



# Chapter 14

## Automated Functional Screening for Modulators of Optogenetically Activated Neural Responses in Living Organisms

Ross C. Lagoy, Eric Larsen, and Dirk R. Albrecht

### Abstract

All-optical methods of probing in vivo brain function are advantageous for their compatibility with automated microscopy and fast spatial targeting of neural circuit excitation and response. Recent advances in optogenetic technologies allow simultaneous light activation of specific neurons and optical readout of neural activity via fluorescent calcium reporters, providing an attractive opportunity for high-throughput screening assays that directly assess dynamic neural function in vivo. Here we describe a method to automatically record optogenetically activated neural responses in living, hydrogel-embedded organisms over many hours in a multiwell plate format. This method is suitable for screening the neural effects of hundreds of chemical compounds and assessing the time course of bioactivity over 12 h or more. As examples, we show the suppression of neural responses over time with various concentrations of two voltage-gated calcium channel blockers and a full-plate screen of 320 chemicals with positive and negative controls in a single experiment.

**Key words** Neural activity, Optogenetic stimulation, Hydrogel immobilization, Multiwell plate, High-content functional screening, Fluorescent reporter, Genetically encoded calcium sensor

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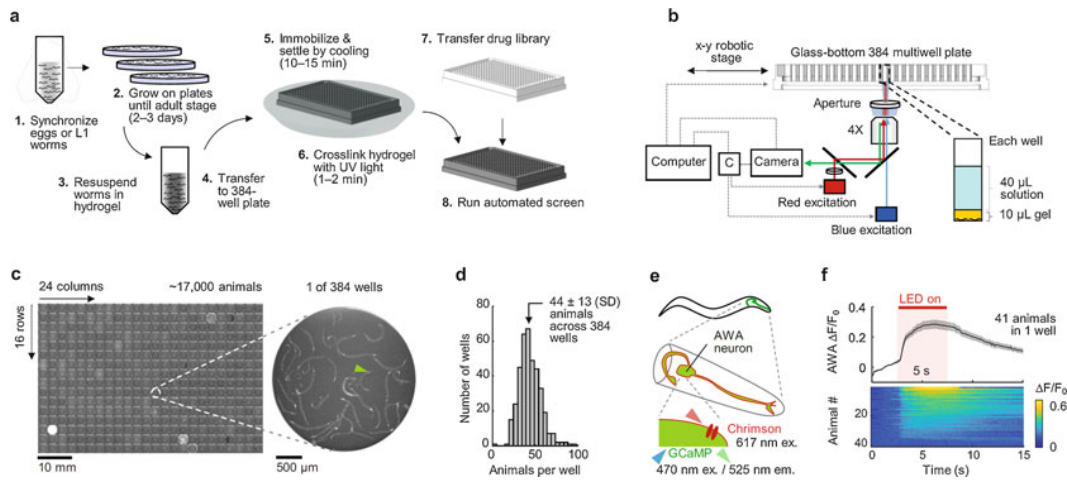
### 1 Introduction

Automated compound screening technologies are essential for drug discovery and for rapid assay development and optimization. Multiwell plates are commonly used to identify hundreds to thousands of compounds that modulate cellular function in vitro by static images of fixed cells at specific time points or by dynamic live cell imaging [1]. Increasingly, screening platforms are transitioning from single cell assays to multicellular organoids and whole organisms, which can mimic in vivo responses more accurately and capture multicellular phenomena [2–4]. However, technical challenges such as keeping living organisms immobilized and healthy during long-term compound exposure has limited the development of such in vivo functional screens in the past [5]. Further,

many biological phenomena are best observed following precise stimulation to access different functional states [6]. In this chapter, we present a cost-effective and versatile screening method to address both live sample immobilization in microwell plates using an inert hydrogel [7] and stimulation via light and optogenetic reagents [8] during live fluorescence imaging.

