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Real-time multimodal optical control of neurons and muscles in freely-behaving *Caenorhabditis elegans*

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Abstract

The ability to optically excite or silence specific cells using optogenetics has provided a powerful tool to interrogate the nervous system. Optogenetic experiments in small organisms have mostly been performed using whole-field illumination and genetic targeting, but these strategies do not always provide adequate cellular specificity. Targeted illumination can be a valuable alternative but to date it has only been shown in non-moving animals without the ability to observe behavior output. We present a real-time multimodal illumination technology that allows both tracking and recording the behavior of freely moving *Caenorhabditis elegans* while stimulating specific cells that express Channelrhodopsin-2 or MAC. We use this system to optically manipulate nodes within the *C. elegans* touch circuit and study the roles of sensory and command neurons and the ultimate behavioral output. This technology significantly enhances our ability to control, alter, observe, and investigate how neurons, muscles, and circuits ultimately produce behavior in animals using optogenetics.

Understanding the cellular and genetic basis of neural function and behavior of an organism is a central problem in neuroscience. Recently developed optogenetic methods have

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AUTHOR CONTRIBUTIONS

J.N.S., M.M.C., A.G., and H.L. designed the experiments. J.N.S. and M.M.C. wrote the software. J.N.S. constructed the illumination system, performed experiments and analyzed the data. S.J.H., S.W. and C.S. contributed to reagents and provided valuable discussions. J.N.S., M.M.C., S.J.H., A.G., and H.L. prepared the manuscript.

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contributed enormously to our experimental toolbox[1–8]. Using these tools, neurons and muscles can be optically excited or inhibited with millisecond-precision in cultured cells as well as intact animals[4–7,9–12]. The nematode *C. elegans* is an ideal organism for optogenetic studies as it is transparent, has a well-characterized nervous system composed of 302 neurons of which the wiring connections are well known, and has a battery of genetic tools available that facilitate its study[13,14]. Probing the neural circuit of *C. elegans* can be performed by ablation experiments or by genetically manipulating neurotransmitters, but these methods have limitations such as their low temporal control and the risk of circuit compensation during development. Optogenetics overcomes many of these drawbacks and has already been successfully applied to investigate neural circuits, synaptic transmission, and the cellular basis of behavior in *C. elegans*[5,15–17].

Most optogenetic experiments are currently done using either whole-field illumination,[5] by positioning an optical fiber directly in the vicinity of the neurons[18,19] or by focusing light onto specific neurons in immobilized animals[20]. Thus, the illumination is either spatially non-specific, or it can only be applied to larger or non-moving animals. The expression of transgenes in a subpopulation of cells is routine in *C. elegans* but precise single-cell expression is often difficult; therefore whole-body illumination generally does not permit the cell specificity required to interrogate circuits at the single-neuron level. To truly understand a specific circuit, one would ideally probe multiple distinct nodes (cells) with temporally separate signals. It has been shown that in constrained worms, Channelrhodopsin-2 (ChR2), a blue light-activated cation channel that can depolarize excitable cells[2,4,5], can be used to stimulate specific spatially separate neurons while Ca^{2+} transients are recorded from neurons connected to them using a commercial digital micromirror device (DMD)[20]. This technique allows the interrogation of neural circuits in a cell-specific manner. However, many behavioral neuroscience problems would additionally benefit from the ability to control and monitor a particular behavior in a freely moving animal. Furthermore, assembled commercial systems are often prohibitively expensive, and custom-modified DMDs additionally require considerable expertise, making their wide translation to biology laboratories difficult.

Here we demonstrate a method to optogenetically stimulate and silence specific neurons in freely behaving *C. elegans* with spatial, temporal and chromatic precision. Our illumination system allows us to project an illumination pattern onto an animal and maintain the illumination in the intended anatomical position while the animal is moving. We perform simultaneous multi-color illumination, which allows us to activate some cells while simultaneously silencing others. Furthermore, it allows for high-resolution spatiotemporal and light-intensity control, with changes to stimulation location, intensity and color updated in < 40ms. Finally, because the central component of the illumination system is a modified off-the-shelf liquid crystal display (LCD) projector, the cost and complexity of the system are dramatically reduced, compared to commercially available illumination systems, while maintaining high performance. To demonstrate the capabilities of this new multimodal illumination technology, we optically manipulate nodes within the *C. elegans* touch circuit to study the roles of sensory and command neurons and the ultimate behavioral output.

