.
NOTE: For example, line NZ1091 (*kyIs587 [gpa-6p::GCaMP2.2b; unc-122p::dsRed]; kyIs5662 [odr-7p::Chrimson:SL2:mCherry; elt-2p::mCherry]*) expresses GCaMP and the red light-sensitive cation channel Chrimson in the AWA sensory neuron pair⁶. Both transgenes are integrated into the genome for stable expression in every animal.
- One day before experimentation, place at least 20 L4 larval stage *C. elegans* per experiment onto a nematode growth medium (NGM) agar plate seeded with an OP50 *E. coli* lawn. When maintained at 20 °C, this will synchronize the wild-type animals at the young adult stage the next day.
NOTE: For array transgenes, pick animals using a fluorescence stereoscope to ensure transgene expression in the chosen animals.

4. Solution preparation

- Prepare 1x S Basal buffer (100 mM NaCl and 0.05 M KPO₄, pH 6.0) from a 10x stock solution.

- Prepare 1 mM tetramisole buffer by diluting 1 M stock in 1x S Basal. Use this paralytic buffer to prepare all the stimuli. An experiment typically uses about 150 mL.
- Add 0.1-1 µg/mL fluorescein to the "control" buffer reservoirs to visualize the flow.
- Create stimulus solutions by serial dilution to the desired final concentration. For example, create a 10⁻⁷ dilution of diacetyl attractant by first creating a 10⁻³ stock.
NOTE: A small amount of fluorescein (0.1–1 µg/mL) may be added to the stimulus or buffer solution to verify stimulus timing, but concentration should be minimal to avoid artifacts in neural fluorescence.

5. Microfluidic device preparation

NOTE: See Reilly et al.⁹ for a video protocol showing the reservoir generation, device setup, and the loading of the animals. See also Lagoy et al.¹⁷ for a written protocol including many helpful tips.

- Prepare three or more fluid reservoirs. For each, attach a 30 mL or 60 mL syringe reservoir, a 3 mL priming syringe, and a needle stub to a three-way Luer valve (**Figure 2D**). Connect the needle to microbore tubing fitted with a metal tube at the end. Label the reservoirs, mount them onto a rack attached to a ring stand, and fill them with the corresponding buffer or stimulus fluids (**Figure 2E**).
- Degas the assembled microfluidic device in a vacuum desiccator for ~1 h.
- Fill and remove the air bubbles from the reservoir tubing using the priming syringe. Fill the outflow tubing with buffer.

4. Remove the microfluidic device from the vacuum, and quickly insert the outlet tubing and inject the fluid through the device until a droplet emerges from one inlet.
5. At this inlet, use a "drop-to-drop" connection¹⁷ to insert the corresponding fluidic inlet tube (**Figure 2F**). Ensure that liquid drops are present on both the inlet tubing and the device port hole to avoid introducing a bubble.
6. Inject more fluid from the outlet, connect the next inlet tube, and repeat until all the inlets are filled. Insert a solid blocking pin at any unused inlets and the worm loading port.
7. Initiate flow by opening the inlet and outlet Luer valves. Inspect the device for leaks at the inlets and glass base. Inspect the device for any bubbles within the flow channels or inlets using the microscope image capture software in live mode.

NOTE: If bubbles are present, wait for them to absorb into the PDMS material.

6. Animal loading

NOTE: See Lagoy et al.¹⁷.

1. Transfer young adult animals onto an unseeded NGM agar plate using a wire-tipped "pick".
2. Flood the plate with approximately 5 mL of 1x S Basal buffer such that the animals are swimming.
3. Draw the worms into a loading syringe (1 mL or 3 mL syringe with attached tubing that has been prefilled with 1x S Basal).
 1. Using a stereoscope, move the tubing end below the liquid surface with one hand to each desired animal, and draw it into the tubing using the syringe held in the other hand.
 2. Draw the worms only into the tubing, not into the syringe.

NOTE: The tubing typically holds only about 100 μ L. The animals can be expelled into a local area and then drawn again into the tubing with a small volume.
4. Close the outlet line, remove the worm loading pin, and connect the worm loading syringe to the device using a drop-to-drop connection.
5. Gently flow the animals into the arena, establish buffer flow, and allow up to 1 h for immobilization by tetramisole.

NOTE: During the immobilization period, ensure that only buffer fluid enters the arena. The stimulus and control reservoirs can be turned off during this period but open them and verify the correct flow before running the stimulation trials.

7. Automated stimulation and neuronal recording

1. Create a stimulus definition text file called "**User Defined Acquisition Settings.txt**" with a text editor (e.g., Notepad) containing the stimulation settings for the automated image acquisition (see examples in **Figure 3**). The settings are divided into two sections:
 1. **Microscope acquisition settings:** Define the experiment type (Single-Stimulus or Multi-Pattern), exposure and excitation timing, trial duration and intervals, and save directory.
 2. **Stimulation settings:** Define the stimulus control parameters. A "stimulation command" specifies the actions occurring at certain video frames with the syntax <letter code><frame number>, where the letter codes are A = valve1, B = valve2, C = valve3, L = LED light; additionally, uppercase = on and lowercase = off. The LED intensity is set with the

letter code "i" and a value of 0 (off) to 255 (maximum brightness).

NOTE: The LED intensity value from 0 to 255 sets the output analog voltage from 0 V to 5 V. The current controller used here has a linear intensity scaling, and others should be calibrated with a light power meter.

1. For a Single-Stimulus experiment with only one repeated stimulation command, use the format in **Figure 3A**.
2. For a Multi-Pattern experiment with multiple stimulation commands, use the format in **Figure 3B**. Include a "Pattern Sequence" of digits that represents the order of the stimulus patterns. For each pattern, enter a stimulation command on a separate line.

NOTE: A pseudorandom sequence or m-sequence can be useful to investigate stimulus history dependence.

2. Run the microscope control software. Verify that all the fluidic inlets are open, the flow is as desired within the arena (see **Figure 2H**), and the neurons of interest are in focus within the live window.
 3. Close the live window and run the script "**MultiPattern_RunScript.bsh**" within the software.
- NOTE:** It is useful to run a test experiment without animals to verify proper flow and stimulation. Substituting a different fluorescein concentration for each stimulus fluid can help visualize and document new stimulus patterns.
4. After experimentation, disassemble the microfluidic device, and rinse all the surfaces, tubing, and reservoirs with water.