Drug discovery for activity-dependent neuropsychiatric disorders [9–11] in particular requires a system that can screen for neuroactive chemicals by recording stimulated neural responses [12–14]. Assessment of circuit-level phenomena further requires multicellular preparations such as cultured neuronal networks [14] or, preferably, *in vivo* measurements in intact organisms that include all physiological systems that modulate neurological function. All-optical methods are experimentally advantageous as they use light for noninvasive and noncontact stimulation and recording of neural activity [15], which can be done in intact and even freely behaving organisms [16–18]. The nematode *C. elegans* is an attractive organism for neuroactive chemical screens, due to its optical transparency, ease of genetic manipulation, neurochemical homology with other species including humans, and compact nervous system with 302 identifiable neurons. Further, they are convenient for high-throughput screening, owing to their small size (50  $\mu\text{m}$  diameter  $\times$  1 mm length), rapid life cycle, and large brood size to generate thousands of clonal animals within days [5, 19].

This *in vivo* high-throughput and high-content functional screening technology is cost-effective (<\$100/plate) and can be performed in about 1 week, requiring just a few hours of active user preparation across multiple days (Fig. 1). One to four 384-well plates can be screened per day, depending on drug exposure duration. Here, we demonstrate two neural screen examples using a worm strain expressing a genetically encoded calcium sensor GCaMP and a red-shifted light-gated ion channel Chrimson [8] in the AWA pair of chemosensory neurons. In the first example, we characterized two voltage-gated calcium channel (VGCC)-blocking drugs [20] over 12 h, finding nemadipine-A more potent than nemadipine-B and a dose-dependent acceleration of drug effects (Fig. 2). In a second example, we automatically screened 320 small-molecule drugs for effects on optogenetically activated neural responses in living *C. elegans* worms, identifying three potential suppressors and one potential enhancer during 12 h of compound exposure (Fig. 3). This method of immobilization and illumination can be extended to monitor other stimulated cellular and organismal processes by strategic localization of optogenetic activation (Chrimson or similar) and optical readouts (GCaMP or other fluorescent reporters).



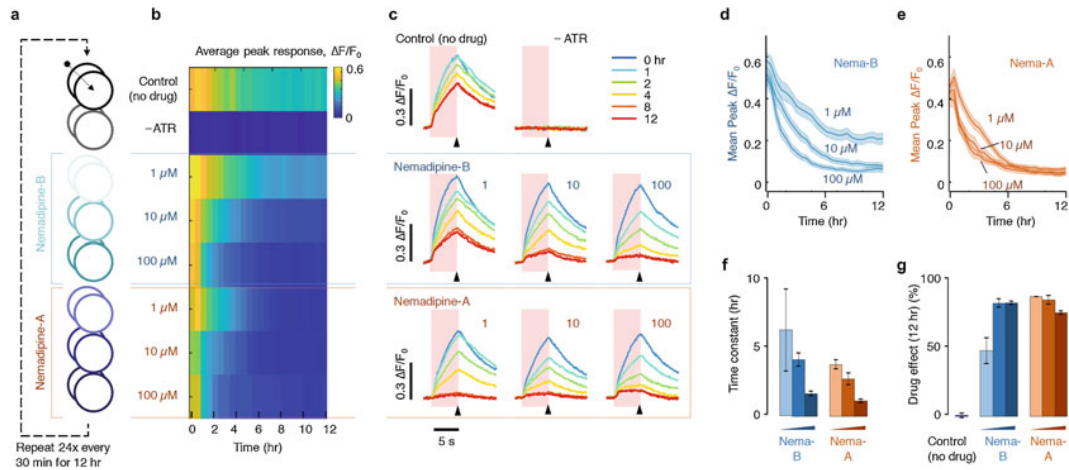
**Fig. 1** Method for recording neural responses to optogenetic activation in a multiwell plate format. **(a)** Flow diagram to prepare age-synchronized animals, immobilize them in a multiwell plate, and add drug conditions per well. **(b)** Diagram of the control system for optogenetic stimulation (red light) and neural recording via fluorescent calcium sensor (blue light excitation). The computer synchronizes camera exposure and illumination via a controller (labeled c). Animals are embedded within a hydrogel at the bottom of each well (inset). **(c)** Montage image of a 384-well plate containing ~17,000 *C. elegans*. Arrowheads indicate neurons in inset magnifying one well. **(d)** Histogram of number of animals per well from the 384-well plate in **c**. **(e)** Schematic of animals co-expressing GCaMP and Chrimson in the AWA chemosensory neurons in the head. **(f)** Example neuronal activity response to a 5 s red light pulse, displayed above as average relative change in fluorescence ( $\Delta F/F_0$ ), with shading indicating SEM, and below as individual animal responses sorted by peak calcium response