RESULTS

Experimental setup

To set up a structured illumination system for exciting and/or silencing optically activatable cells in a moving animal, we used an off-the-shelf three-color LCD projector integrated with an inverted epi-fluorescence microscope. Simple modifications were made to both the projector and the microscope (Fig. 1a,b; and Supplementary Note 1). The projector was placed at the epi-fluorescence illumination port and the primary image of the projector was demagnified and translated to the specimen plane by a relay lens (Supplementary Fig. 1). A three-color LCD was selected as it contains three (Red-Green-Blue, or RGB) distinct light paths, each processed using individual LCDs, thus ensuring simultaneous multi-color illumination. We further filtered each color within the projector to limit the large spectral spread and overlap of the native RGB colors (Supplementary Fig. 2a). The intensity of each color is determined by an 8-bit (0–255) value. We calibrated the intensity (with filters inserted) versus pixel value (Supplementary Fig. 2b) and found the maximal intensities across the field (Supplementary Fig. 3) to be sufficient for most optogenetic experiments[5,7,17].

In order to track a freely moving animal while stimulating specific neurons, a relatively low-magnification objective (4x) was selected. This provided sufficient spatial resolution (14 μm) (Supplementary Fig. 4) for both stimulation of groups of cells and whole animal tracking. Using this system multicolor optogenetic stimulation and inhibition can be performed with high spatiotemporal resolution while monitoring subsequent behavioral outputs in real-time (25 Hz) (Supplementary Note 2). Ultimately the accuracy in illumination depends on the speed of the animal and the update rate of the system. In our experiments the animals' forward velocity is about 250 μm s^{-1} allowing an accuracy of 10 μm when operating at 25 Hz (Supplementary Note 2). In order to track and illuminate the animals, we created three independent software modules in LabView (2009 with Vision) (Supplementary Software).

Qualitative behavior elicited by structured illumination

We performed two simple experiments to show spatiotemporal control over gross *C. elegans* behaviors using structured illumination and ChR2-expression. First, we tracked animals expressing ChR2 in the cholinergic motor neurons (ZX460). While the animals were moving forward, we illuminated the head with blue light (430–475 nm) at regular intervals. This produced a dorsal coiling effect[15] when the head was illuminated and resulted in an animal moving in a triangle (Fig. 1c and Supplementary Video 1). In the second experiment, we controlled the muscles of neuronally-paralyzed animals that express ChR2 (ZX299)[7] using structured illumination. Ivermectin (0.01 mg ml^{-1} solution), a nematocidal agonist of glutamate-gated Cl- channels, which causes neuronal hyperpolarization, was delivered to the animals; this eliminates the activities of motor neurons —which are known to express ivermectin-sensitive channels— while muscles remained excitable[21] and were controlled with the light pulses. Partitioning the paralyzed animal into four quadrants (Dorsal-Anterior, Dorsal-Posterior, Ventral-Anterior, and Ventral-Posterior) and exciting the muscles in alternating patterns, we were able to produce S-shaped body postures, suggestive of

locomotion patterns during crawling (Fig 1c and Supplementary Video 2). Although non-quantitative, these experiments together demonstrate that illumination of optically controllable cells can be well defined, easily controlled, and dynamically alterable using the projector system.

Spatial activation of sensory and command neurons

To determine the spatial resolution of our system, we performed experiments analyzing the mechanosensory behavior in *C. elegans*. There are six major mechanosensory neurons in *C. elegans*: AVM and ALML/R (anterior), and PVM and PLML/R (posterior)[22,23]. Animals carrying *pmec-4::ChR2* (AQ2334) express ChR2 in these six touch neurons (Fig. 1d). By traditional touch assay and laser ablation, it has been established that stimulating the anterior neurons causes the animal to move backwards, whereas stimulating the posterior neurons causes forward movement or acceleration[22]. In our experiment, we used a 20 μm-wide bar of blue light and scanned it along a *pmec-4::ChR2* animal's anterior-posterior axis at a relative velocity of 12.5% body length per second (~100 μm s^{-1}) while monitoring the locomotor behavior of the animal (Fig. 1d). The line was scanned in both the head-to-tail and tail-to-head directions (Fig. 1e; Supplementary Video 3). As expected, while illuminating from tail to head, as long as the illumination was in the posterior half of the animal, no reversals were elicited, and as soon as the bar reached the anterior half, animals reversed (Supplementary Video 3). It was also evident that illuminations in the posterior initiated acceleration. We quantified the exact body position at which these behaviors were initiated, as well as the anatomical positions of the touch neurons (Fig. 1f). Reversals were initiated most often within the range 0.40–0.48 along the A-P axis in tail-to-head scans, consistent with the anatomical data (i.e. the positions of the ALM/AVM cell bodies[14]), and with our quantification of neural cell body locations (Fig. 1f and Supplementary Fig. 5). In the head-to-tail scans, animals showed a high probability of reversal well before reaching the AVM or ALM cell bodies (Fig. 1f), indicating that activation of the ChR2 in the processes is sufficient to elicit a response. This is likely because enough ChR2 is present in neuronal processes to allow sufficient photo-depolarization of the cell (Supplementary Note 2). This experiment suggests that the spatial resolution of the system can be used for precise interrogation of the neuronal network at the single-cell level, provided that the cells expressing ChR2 have cell-bodies or processes farther apart than the spatial resolution of the system.