NOTE: All the components can be reused dozens of times if kept clean. Ensure that reservoirs, tubing, and microfluidic devices are maintained wet or fully dried in an air stream to avoid salt crystallization, which can clog and is difficult to remove. The devices can be kept in ethanol to sterilize but should be fully dried before reuse (>1 h at 65 °C)¹⁷.

8. Data analysis using NeuroTracker

NOTE: NeuroTracker^{4,18,19} is an ImageJ/FIJI²⁰ software plugin for tracking the fluorescence intensity of multiple neurons and animals, even as they move during trials. This plugin saves data as text files with each neuron's position and background-corrected fluorescence intensity (F). The fluorescence data are normalized to the baseline fluorescence (F_0), for example, the average of several seconds prior to stimulation, as $\Delta F/F_0 = (F - F_0)/F_0$, which can be averaged across populations.

1. Install NeuroTracker scripts as instructed (github.com/albrechtLab/NeuroTracker).
 2. Run NeuroTracker by clicking on **Plugins | Tracking | NeuroTracker**.
 3. Select the folder containing the .TIF video files to be tracked, and select the desired settings.
- NOTE:** If only a subset of the video files are to be tracked, select the numerical range of the files to analyze at the prompt. Default tracking settings are appropriate for nonmotile animals at 250 pix/mm resolution. Adjust the parameters proportionally for other resolutions. See the User Guide¹⁹ for further settings information.
4. Set the intensity threshold such that the neurons are visible for selection (**Figure 4**).

5. Open the **Brightness/Contrast (B/C)** and **Threshold** control windows using the **Image | Adjust** menu.
6. In the **B/C** window, adjust the **Minimum** and **Maximum** sliders until the neurons are clearly distinguishable (**Figure 4A**).
7. In the **Threshold** window, check **Dark background** and **Don't Reset Range**.
8. Slide the frame slider to observe the neuron movement and intensity changes, noting any animals to exclude from tracking, such as due to overlap with other animals.
9. Identify the neurons for tracking.
10. For each animal, adjust the threshold level such that the red threshold area above the neuron is visible in every frame before, during, and after stimulation.
11. Click on the neuron to record its position and threshold level.
12. Repeat the threshold adjustment and selection for each animal and neuron to track. See **Figure 4C** for a good threshold level example and **Figure 4D,E** for over-threshold and under-threshold examples. Previously selected neurons are indicated by a small box.
13. When all the neurons are selected, press the **spacebar** to begin tracking.
NOTE: For additional NeuroTracker examples, see previous references^{4, 19}.
14. Monitor the tracking process for each animal, and make any necessary corrections.
15. If NeuroTracker pauses, it has lost the neuron. Reclick on the neuron, adjusting the threshold level as needed.
16. If the integration box jumps to another nearby animal or non-neuronal structure, press the **spacebar** to pause,

move the **slider** back to the first erroneous frame, and relick on the correct neuron location.

9. Data exploration and visualization

NOTE: The MATLAB analysis script is used for data processing and visualization and to generate summary PDFs for each analysis.

1. Run the **"NeuroTrackerSummary_pdf.m"** file in MATLAB, and select the folder containing the NeuroTracker data text files.
2. Wait for a summary PDF to be produced, allowing the verification of the tracking process (**Figure 5**). Animals are identified by a number (**Figure 5A**), and the neural responses from each animal and trial can be viewed to assess the population variability (**Figure 5B**).
3. Use the function **"databrowse.m"** to explore the neural data, for example, grouping by trial number (**Figure 5C**), by animal number (**Figure 5D**), by stimulation pattern, or by another category.

Representative Results

We present several examples of stimulus patterns that assess different neural phenomena, including temporal inhibition, adaptation, and disinhibition. **Temporal inhibition** is the momentary suppression of a neural response to a second stimulus presentation occurring shortly after the initial presentation¹⁴. To test this phenomenon, in a paired-pulse experiment, eight patterns consisting of two 1 s odorant pulses separated by an interval ranging from 0 s to 20 s were presented (**Figure 6B**; see **Figure 3B** for the corresponding stimulus control file). The arena was set up with a single stimulus fluid (1.1 μ M diacetyl), buffer, and control flow tubing, with the unused inlet port blocked with a solid pin (**Figure**

6A). By switching valve 1 on, the control fluid enters the *ctrl1* inlet, shifting the fluid streams such that the stimulus enters the arena; when valve 1 is off, the control fluid enters the *ctrl2* inlet, and buffer flows through the arena (see **Figure 2G,H**). Whereas the first 1 s odor pulse elicited an equal response magnitude in the diacetyl-detecting AWA neurons, responses to the second pulse varied with the interstimulus interval (**Figure 6D**). For pulse intervals of less than 10 s, the second response was weaker, demonstrating the temporal inhibition phenomenon. Using this experimental setup, the disruption of the dynein component CHE-3 was found to prevent temporal inhibition, implicating axonal transport in this phenomenon⁵.

Adaptation is the gradual decrease in neural responses to repeated presentations of the same stimulus. This phenomenon can be caused by different processes, such as active inhibition, which can be rapidly reversed, or other processes that are slow to reverse. One experiment to assess rapid reversibility is a "catch" trial, in which one stimulus is repeated to establish adaptation, followed by a "deviant" or novel stimulus, and then a return to the original stimulus. **Disinhibition** is observed if the neural responses increase following the novel "disinhibiting" stimulus. **Figure 7** demonstrates an example using two odorants, diacetyl and 2-methylpyrazine (2-MP), detected by the AWA chemosensory neurons *via* different receptors⁴. Two stimulus reservoirs and tubing were connected to the microfluidic device, with an additional three-way pinch valve to determine which stimulus enters the arena (**Figure 7A**). The stimulus patterns were defined to present chemical pulses for 4 s *via* valve 1 for all 30 trials and to select between stimulus S1 or S2 *via* valve 3, which was turned on after the 25th trial and off after the 26th

(**Figure 7B**). This arrangement ensured sufficient time for the different stimulus fluids to flow through the microfluidic device before presentation to the animals. Adaptation was observed to the diacetyl stimulus, but the presentation of the novel 2-MP stimulus did not elicit a strong disinhibition effect (**Figure 7C**).