## 2 Materials

Prepare all solutions using analytical grade reagents and ultrapure water ( $\text{diH}_2\text{O}$ ). Prepare and store all reagents at room temperature unless indicated otherwise. Follow all waste disposal reagents when disposing hazardous materials, and prepare any chemical library plates, particularly those with potential neuroactive effects, with appropriate protective equipment and fume hood.

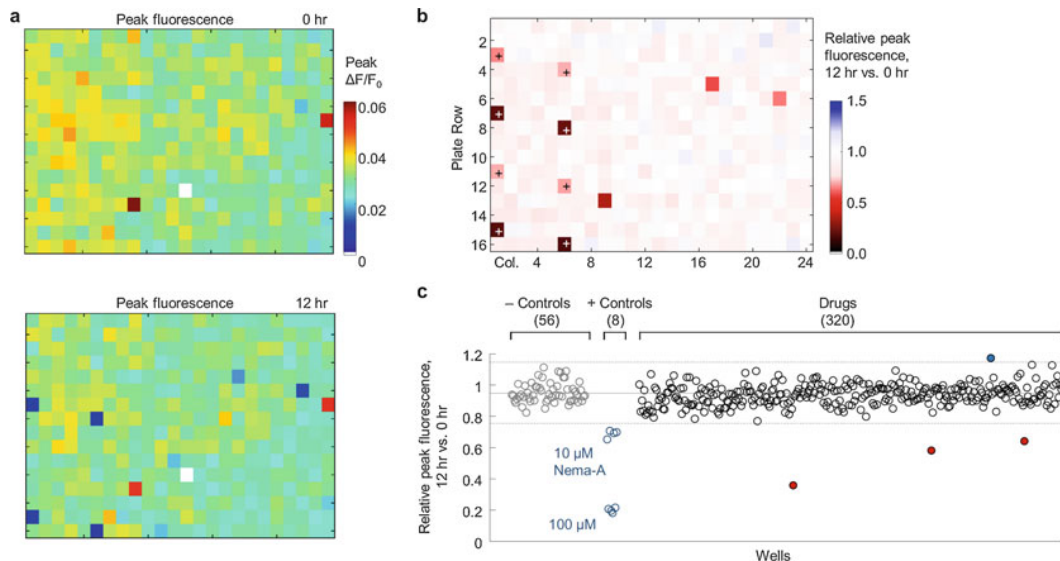
### 2.1 *C. elegans* Culture [22]

1. Worm M9 buffer: 22 mM  $\text{KH}_2\text{PO}_4$ , 42.3 mM  $\text{Na}_2\text{HPO}_4$ , 85.5 mM NaCl, and 1 mM  $\text{MgSO}_4$ . Combine 3 g  $\text{KH}_2\text{PO}_4$ , 6 g  $\text{Na}_2\text{HPO}_4$ , 5 g NaCl, 1 mL of 1 M  $\text{MgSO}_4$ , and  $\text{diH}_2\text{O}$  up to 1 L, and autoclave.
2. Worm lysis buffer (3.3× concentrated): 1.67 N NaOH, 3.3% bleach. Prepare a 5 N NaOH solution by adding 20 g NaOH to 100 mL water. Combine 1 part 5 N NaOH with two parts household bleach (~5% solution of sodium hypochlorite) just prior to use (*see Note 1*).
3. Bacterial growth medium such as LB (Luria-Bertani) medium.