In the *C. elegans'* touch circuit, command interneurons integrate signals from sensory neurons and ultimately produce locomotor behaviors[22–25] (Fig. 2a,b). To quantify these behaviors, we excited the head- or tail-touch neurons and the head- or tail-interneurons (Fig. 2b) using ChR2 and measured the animals' velocity. First, *pmec-4::ChR2* animals were stimulated either in the second 25% or the last 25% of the body. We illuminated a quarter of the body length because this resolution is sufficient to distinguish the anterior and the posterior sensors, and it ensures illumination of the relevant cell bodies in all animals (Supplementary Note 2 and Supplementary Video 4). When the last quarter was illuminated with blue light, thus exciting PLML/R neurons, we observed the expected velocity increase (Fig. 2c). Conversely, when the second quarter of the body was illuminated, exciting AVM

and ALML/R neurons, we observed a large velocity decrease followed by a reversal (Fig. 2c).

We performed similar experiments on *pglr-1::ChR2* animals, which express ChR2 in the command interneurons as well as in other neurons[26]. Illuminating the first quarter of the body with blue light excites the interneurons in the head, including AVA, AVD, and AVB (Supplementary Video 4). Although this stimulation includes interneurons for both backward and forward movements, the predominant effect is the backward command. The velocity profile (Fig. 2d) shows a robust reversal upon stimulation using this light pattern. Similarly, when the last quarter of the *pglr-1::ChR2* animals was illuminated and PVC excited, there was a small but appreciable acceleration. Although we cannot exclude the effects of photostimulation of the other *glr*-1 expressing cells, the experiment shows specific illumination in freely moving animals in ways that is not possible to perform with previous methods. The behavior is consistent with the known roles of the locomotive interneurons and the illumination scheme and measured behaviors are reproducible (Fig. 2c, d and Supplementary Fig. 6).

Spatiotemporal control of the illumination intensity

Traditionally, the study of *C. elegan's* touch circuit has been largely performed using a manual assay that consists in either touching the head or tail of a freely moving animal with an eyelash or tapping on the agar plate that containins the animals[22,25]. One difficulty associated with this assay is controlling and standardizing the force with which animals are stimulated. Micro force transducers have been fabricated to allow control of forces[27]. While precise, these systems are technically demanding, particularly when used on behaving animals and when applied in different positions simultaneously. Using light to drive ChR2, the stimulus intensity (which translates into signal strength in neurons[17,28]) can be easily controlled over a wide range with spatial specificity and in a variety of illumination and intensity profiles. Changing the light intensity in optogenetic experiments normally requires changing the lamp voltage or introducing neutral density filters, which change the light-intensity over the entire field of view. With the illumination method described here, one can easily control the local intensity by varying the pixel values.

First we show that illumination using graded intensities elicits differential behaviors when stimulating the second anterior quarter of *pmec-4::ChR2* animals with blue light. We recorded the animals' responses to 0.29, 1.17, and 4.67 mW mm ^{2} illumination intensities and reordered whether different stimulation strengths produce reversals with different probabilities (Fig. 3a). We grouped the behavior of all the animals analyzed into four categories: a robust large reversal (R, defined as reversals with three or more headswings[29]), a small reversal (r, defined as reversals with less than three headswings[29]), a slowing or pausing response but no reversals (Sl/P), and no measurable responses (NR) (Supplementary Video 5). Regardless of the illumination intensities, we observed that these four categories always exist and are distinguishable (Fig. 3b). Grouping the behavioral responses by the illumination intensities, we show that the low intensity stimulation produces a higher probability of no-response and slow-response in the animals, while the animals are much more likely to reverse upon stimulation at higher intensities (Fig. 3c). This suggests that the

illumination intensities affect the sensory neuron responses and ultimately modulate the distribution of the behavioral responses.

Next we asked whether it is possible to simultaneously stimulate neurons in spatially distinct locations and with sophisticated light-intensity patterns. We were interested in the animals' responses to simultaneous stimuli in anterior and posterior regions (i.e. past which intensity threshold a reversal is produced, and how this changes when a competing signal is present), something that would be thus far impossible using the traditional manual approach. We compared *pmec-4::ChR2* animals that were stimulated only in the head in an increasing step function (to a maximal intensity of 1.17 mW mm^{-2}) (Fig. 4a), to animals stimulated with an identical pattern in the head but that additionally were being stimulated in the tail at a constant intensity (1.17 mW mm^{-2}) (Fig. 4b and Supplementary Video 6). When the thresholds for a population of animals were compiled, we observed that holding constant tail illumination intensity increases the average head intensity at which animals respond (Fig. 4c). To further investigate the integration of competing signals, we stimulated one set of animals with a single light pulse in the anterior, and another set with anterior and posterior pulses with the same intensity. The behavioral response based on illumination modes (Fig. 4d) show how the combined probability of reversals decreases while the probability for no-response increases when the posterior sensory neurons PLML/R are excited. This suggests that the signals from the anterior and posterior sensors are integrated at all times to produce the proper behavior. Additionally, certain combinations of anterior and posterior illumination intensities appeared to be conflicting sensory signals and resulted in conflicting commands as the animals quickly alternated between forward and reverse locomotion (Supplementary Video 7).