Optogenetic stimulation uses light to activate neurons *via* light-sensitive ion channels. While convenient to use, optogenetic activation bypasses sensory receptors and some regulatory pathways, so some neural phenomena may differ compared with activation by natural stimuli. To test these differences, multimodal experiments are useful for presenting different types of stimulation within a single experiment. Neurons expressing the channelrhodopsin variant Chrimson are activated by red light exposure⁵. To assess whether the AWA neurons adapt similarly to chemical and optogenetic stimulation, the NZ1091 strain expressing both Chrimson and GCaMP in the AWA neurons was maintained on a plate containing the cofactor all-trans-retinal (ATR, 100 μ M) for 14-28 h. A 615 nm red LED illuminated the microfluidic arena from above (**Figure 8A**), and a set of multimodal stimulus patterns were generated by providing 5 s pulses of diacetyl, red light, or both within the same trial (**Figure 8B**). Chemical stimulation alone caused adaptation, whereas optical stimulation alone did not (**Figure 8C**). However, the combined multimodal stimulus pattern demonstrated that optogenetic responses were susceptible to chemically-induced adaptation (**Figure 8C,D**). These experiments suggest that adaptation is initiated at sensory receptors or dendritic processes, but its effects spread throughout the neuron.

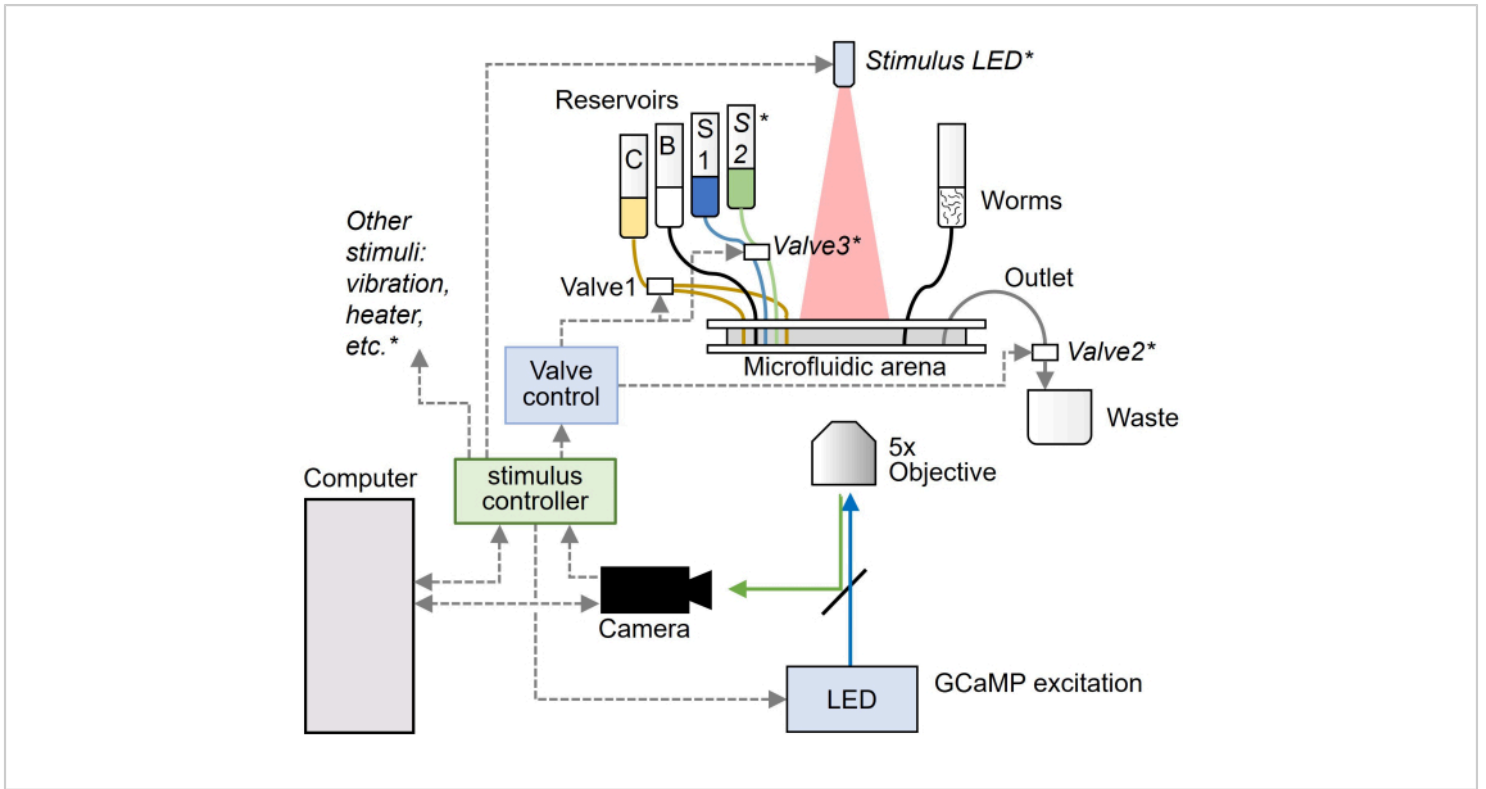


Figure 1: Schematic of the neural calcium recording and stimulation equipment. The microscope image capture, fluorescence excitation light, and stimulation are controlled by a computer and an open-source Arduino microcontroller. The chemical stimulation in the microfluidic arena switches between buffer **B** and stimulus **S** solutions *via* control fluid flow **C**. The optional system elements are indicated by an asterisk (*), such as additional valves and optical or other stimulation modalities. The fluorescence microscope images are used to measure the neural activity *via* calcium transients, for example, using the calcium indicator GCaMP. The dashed lines represent the electrical connections (digital or analog), the curved lines are the fluid tubing, and the straight solid arrows are the optical light paths. Abbreviation: LED = light-emitting diode. This figure is modified from Lawler and Albrecht¹⁵. [Please click here to view a larger version of this figure.](#)