**Fig. 2** Example time course of drug effects on in vivo neural responses over 12 h. **(a)** Pattern of duplicate wells containing control conditions, 1% DMSO (no drug) and all-trans-retinal negative (–ATR), or 1–100  $\mu\text{M}$  L-type voltage-gated calcium channel (VGCC) blockers nepadipine-A and nepadipine-B. Wells were retested every 30 min for 12 h. **(b)** Heatmap of peak calcium response over 12 h, averaged over duplicate wells containing  $\sim 49$  animals each. **(c)** Average calcium response dynamics over 15 s with 5 s red light stimulation (shading). Peak values in **(b, d, e)** were obtained at the end of light stimulation, arrowheads. Colored traces represent different drug exposure times from 0 to 12 h. **(d, e)** Time course of VGCC drug effects over 12 h plotted as peak calcium response for 1, 10, and 100  $\mu\text{M}$  nepadipine-B and nepadipine-A. **(f)** Time constant of exponential decay fit for each drug and concentration. Bars represent best fit; error bars represent 95% confidence limits. **(g)** Peak calcium response suppression relative to mean negative control for each drug and concentration. Bars represent best fit; error bars represent 95% confidence limits

4. 100 mm NGM (nematode growth medium) culture plates (*see Note 2*).
5. 100 mm NGM culture plates seeded with a bacterial lawn of OP50 *E. coli* (*see Note 3*).
6. *C. elegans* worms expressing Chrimson and GCaMP in desired neurons (*see Note 4*). Ideally, these optogenetic reagents should be integrated into the genome.
7. 50 and 15 mL sterile conical centrifuge tubes.
8. All-trans-retinal (ATR) stock solution: 100 mM ATR. Dissolve 28.4 mg ATR in 1 mL 100% DMSO, and store in  $-20^\circ\text{C}$  freezer protected from light.
9. Worm growth plates containing ATR and OP50 bacterial lawn. Prepare an overnight OP50 culture by inoculating  $\sim 100$  mL LB medium with a single OP50 colony ( $\sim 1$  mL is needed per plate). Centrifuge at 3900 rpm for 10 min, aspirate medium, and resuspend OP50 pellet in M9 buffer to 1/5 the original volume. Add 0.5  $\mu\text{L}$  of 100 mM ATR stock per 1 mL bacteria suspension for a 50  $\mu\text{M}$  final ATR concentration. Seed NGM plates in the center with the ATR + OP50 suspension to 1:100 agar volume (e.g., 220  $\mu\text{L}$  to a 100 mm plate containing 22 mL



**Fig. 3** Example automated 384-well chemical screen for suppressors of in vivo neural responses over 12 h. **(a)** Plate heatmap of peak GCaMP fluorescence after 5 s red light stimulation immediately after adding drugs (0 h, above) and 12 h. later (below). **(b)** Heatmap of relative peak GCaMP fluorescence at 12 h vs. 0 h. Suppressed responses are indicated by red shading. Positive controls (10 and 100  $\mu$ M nemadipine-A) are indicated by + symbols. **(c)** Quantification of relative peak GCaMP fluorescence indicating change in neural response in 56 negative controls (gray, 1% DMSO), 8 positive controls (blue), and 320 drugs. Horizontal gray lines represent mean and  $\pm 3$  standard deviations of the negative control group. Three potential suppressor hits (red points) and one potential enhancer (green point) lie outside this range

NGM agar; *see Note 2*). Cover plates with foil to protect from light.