Simultaneous multi-color illumination

Because many of the currently available light-sensitive proteins used in optogenetics[2,7,8] are spectrally distinct, an illumination system that can be used to illuminate at different wavelengths would be valuable. For instance, ChR2 is activated in the blue region while NpHR[7] and MAC[8], which both hyperpolarize and silence cells, are both activated in the green-yellow region. Simultaneously exciting and inhibiting different cells in a circuit, particularly in behaving animals, can greatly enhance our ability to understand circuits and their functions.

By using an LCD projector, we have three independently controllable LCD panels that can be used for three independent illuminations. We used two of these channels to interrogate the mechanosensory circuit using *pmec-4::ChR2; pglr-1::MAC* animals. In these animals, MAC inactivates the *glr-1* interneurons when illuminated by green light (550 nm). Because MAC can also be activated (although less efficiently) by blue light[8], we illuminated the second quarter of the body along the A-P axis (i.e. avoiding illumination of *glr-1* neuron cell bodies) using blue light and the first quarter of the body using green light. This allowed exciting the ALM/AVM sensory neurons while inhibiting the *glr:1* neurons only in the anterior part of the animals (inhibiting all backward command neurons but only one of two pairs of forward command neurons) (Supplementary Video 8). As processes of the *glr-1::MAC*-expressing cells pass the region of *mec-4::ChR2* expressing neurons, behaviors

evoked in *mec-4* neurons may be slightly dampened (Supplementary Note 2). The behavior of the animals was tracked over time while they were manipulated following the two photostimulation schemes depicted in Fig. 5a. Velocity averages from multiple animals are shown in Fig. 5b. When the anterior sensory neurons (ALM/AVM) were stimulated by blue light for 4 seconds at 1.17 mW mm^{-2} intensity (Scheme 1; Fig. 5a–c), the animals produced a robust reversal behavior. When ALM/AVM neurons were illuminated the same way while the head interneurons were inhibited by green light 2 seconds after the blue light was on (Scheme 2; Fig. 5a–c), the animals first produced the expected reversals but upon silencing of the interneurons, the reversals were inhibited and the velocity became positive (Fig. 5b). Spontaneous reversals could also be inhibited by green illumination (Supplementary Video 8), suggesting that this is not an artifact by the optogenetic stimulation but a direct interference with the neuronal circuit. This experiment illustrates our ability to illuminate a behaving animal with spatial, temporal, spectral and intensity control. The method yields quantitative behavior data that cannot be obtained by manual touch assays, laser cell ablation, or genetic manipulation of neurotransmitters.

DISCUSSION

Optogenetics has received significant attention due to the potential for fast, repeatable stimulation of genetically defined neurons. We have shown here for the first time that it is possible to track a freely moving *C. elegans* and spatiotemporally excite and/or inhibit specific nodes of neural networks. This illumination system is capable of delivering light stimuli to genetically modified, optically excitable cells with high repeatability and light intensity control. It also enables the use of combinations of optogenetic tools with non-overlapping activation spectra. By using a three-color LCD, we were able to achieve simultaneous multi-color illumination, allowing this spatial and spectral separation to probe neuronal networks more precisely.

Our system uses a modified off-the-shelf projector coupled with a standard microscopy setup, and thus facilitates its adoption by other labs. We note that single DMD or LCD systems can be similarly applied for single-color illumination. The structured illumination system combined with video tracking could be used to study a variety of biological questions related to the behavior and neuronal function of *C. elegans* (and other transparent animals, e.g. *Danio rerio* or *Drosophila* larvae). The ultimate resolution of the system depends on the behavior tracking requirements (e.g. speed of the animal) and hardware, and one would also have to consider where the light-sensitive proteins are expressed (Supplementary Note 2)

In addition to the experiments shown here, multimodal real-time optogenetic control will allow further studies of other sensory circuits. Furthermore, studies related to the integration of different sensory modalities and behaviors will be considerably advanced by the ability to track and stimulate freely moving animals. Real-time illumination and behavior tracking as presented here can also be combined with calcium imaging or with other methods capable of perturbing the circuit, such as using microfluidic devices to deliver well-defined sensory stimuli, analyzing animal mutants for particular neurotransmitters or performing laser ablation of cells, axons, or synapses to remove single nodes or connections within the

circuit. Lastly, one could imagine using the illumination system with other photostimulation methods such as uncaging of small molecules.