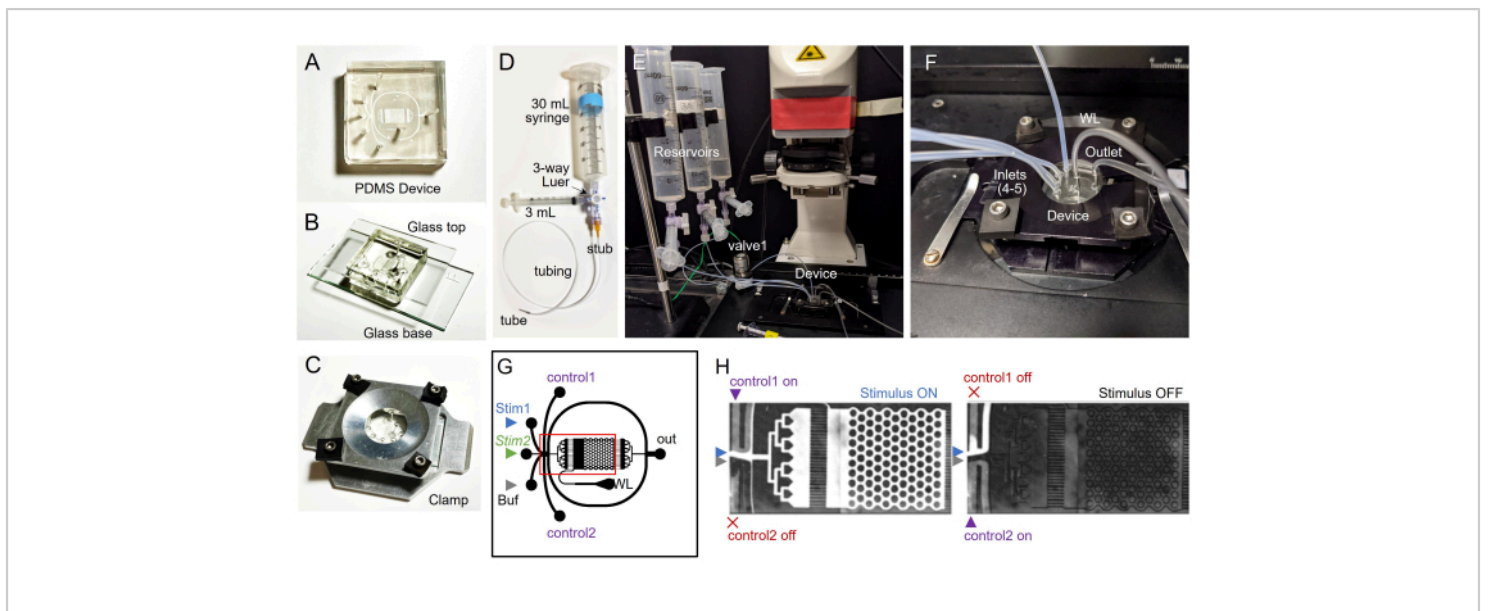


Figure 2: Microfluidic device assembly and setup. The microfluidic device is comprised of a (A) micropatterned PDMS device sandwiched between a (B) drilled glass top and hydrophobic glass base and (C) clamped. (D) The reservoirs are positioned above the device with a (E) tubing rack stand. The tubing from the reservoirs is connected either directly into the device or through actuated valves for fluidic control. The device is positioned above the objective on the microscope. (F) Close-up image of the microfluidic device with all the tubing attached in the inlets, the worm loading port, and the outlet. (G) Schematic of the 20 mm x 20 mm microfluidic device. The black lines represent the 55-70 μm tall fluid channels. This geometry is designed to allow for precise spatiotemporal control with a stimulus front perpendicular to the flow while keeping the animals within the arena and microscope field of view. The Buffer stream and one Stimulus stream (or optionally Stim2, if used) flow continuously, whereas only one control fluid inlet flows at a time, shifting which fluid enters the arena. (H) When Valve 1 is energized, control fluid flows from the normally closed port to the control1 inlet, and the stimulus turns on and flows through the arena. When Valve 1 is off, fluid flows from the normally open port to the control2 inlet, and the buffer enters the arena. The proper flow balance is shown, with fluorescein dye used as the stimulus fluid. Note that some of the fluid entering the arena also bypasses it *via* the upper and lower curved channels. These streams should be narrow and have equal width in the upper and lower channels. The reservoir heights can be adjusted as needed. Abbreviations: PDMS = polydimethylsiloxane; WL = worm loading; Buf = buffer; Stim1 = first Stimulus; Stim2 = second stimulus; NC = normally closed; NO = normally open. This figure is modified from Lawler and Albrecht¹⁵. [Please click here to view a larger version of this figure.](#)

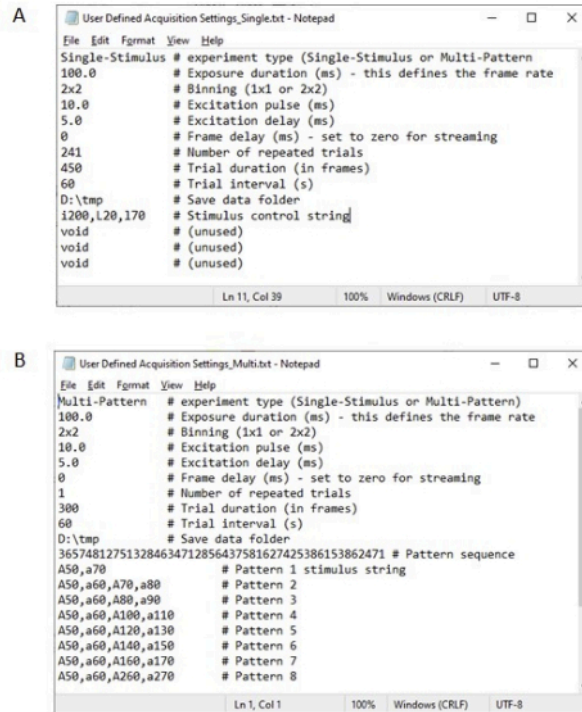


Figure 3: Example stimulus definition files. (A) Example single repeating stimulus experiment: a 5 s light pulse at intensity 200 from 2 s to 7 s (frame 20 to 70). (B) Example multi-pattern stimulus experiment with eight different stimulus patterns, corresponding to **Figure 6**. Two 1 s chemical pulses are presented with an interval of 0 s to 20 s between them. [Please click here to view a larger version of this figure.](#)