10. P20 micropipette and 20 or 200  $\mu$ L tips (*see Note 5*).
11. Microfuge tube rotator (optional).

## 2.2 Hydrogel Embedding in Microwell Plates

1. Photoinitiator solution (*see Note 6*): 1% (w/v) Irgacure 2959. Dissolve 10 mg Irgacure 2959 in 1 mL diH<sub>2</sub>O or buffer. Store at 4 °C in an amber vial or covered in foil to protect from light.
2. Hydrogel solution: 20% (w/v) Poly(ethylene glycol) diacrylate (PEGDA), MW 3300 g/mol. Dissolve 100 mg PEGDA in 500  $\mu$ L 0.1% (w/v) Irgacure (diluted from **step 1**). Store at 4 °C protected from light.
3. UVB illuminator, 308/312 nm, handheld or benchtop (*see Note 6*).
4. 384-well glass-bottom microtiter plates (e.g., Corning 4581).
5. Microwell plate sealing film (e.g., Thermo Scientific 232,702).

**2.3 Neural Imaging**

1. Epifluorescence microscope with a 2.5–10× objective, EGFP filter set, blue (470 nm) excitation source, and microscope camera (*see Note 7*).
2. Motorized *x-y* microscope stage (*see Note 8*).
3. Stimulation LED, red 617–630 nm, with aperture (*see Note 9*).
4. Computer with MicroManager microscopy software (*see Note 10*).
5. Controller hardware to synchronize light stimulation with camera recording and microscope *x-y* stage movement (*see Note 11*).

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**3 Methods**

Carry out all procedures at room temperature unless otherwise specified.

**3.1 Synchronous Plate Culture [21, 22]**

This procedure can yield over 50,000 young adult animals from one starved 100 mm plate in about 4–5 days.

1. Evenly chunk a starved 100 mm plate to eight seeded 100 mm plates. Use a flame-sterilized spatula to divide the agar into eight slices. Place one slice onto the edge of each new 100 mm plate seeded with a large OP50 bacterial lawn in the center, such that animals crawl toward the bacteria. Plates are ready for the next step after ~48 h when kept at 20 °C (or ~32 h when kept at 25 °C) and yield about 5000 animals per 100 mm plate (*see Note 12* for suggested scheduling).
2. Collect animals into liquid. Wash *C. elegans* plates containing many gravid hermaphrodites and eggs with 6 mL diH<sub>2</sub>O using a serological pipette. Use a sterile plate spreader to loosen eggs if necessary. Collect the liquid into one sterile 50 mL conical centrifuge tube, allowing worms to settle to the bottom for 10 min. Aspirate cloudy liquid to remove bacteria (*see Note 13*). Fill tube to 42 mL with diH<sub>2</sub>O, and then divide the liquid evenly into two 50 mL conical tubes (21 mL each).
3. Lyse animals. Add 9 mL freshly prepared worm lysis buffer to each tube (premix 3 mL of 5 N NaOH with 6 mL bleach per tube). Shake well for 5 s. Repeat shaking every 2 min for a total of 6–10 min (*see Note 13*).
4. Wash animals. Spin for 2 min at 2000 rpm to pellet eggs. Aspirate supernatant, and add sterile M9 buffer to 10 mL in each tube, and mix by pipetting, vortex, or shaking. Combine and mix all liquid, and then split into two sterile 15 mL conical tubes (10 mL each). Repeat the spin, aspiration, and buffer addition to complete three washes (*see Note 14*).

5. Add  $\leq 2$  mL M9 to each egg pellet in each tube, resuspend by pipetting, and combine all liquid into one tube. Measure egg concentration by depositing three 10  $\mu$ L samples using a P20 micropipette to an unseeded plate, counting eggs, and averaging the three measurements. Rotate egg preparation slowly overnight at 20 °C to obtain starved L1 (larval stage 1) animals 24 h. later (*see Note 12*). Alternatively, plate eggs onto one or more unseeded plates ( $\leq 5000$  per plate), and keep at 20 °C overnight (*see Note 15*), and skip to **step 7**.
6. After 24 h (*see Note 12*), examine the egg preparation for synchronized L1 animals. Remeasure animal concentration as in **step 5** but count only L1 animals.
7. Treat animals with all-trans-retinal (ATR). Deposit  $\leq 5000$  L1 animals from the tube to each previously prepared ATR + OP50 plate (*see Note 15*). Alternatively, for animals starved on plates, spot 220  $\mu$ L of OP50 bacteria with 50  $\mu$ M ATR solution (*see Note 2*), and spread thin by tilting the plate. Cover plates with foil, and store them upright at 20 °C overnight for up to 48 h.