METHODS

Methods are available in the online version of the paper at http://www.nature.com/naturemethods/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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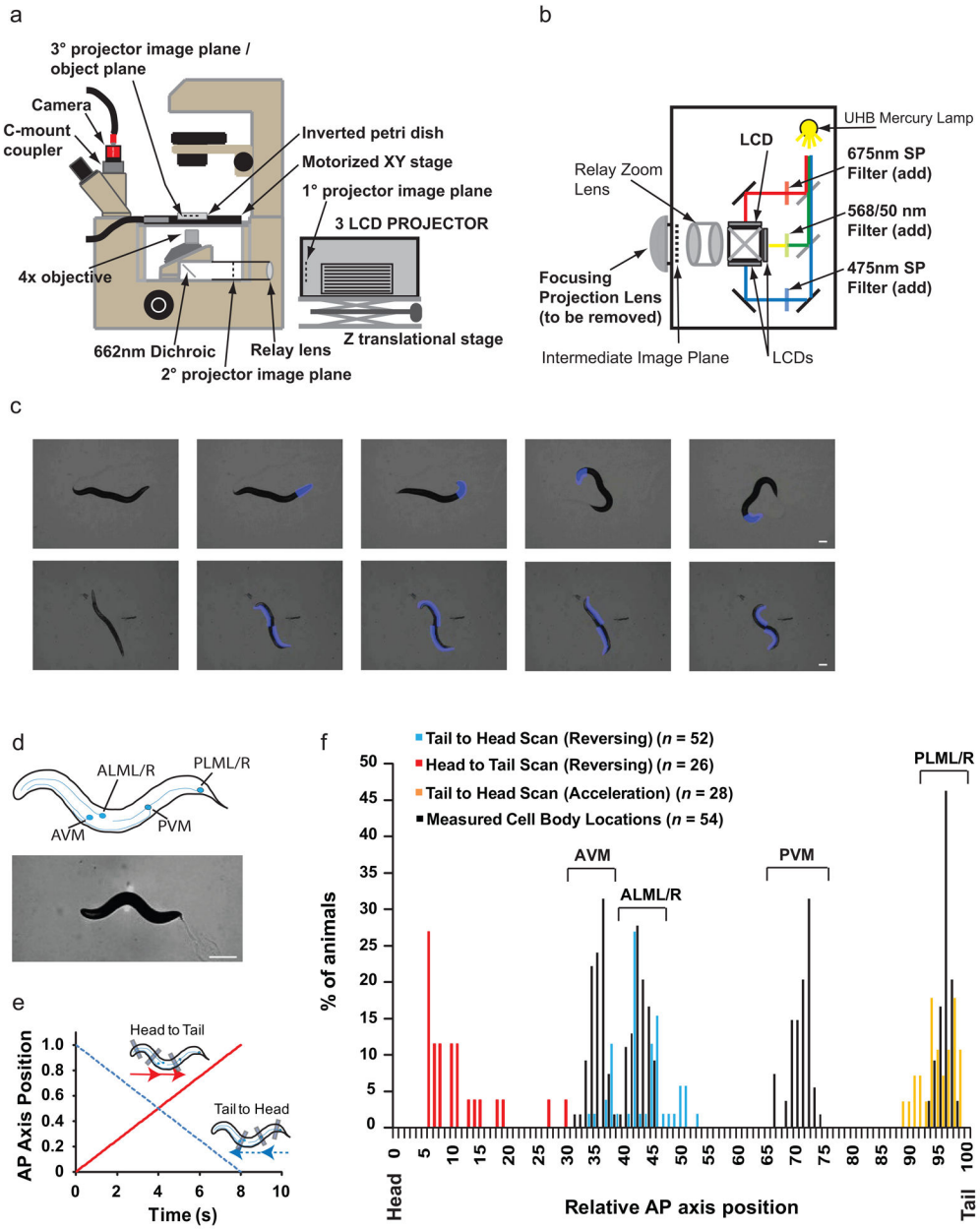

Figure 1.