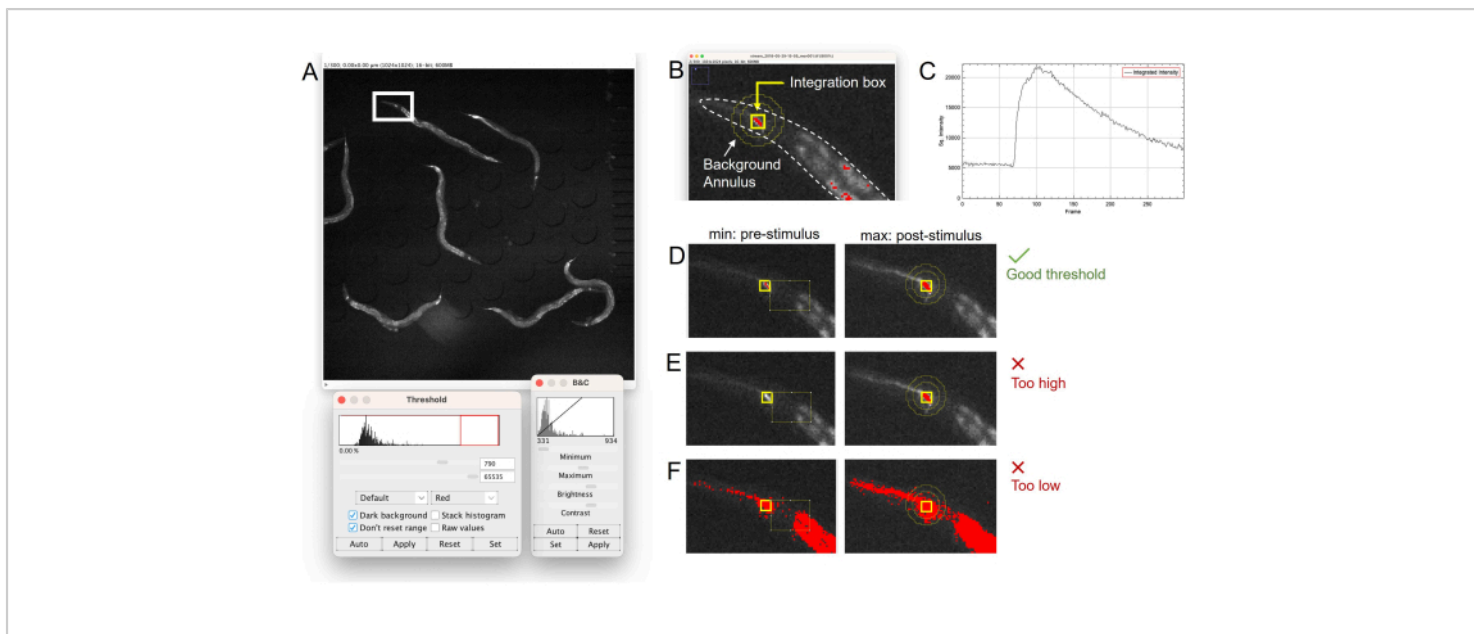


Figure 4: Selection of neurons and threshold levels in NeuroTracker. (A) Full microscope field of view, with the brightness and contrast adjusted to clearly see the AWA neurons. (B) Magnified view of an animal's head region depicting the integration box from which the fluorescence intensity is calculated and the background annulus for background subtraction. (C) Integrated intensity plot showing a robust neural response. (D) A good threshold selection will result in a red threshold region that is above the **Min Size** parameter and below the **Max Size** parameter for every frame in the image stack and includes no nearby regions, such as gut autofluorescence (see panel B). (E) A threshold set too high may appear good when the neuron is active, but when inactive, the neuron may be lost. It is, therefore, important to check both the pre-stimulation and post-stimulation frames when selecting a threshold. (F) A threshold set too low may highlight other non-neuronal structures. Over-threshold or under-threshold selection can lead to NeuroTracker losing the neuron and pausing for user feedback to correct the threshold and neuron position. Abbreviation: B&C = brightness and contrast. [Please click here to view a larger version of this figure.](#)