### 3.2 Multiwell Plate Preparation and Microscopy

This section should be performed on the day of the imaging experiment, 48 h later, when animals are at young adult stage (*see Note 12*).

1. Prepare “100 $\times$ ” drug solutions (*see Note 16*). Make 0.1–10 mM stock solutions in 100% DMSO of each drug, and keep stored at  $-80$  °C. On the day of imaging, thaw stock solutions at room temperature. Add 126  $\mu$ L H<sub>2</sub>O to individual tubes or microwell plate wells and 1.6  $\mu$ L of stock drug (to make “1.25 $\times$ ” drug solutions in 1.25% DMSO). Vortex to mix for 20–30 s. Also prepare 1.25% DMSO in H<sub>2</sub>O solution for buffer controls (*see Note 17*).
2. Verify worms are young adult stage, and wash each plate with  $\sim 6$  mL M9 buffer using a serological pipette. Loosen worms by sliding the plate gently back and forth on the benchtop several times. Collect all worm suspension into multiple 15 mL conical tubes to equal volume. Let animals settle for 10 min, and then aspirate the cloudy supernatant. Add 2.5 mL M9 buffer to each tube and mix. If the solutions are still cloudy with bacteria, repeat the wash and aspiration steps. Combine liquid from all tubes into one 15 mL conical tube. Measure the final average animal concentration (three 10  $\mu$ L samples, as above). Adjust concentration to ten animals/ $\mu$ L (either dilute with M9 buffer or concentrate by spinning and aspiration).
3. Add an equal volume of hydrogel solution (20% w/v PEGDA + photoinitiator) to the worm suspension, for a final concentration of 5 animals/ $\mu$ L. About 10  $\mu$ L of worm hydrogel suspension is needed per well (*see Note 18*).



4. Use a P20 micropipette to load animals/hydrogel into plate wells while observing under a dissecting scope (*see* **Notes 5 and 16**). Flick tube containing animals in solution, and mix by pipetting gently three times every 2–3 wells to keep worm suspension even. Pipette 10  $\mu\text{L}$  to the edge of the well (about one-half depth), and then gently tap plate several times so the volume fills the bottom of the well (*see* **Note 19**).
5. Cover the well plate, and place it in a  $-20\text{ }^{\circ}\text{C}$  freezer for 10–15 min on a raised shelf support to allow worms to settle (*see* **Note 20**). Wipe any condensation from glass bottom, and then crosslink the hydrogel by illuminating from below using a 308 nm light source ( $\sim 3\text{ mW}/\text{cm}^2$ ) for 45–60 s (*see* **Note 6**). Use UV shield and eye protection or cover with foil.
6. Under the dissecting scope, confirm that animals are immobilized and located at the same focal plane (*see* **Note 20**). Add 40  $\mu\text{L}$  of each “1.25 $\times$ ” drug solution to each well (*see* **Note 16**). Gently pipette to the edge of the well (about one-half depth), which should fill the well and contact the gel solution (*see* **Note 19**). Discard tips between new drug concentrations. Once drugs diffuse into the hydrogel, their final “1 $\times$ ” concentration is 1–100  $\mu\text{M}$  in 1% DMSO.
7. Seal the multiwell plate with plastic sealing film. Place the multiwell plate on the microscope stage with well A1 in top left orientation.
8. Set up the microscope and the MicroManager software (*see* **Note 21**). Move the stage to the center of well A1, and focus the z-axis on neurons via the focus wheel.  
Run the *SCREEN\_GUI* script, and enter input parameters as described in software documentation (*see* **Note 10**).
9. Run the *SCREEN\_RUN* script to begin automated well movement, optogenetic stimulation, and fluorescent image capture.