Illumination system for live animal tracking and optogenetic stimulation and quantification of behavior elicited by targeted illumination. (**a**) Optical configuration for using a projector for illumination. The normal epi-fluorescence optical train was replaced by a projector and a relay lens. Projector image planes are indicated, and a motorized X-Y translational stage is used to track animals. (**b**) Modification of the 3-color LCD projector to further narrow the spectrum is accomplished by the addition of filters into the individual RGB light paths. (**c**) Sequential frames from Supplementary Video 1 and 2 showing qualitative behavioral responses. Top, using the dorsal coiling effect to cause a worm to crawl in a triangle, and bottom, showing direct muscular control of a paralyzed worm. Images are falsely colored to

show illumination pattern. (**d**) Illustration of the positions of the six sensory neurons, and a frame from Supplementary Video 3 showing the 20 μm bar of blue light, perpendicular to the animal's longitudinal axis, which was scanned at a rate of 12.5% animal body length per second (~100 μm s^{-1}). (**e**) Two scanning schemes along the AP axis: head-to-tail and tail-to-head. (**f**) Histograms showing the distributions of positions along the AP axis at which point the blue light elicited a reversal response. Shown are the distribution of positions where accelerations elicited by the tail-to-head scan were observed (28 out of 52 animals showed an increase in speed two standard deviations greater than the average speed prior to illumination), and the distributions of the anatomical positions of the touch neurons in *pmec-4::GFP* animals. Scale bars are 100 μm.

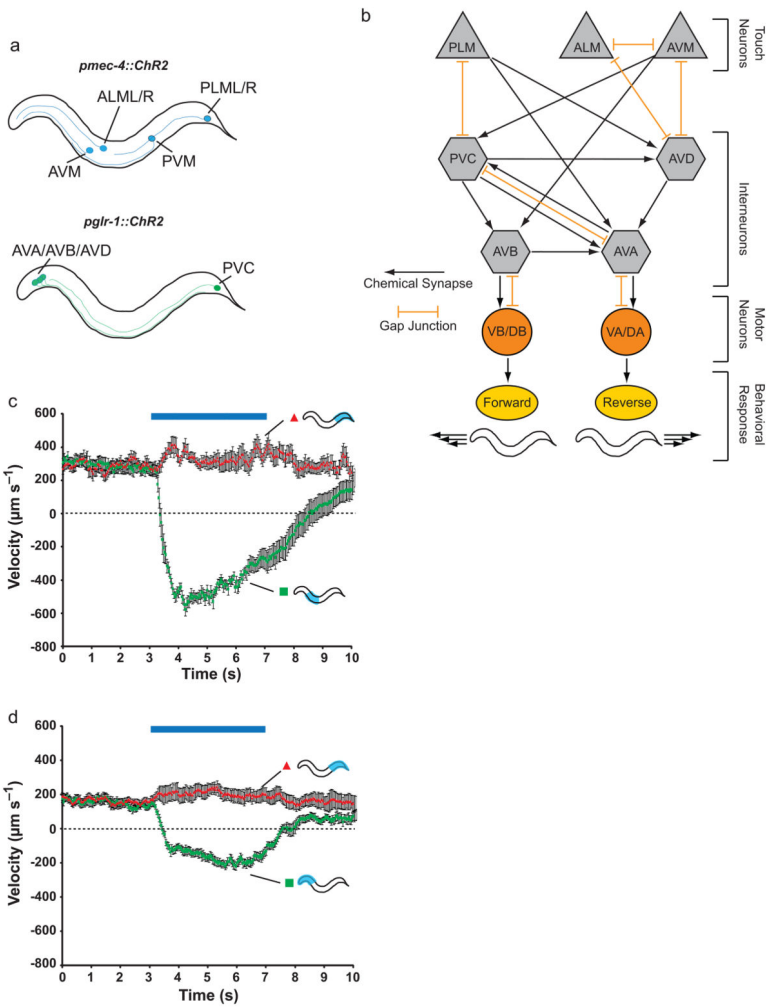

Figure 2.

Optical stimulation of anterior/posterior mechanosensory neurons or forward/backward command interneurons. (**a**) Illustration of the positions of neurons expressing ChR2 in *pmec-4::ChR2* and *pglr-1::ChR2* transgenic worms. (**b**) The touch circuit showing receptors, command neurons and the resulting behaviors. (**c**) Average velocity plots of *pmec-4::ChR2* animals under illumination conditions (shown as a blue bar above). *n* = 13 (posterior illumination); *n* = 15 (anterior illumination). Error bars = s.e.m. (**d**) Average velocity plots of *pglr-1::ChR2* animals under illumination conditions (shown as a blue bar above). *n* = 24 (posterior illumination); *n* = 12 (anterior illumination). Error bars = s.e.m.