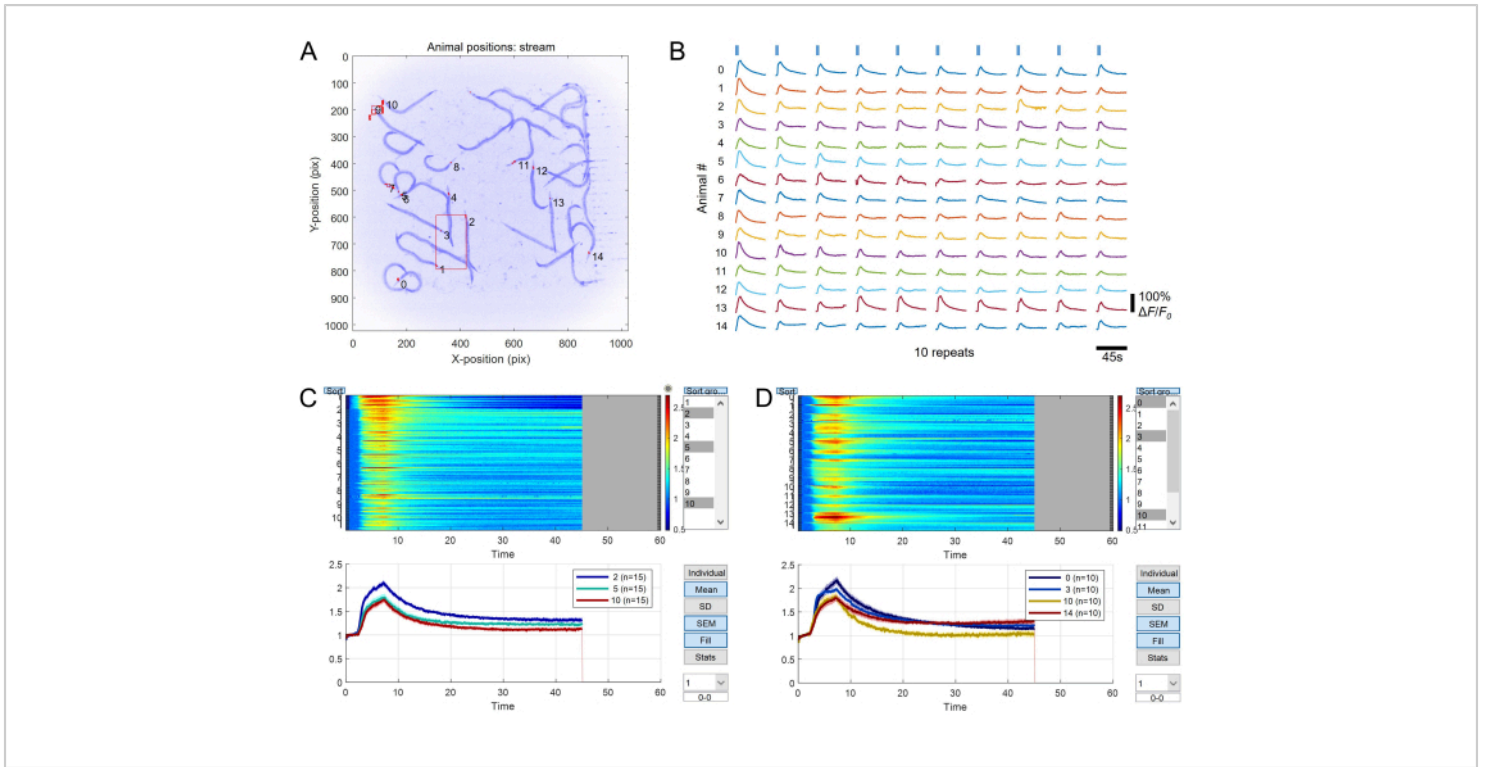


Figure 5: Example data analysis. Neural responses of 15 animals exposed to 10 repeats of a 5 s duration optogenetic stimulation once per minute. **(A)** Summary image indicating the animals tracked and the movement of the neurons within the trial (red boxes). Animal 2 moved during the trial. **(B)** The individual animal responses over time show a relatively consistent response to red light stimulation. **(C)** Data exploration output using the **databrowse** function. The traces are grouped by trial repeat (above), and averages are shown below for user-selectable trials. **(D)** Data grouped by individual animal number and averaged across repeated trials. This dataset shows minimal inter-animal variability, justifying averaging responses across the population. [Please click here to view a larger version of this figure.](#)

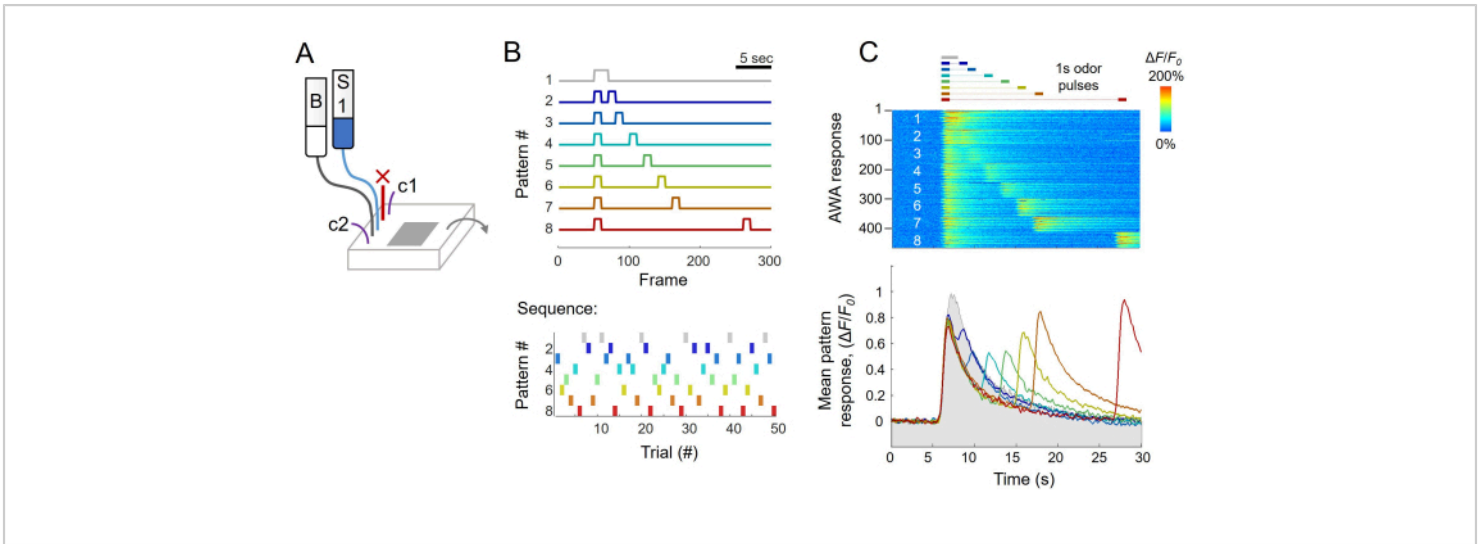


Figure 6: Paired-pulse experiment with a variable interstimulus interval to assess temporal inhibition. (A) Microfluidic device inlet connections. The unused inlet is blocked with a solid pin (X). The control fluid reservoir and valve1 are connected to the c1 and c2 inlets, omitted here for clarity. (B) Stimulus timing for eight patterns. The pulses are 1 s in duration and separated by 0-20 s. The sequence of patterns is depicted below; each pattern was presented six or seven times. (C) AWA neural activity from 50 trials and nine animals. A heatmap of responses sorted by stimulation pattern is shown above; the mean neural activity for each pattern ($n=56-63$ responses) is shown below. Temporal inhibition occurs for ~ 10 s following the initial pulse. [Please click here to view a larger version of this figure.](#)

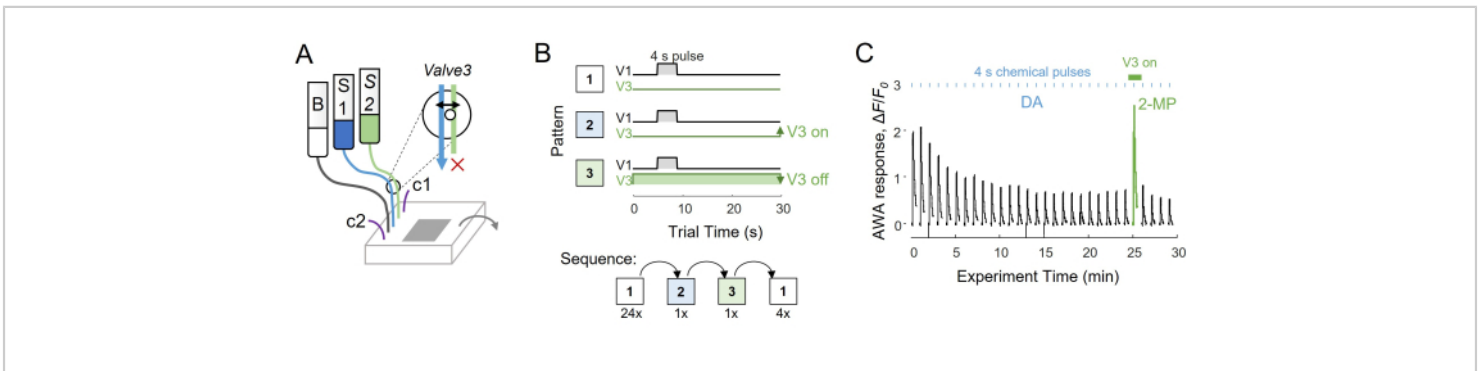


Figure 7: Catch trial experiment to assess disinhibition. (A) Microfluidic device inlet connections. A three-way pinch valve allows only stimulus S1 to pass at rest and only S2 to pass when energized. (B) Diagram of the three pattern sequences used to deliver the repeated diacetyl odor pulses, with the novel stimulus 2-methylpyrazine presented in place on the 26th trial. (C) Mean AWA neural activity responses across 20 animals. Abbreviations: DA = diacetyl; 2-MP = 2-methylpyrazine. [Please click here to view a larger version of this figure.](#)