### **3.3 Analysis of Optogenetically Activated Neural Responses**

Refer to detailed documentation for software analysis code (*see* **Note 10**).

1. *Image processing (ImageJ)*: After the automated experiment is complete, transfer image data to a server for secure storage. Organize video files into individual well folders (e.g., using the ImageJ script *organizeWells*).
2. Select neuron positions (e.g., using the ImageJ script *neuron-Select*) for each animal (*see* **Note 22**).
3. Begin automated neuron quantification using the ImageJ script *neuronTrack*. This uses the neuron positions from **step 2** and measures integrated pixel intensity with background correction (*see* **Note 23**), saving one text file per animal per trial.



4. *Quantification and visualization (MATLAB)*: Run the *analyzeNTdata* MATLAB script, and select the desired folder (*see Note 24*). This script imports raw text file data into an organized MATLAB workspace and computes parameters such as the relative change in GCaMP fluorescence compared to baseline ( $\Delta F/F_0$ ). Save the workspace for data visualization in **step 5** (*see Note 25*).
5. Run the *visualizeNTdata* MATLAB script to further process and display imaging data (*see Note 25*). For example, plot individual animal responses (Fig. 1f), single well average responses (Fig. 1f), replicate average responses (Fig. 2c), average peak responses (Fig. 2b, d, e; Fig. 3a), or changes in these parameters over time (Fig. 2f, g; Fig. 3b, c) or relative to controls (Fig. 2g). For drug screening, choose appropriate cutoff values for potential hit identification based on the number of conditions and desired selectivity.

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## 4 Notes

1. Worm lysis buffer should be freshly prepared or stored protected from light at 4 °C for up to 1 week.
2. Other nematode culture plate sizes (such as 150, 60, or 35 mm) can be used depending on the number of animals needed. Volumes of ATR and bacteria should be scaled according to the volume of NGM agar used.
3. Add OP50 bacteria from an overnight culture in LB medium picked from a single colony, to avoid contamination by other bacterial strains.
4. *C. elegans* strains can be prepared by standard microinjection methods [23] or by obtaining them from the *C. elegans* Genomics Center (CGC) or from the *C. elegans* research community. Integrated Chrimson and GCaMP transgenes simplify nematode growth by ensuring all progeny express both genes. Extrachromosomal arrays can work as well, but many progeny will not express both genes. During analysis, animals without GCaMP expression and those non-responsive to red light (without Chrimson expression) must be excluded.
5. Use large-bore pipette tips to prevent damage to worms. Standard 10  $\mu$ L tips are not recommended. While worms generally stick to standard plastic tips when in buffer, we find the PEG hydrogel solutions prevent worm sticking, so special nonadherent tips (or silanization treatments) are not needed.
6. Other photoinitiators compatible with acrylate polymerization can be used. Adjust light exposure duration, intensity, and wavelengths as needed [7]. A 308 or 312 nm DNA gel UV transilluminator generally works well for Irgacure 2959.