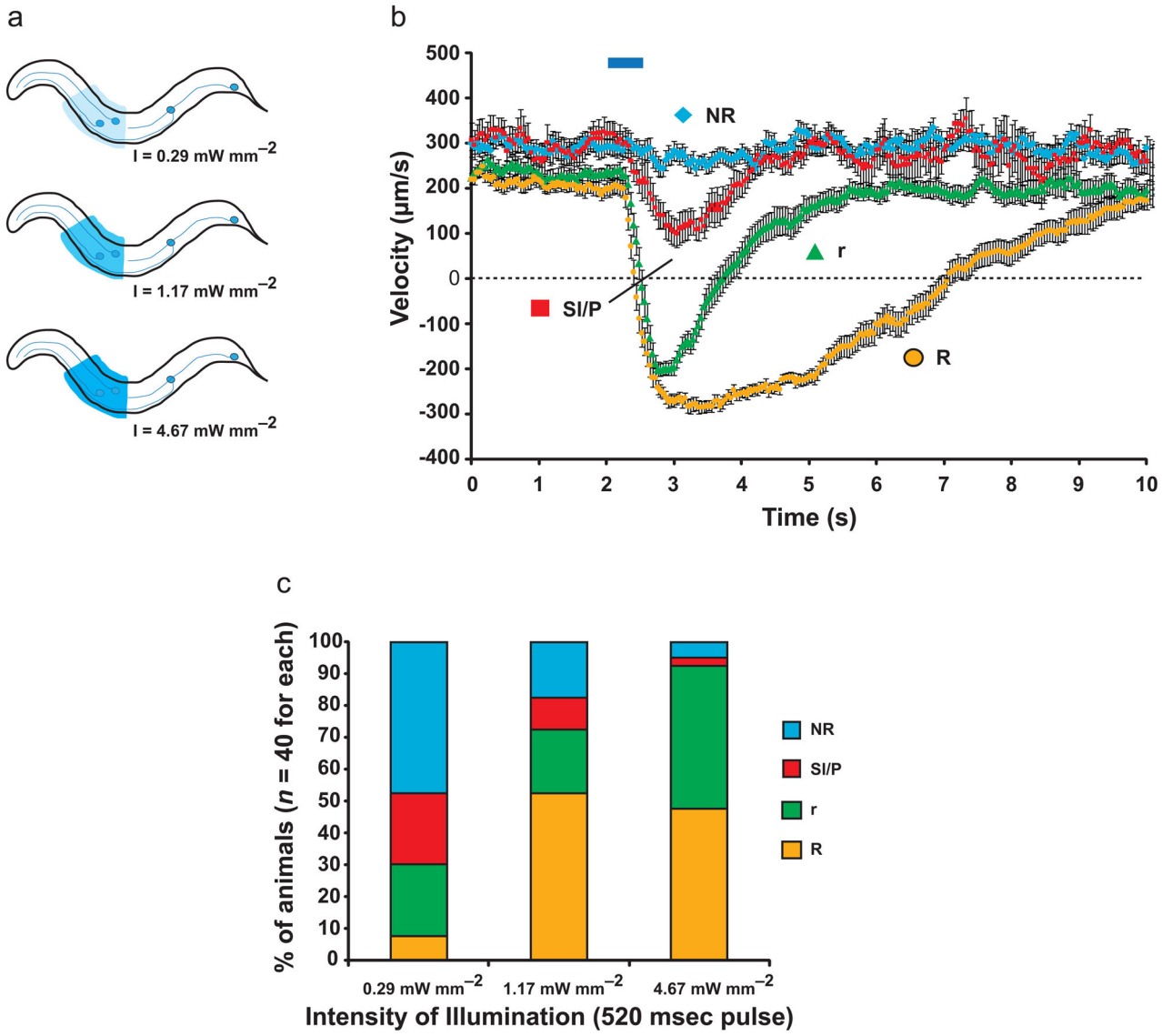

Figure 3.

Quantification of behavioral responses elicited by different anterior illumination intensities. (**a**) Patterns used for illumination location and their intensity. (**b**) Velocity plots from pooled data from animals receiving different illumination intensities (also see Supplementary Video 5). 'NR' = No Response; 'Sl/P' indicates a slowing or pausing of the animal with no negative velocity; 'r' is a small reversal; and 'R' is a large reversal. *n* = 40 for each of the three illumination levels. The number of animals showing NR, Sl/P, r, and R are 28, 14, 35, and 43 respectively. Error bars = s.e.m. (**c**) Distribution of the four responses observed at the three intensity levels.