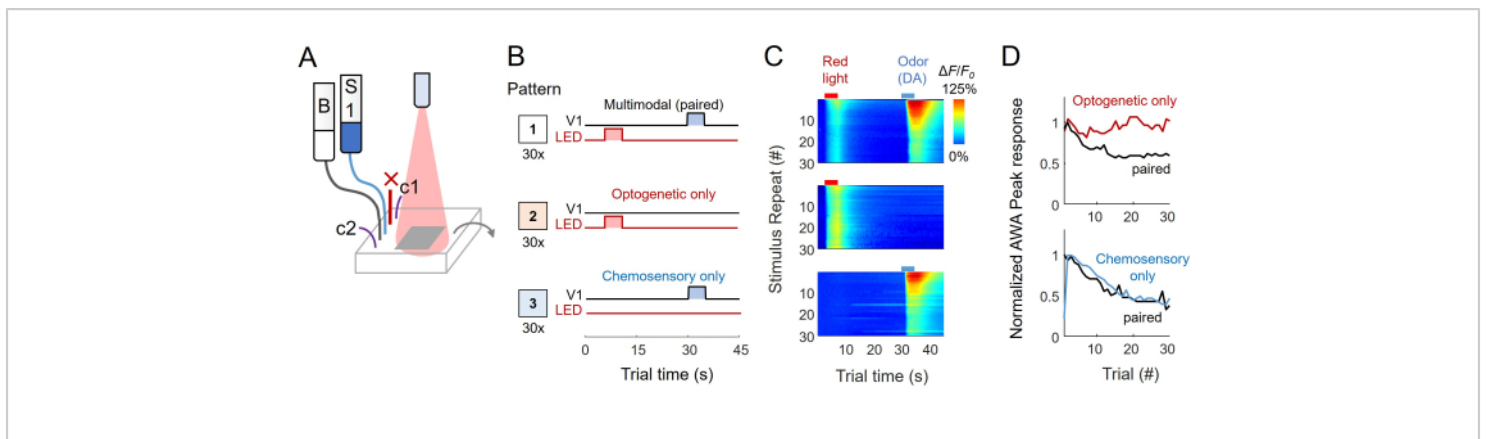


Figure 8: Multimodal stimulation example. (A) Microfluidic device inlet connections, with an unused inlet blocked with a solid pin. A red LED illuminates the arena from above. (B) Patterns for optical, chemical, or multimodal stimulation. (C) Mean AWA neural activity ($n = 11$ animals) for 30 repeated trials with paired optogenetic and chemosensory (top), optogenetic only (middle), and chemosensory only (bottom) stimulation. (D) Peak neural response average for each trial for optogenetic pulses above and chemical pulses below. The optogenetic pulses do not directly cause adaptation, but their responses are susceptible to chemosensory adaptation. Abbreviations: DA = diacetyl. [Please click here to view a larger version of this figure.](#)

Discussion

In this protocol, we describe an open-access microscopy system for the assessment of neural activity phenomena using the temporally precise delivery of different stimulus patterns. The microfluidic platform delivers repeatable stimuli while keeping tens of animals in the microscope field of view. Few commercial microscopy software packages allow for the easy programming of various stimulus timing patterns, and those that do often require the manual entry of each pattern or proprietary file formats. In contrast, experiments are defined in this system by text files, which can be computer-generated and are readable by analysis software. Here, we demonstrate the utility of employing variable stimulus patterns for assessing neuronal phenomena such as temporal inhibition, adaptation, and crosstalk during multimodal stimulation. The data analysis pipeline reduces

large microscopy video files to neural activity data that can be visualized and analyzed for population variability (Figure 5B), changes over time (Figure 7C and Figure 8C,D), and changes after perturbation (Figure 7C).

A key benefit of automation is the reproducibility of stimulation across trial repeats and between experiments, and the microfluidic arena format allows at least a 20-fold increase in throughput compared to previous single-animal methods^{7,9,13}. Subjecting a population of animals to the same stimulus and environmental conditions ensures that the data are comparable and avoids potential day-to-day variations in solution preparation, temperature, animal sources, or other factors. However, a limitation of this approach is the low-magnification, low-resolution microscopy that keeps all the animals in the field of view. Only one neuron should be monitored per animal to minimize tracking

errors, although neurons separated by at least 50 μm can, in principle, be distinguished. Bilateral neuron pairs can be imaged together if the responses are expected to be symmetric. It is important to first identify which specific neurons are involved in a neural process of interest, such as by whole-brain imaging⁷, and then to express GCaMP only in the relevant neurons for increased throughput.

Neuronal calcium responses vary by animal due to reporter expression levels or other inter-individual differences. For example, normalized AWA GCaMP responses vary twofold in peak magnitude, even in isogenic wild-type animals with the calcium reporter stably integrated into the genome⁴. Some stimuli show variable responses across the population (e.g., butyl acetate⁴), suggesting variable receptor expression. In practice, at least 20 individual animals should be assessed to determine population variability. It is recommended to make use of individual animal data to increase statistical power in determining response changes, such as by the statistical comparison of individual neural responses paired before and after a perturbation.

With large datasets and automated experimentation, it is important to validate and verify proper experiment function, tracking, and data reduction, especially for unattended steps. Flow patterns should be verified with fluorescent solutions (as in **Figure 2H**) by observing no bubbles or control fluid entering the arena in the recorded videos. Datasets should be validated by, for example, noting any animal movement (as in **Figure 5A**) and verifying smooth neural responses (as in **Figure 4C** and **Figure 5B**). Any problematic data should be excluded. It is also important to verify data grouping before averaging using the "databrowse" function to ensure that the average response reflects individual responses.