7. Microscope equipment should be appropriate for the fluorescent signal expected at the desired magnification level. Scientific CMOS (sCMOS) or electron-multiplying CCD (EM-CCD) cameras are recommended. Solid-state excitation sources are preferred for rapid pulsing of excitation light, reducing photobleaching, and allowing recordings for hours.
8. Any motorized microscope  $x$ - $y$  stage that is controllable by microscope software and capable of holding a multiwell plate is sufficient for automated multiwell experiments.
9. Optogenetic stimulation light can be delivered by LED or laser with wavelength tailored to the optogenetic reagent, such as 610–630 nm for Chrimson. Illumination is best from the imaging objective (below plate) but can also be delivered from above if adjacent wells are protected from light. An adjustable aperture is helpful to adjust the illumination spot size. We used a 3D-printed objective cap that placed a well-diameter aperture just below the microwell plate.
10. All software used here with MicroManager ([www.micro-manager.org](http://www.micro-manager.org)) and MATLAB is available at: [www.github.com/dalbrech/NeuroPlateScreen](https://www.github.com/dalbrech/NeuroPlateScreen). Other microscopy software can be used. It must be able to record video streams to TIFF stacks, control camera settings, and trigger optogenetic illumination at the desired frame numbers.
11. We use an Arduino Uno to read in a digital camera exposure signal, output a digital fluorescence excitation pulse per frame, count frames, and control the optogenetic activation for the desired frames. Other hardware and software that control these outputs can be used, such as Pulse Pal or commercial systems [24].
12. For convenience, the following worm preparation scheduling is suggested. Nematode growth times are about 1/3 faster at 25 °C. Adjust timing according to observed growth rates, which may be slower if genetic mutations are present.
  - (a) Keeping animals at 20 °C: Chunk at 5 pm Sun. (0 h), egg preparation before 5 pm Tues. (48 h), plate and add ATR at 5 pm Wed. (72 h), and prepare for testing 9 am Fri. (112 h) when animals are young adult stage.
  - (b) Keeping animals at 25 °C: Chunk at 9 am Mon. (0 h), egg preparation before 5 pm Tues. (32 h), plate and add ATR at 9 am Wed. (48 h), and prepare for testing after 12 pm Thurs. (>75 h) when animals are young adult stage.
  - (c) A combination of temperatures is also possible: For example, chunk at 9 am Mon. (0 h) and keep at 25 °C, egg preparation before 5 pm Tuesday (32 h), and then follow the schedule from (a) keeping animals at 20 °C.

13. Repeat the wash step if the supernatant remains cloudy, as excess bacteria can slow the worm lysis process. Increase the lysis time up to 10 min if synchronized worm yield is low due to poor lysis, or decrease lysis time if viability is low and many eggs do not hatch.
14. It is convenient to split worm suspensions into two or more tubes with equal volume for balanced spins and rotation.
15. Total liquid volume added (including animal suspension and ATR/OP50) should not exceed 500  $\mu\text{L}$ , to avoid pooling of liquid on the agar surface.
16. A multidispense or liquid handling robot can complete these steps for drug libraries, or it can be done manually using single or multichannel pipettes. We find when using  $>50$  wells it is helpful to use a robotic aid for creating dilutions and for depositing worms.
17. These volumes are suitable for triplicate wells. Adjust volumes according to desired replicates with 40  $\mu\text{L}$  needed per well.
18. Allow for 20% extra volume. For a full 384-well plate, 5 mL total worm hydrogel suspension is recommended.
19. Pipetting with the tip about halfway up the well wall prevents damage to the worms as can occur when pipetting directly on the well floor. However, if the drop is added too high in the well, a bubble might form. If so, remove the bubble by gently pipetting solution back up and depositing again.
20. Observe animal movement with a microscope, and extend the cooling time if animals are not immobilized or located at the same plane.
21. Microscopy software and setup depend on specific hardware configuration. Load the appropriate configuration file, and verify that the camera,  $x$ - $y$  stage, and light sources are properly controlled before installing the microwell plate.
22. Continue selecting neurons until all are complete for each well. Exclude animals that appear abnormal or overlap with adjacent animals.
23. Image processing is automatic and may take several hours for a full microwell plate. Typical analysis throughput with a single standard computer is about 150,000 frames/h (e.g., 20 wells/h  $\times$  50 animals/well  $\times$  150 frames/animal); hence, processing on parallel systems or using more than one computer may be useful to speed analysis. If desired, individual tracking steps can be viewed to verify correct neuron tracking, as described in the software instructions.
24. Image processing scripts make new folders to organize data. Ensure folder permissions permit making new folders.

25. It is helpful to separate data visualization (Subheading 3.3, step 5) from data import (Subheading 3.3, step 4) which can be slow for large datasets. Loading a saved workspace saves time when iteratively modifying the visualization code for desired analyses.

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## Acknowledgment

This work was supported by NSF grants 1605679 and 1724026 and a Career Award at the Scientific Interface from the Burroughs Wellcome Fund to D.R.A.

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