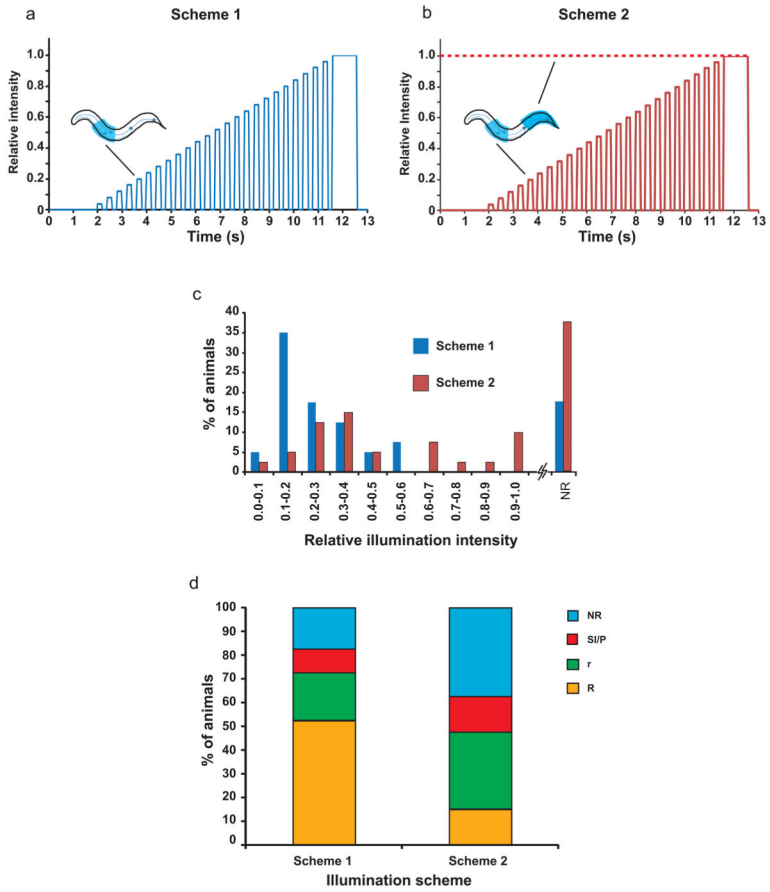

Figure 4.

Illumination patterns used to explore the integration of anterior/posterior signals and behavior generated from the stimulation (also see Supplementary Video 6). (**a**) Illumination locations and plot of the temporal variation of the intensity for the two patterns tested. Normalized intensity of 1 corresponds to blue light of intensity 1.17mW mm^{-2}. (**b**) Histogram distributions of intensity at which animals initiated a reversal under two illumination patterns: anterior alone, and anterior and posterior simultaneously. (n = 40 for each illumination scheme). (**c**) Distributions among the four response states for anterior illumination alone or simultaneous anterior/posterior illumination at the same intensity (1.17mW mm^{-2}). (n = 40 for each).

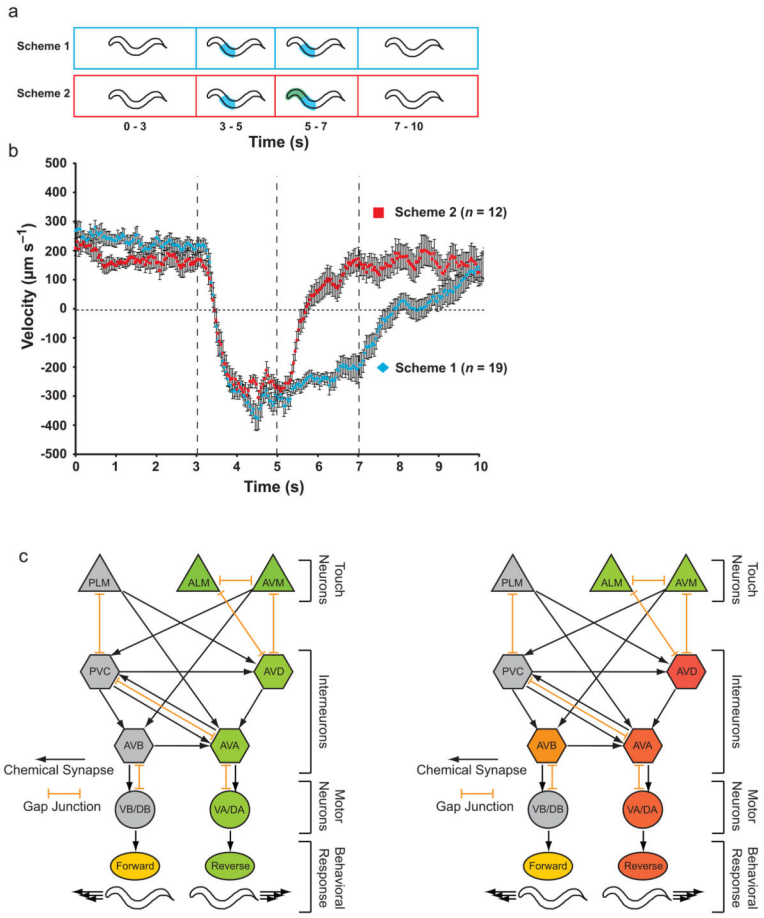

Figure 5.

Simultaneous two color illumination (also see Supplementary Video 8). (**a**) Illustrations of the two illumination schemes (**b**) Velocity plots of *pmec-4::ChR2* and *pglr-1::MAC::mCherry* animals subjected to the illumination schemes in (a). Error bars = s.e.m; *n* = 19 for scheme 1; *n* = 12 for scheme 2. (**c**) The neural gentle touch circuit showing the neurons that are either stimulated or silenced and the resulting behaviors at different points in the two sets of experiments.