The open-source stimulation control system is readily extendable to other stimuli *via* digital or analog signals or serial communication commands. Graded stimuli are typically controllable by analog voltage (0-5 V) inputs, such as LED light intensity or nerve stimulator pulse magnitude. On-off stimuli are typically controlled with digital (TTL) signals, such as electrical stimulator timing. These digital signals can also actuate relay switches to activate other electrical systems, like vibration motors for mechanosensory stimulation. Other equipment uses serial communication, such as by USB connection, with specific character commands transmitted to define actions. For example, dose-response experiments can present several chemical concentrations automatically using a USB-controlled rotational valve⁵ or multi-well plate system⁶. These commands can be added to the open-source microscope control scripts to be sent at specific trial numbers. Time delays can be similarly incorporated; for example, to pause testing for 1 h between two 30 trial stimulation bouts, one would specify a 60 trial experiment and add a single line of code to pause for 1 h when the trial number reaches 31.

The experimental complexity is virtually limitless and can even change during an experiment based on live feedback in a "closed-loop" system. For example, the stimulation could vary in real time based on the neural activity, animal behavior, or another quantifiable parameter. We recently used such a system to assess the sensory neural responses in sleeping versus awake animals by triggering stimulation and neural recording after a change in behavior^{8, 15}. The motion tracking feature of NeuroTracker enables the analysis of neural activity in freely moving animals for correlation to behavioral output. However, tracking individual moving animals requires close attention, such as when they cross paths, and it is, therefore,

recommended to load fewer animals per arena (around five) to minimize these interactions.

Overall, these techniques increase the precision of stimulation and the experimental throughput in exploring population variability and specific neural phenomena. In addition to measuring neuronal activity, these methods are generalizable to other biosystems, including plants, which also communicate *via* calcium signals²¹, neural cell culture, and brain organoids²², as well as to other dynamic fluorescent signals, such as sensors for synaptic activity, neurotransmitter release²³, and transcription²⁴.

Disclosures

The authors have no conflicts of interest to disclose.

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Materials List for

Automated Multimodal Stimulation and Simultaneous Neuronal Recording from Multiple Small Organisms

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jove.com/video/65042

Materials

Name	Company	Catalog Number	Comments
Bacterial strains			
<i>E. coli</i> (OP50)	Caenorhabditis Genetics Center (CGC)	Cat# OP50	
Experimental models: Organisms/strains			
<i>C. elegans</i> strains expressing GCaMP (and optionally, Chrimson) in desired neurons	Caenorhabditis Genetics Center (CGC) or corresponding authors of published work	NZ1091, for example	
Chemicals, Treatments, and Worm Preparation Supplies			
2,3-Butanedione	Sigma-Aldrich	Cat# B85307	diacetyl, example chemical stimulus
Calcium chloride, CaCl ₂	Sigma-Aldrich	Cat# C3881	
Fluorescein, Sodium salt	Sigma-Aldrich	Cat# F6377	
Glass water repellent	Rain-X	Cat #800002250	glass hydrophobic treatment (single-use)
Magnesium chloride, MgCl ₂	Sigma-Aldrich	Cat# M2393	
Nematode Growth Medium (NGM) agar	Genesee	Cat #: 20-273NGM	
Petri dishes (60 mm)	Tritech	Cat #T3305	
Poly(dimethyl siloxane) (PDMS): Sylgard 184	Dow Chemical	Cat# 1673921	
Potassium phosphate monobasic	Sigma-Aldrich	Cat# P5655	
Potassium phosphate dibasic	Sigma-Aldrich	Cat# P8281	
Sodium chloride, NaCl	Sigma-Aldrich	Cat# S7653	
(tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (TFOCS)	Gelest	CAS# 78560-45-9	glass hydrophobic treatment (durable)
Software and algorithms			
Arduino IDE	Arduino	https://www.arduino.cc/en/software	
ImageJ	NIH	https://imagej.nih.gov/ij/	

MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html	
Micro-manager	Micro-manager	https://micro-manager.org/	
Microscope control software	Albrecht Lab	https://github.com/albrechtLab/MicroscopeControl	
Neurotracker data analysis software	Albrecht Lab	https://github.com/albrechtLab/Neurotracker	
Automated Microscope and Stimulation System			
Axio Observer.A1 inverted microscope set up for epifluorescence (GFP filter cubes, 5× objective or similar)	Zeiss	Cat #491237-0012-000	
Excelitas X-cite XYLIS LED illuminator	Excelitas	Cat #XYLIS	
Orca Flash 4.0 Digital sCMOS camera	Hamamatsu	Cat #C11440-22CU	
Arduino nano	Arduino	Cat #A000005	
3-way Miniature Diaphragm Isolation Valve (LQX12)	Parker	Cat #LQX12-3W24FF48-000	Valve 1: Control
2-way normally-closed (NC) Pinch Valve	Bio-Chem Valve Inc	Cat #075P2-S432	Valve 2: Outflow
3-way Pinch Valve	NResearch	Cat #161P091	Valve 3: Stimulus selection
Optogenetic stimulation LED and controller (615 nm)	Mightex	Cat #PLS-0625-030-S and #SLA-1200-2	
ValveLink 8.2 digital/manual valve controller	AutoMate Scientific	Cat #01-18	
Wires and connectors	various	See Fig. 2 of Cell STARS Protocol (Lawler, 2021)	
Microfluidic Device Preparation			
Dremel variable speed rotary cutter 4000	Dremel	Cat #F0134000AB	Set speed to 5k RPM for cutting glass
Dremel drill press rotary tool workstation	Dremel	Cat #220-01	
Diamond drill bit	Dremel	Cat #7134	
Glass slide, 1 mm thick	VWR	Cat #75799-268	
Glass scribe (Diamond scriber)	Ted Pella	Cat #54468	
Luer 3-way stopcock	Cole-Parmer	Cat #EW-30600-07	
Luer 23 G blunt needle	VWR	Cat #89134-100	
Microfluidic device	Corresponding author or fabricate from CAD files associated with this article	N/A	
Microfluidic device clamp	Warner Instruments (or machine shop)	P-2	
Microfluidic tubing, 0.02" ID	Cole-Parmer	Cat #EW-06419-01	
Tube 19 G, 0.5"	New England Small Tube	Cat #NE-1027-12	