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High-Throughput Controlled Mechanical Stimulation and Functional Imaging *In Vivo*

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22 **Abstract:**

23

24 Understanding mechanosensation and other sensory behavior in genetic model systems such as
25 *C. elegans* is relevant to many human diseases. These studies conventionally require
26 immobilization by glue and manual delivery of stimuli, leading to low experimental throughput
27 and high variability. Here we present a microfluidic platform that delivers precise mechanical
28 stimuli robustly. The system can be easily used in conjunction with functional imaging and
29 optical interrogation techniques, as well as other capabilities such as sorting or more
30 sophisticated fluid delivery schemes. The platform is fully automated, thereby greatly enhancing
31 the throughput and robustness of experiments. We show that behavior of the well-known gentle
32 and harsh touch neurons and their receptive fields can be recapitulated in our system. Using
33 calcium dynamics as a readout, we demonstrate the ability to perform a drug screen *in vivo*.
34 Furthermore, using an integrated chip platform that can deliver both mechanical and chemical
35 stimuli, we examine sensory integration in interneurons in response to multimodal sensory
36 inputs. We envision that this system will be able to greatly accelerate the discovery of genes and
37 molecules involved in mechanosensation and multimodal sensory behavior, as well as the
38 discovery of therapeutics for related diseases.

39

40 Introduction

41 Mechanosensation is required for multiple sensory modalities such as touch, hearing, and
42 balance, and is linked to a multitude of disorders including deafness¹⁻⁴. Molecular mechanisms
43 for mechanotransduction have been partially elucidated using a variety of model organisms,
44 including *Caenorhabditis elegans*⁵⁻¹⁶. Conventional mechanosensation experiments with *C.*
45 *elegans* typically involve the manual delivery of a mechanical stimulus to anterior or posterior
46 regions of animals via an eyebrow hair or metal pick^{5,17-19}, and visual scoring of touch avoidance
47 behavior, an assay subject to considerable variability between experimenters. Computer-
48 controlled stimulation methods, for example using a piezo-driven micro stylus, have been used
49 with electrophysiological and functional imaging approaches to deliver more repeatable
50 mechanical stimuli to animals^{20,21}. However, recording of neuronal responses by patch clamping
51 or calcium imaging in response to precisely controlled mechanical stimulation requires animals
52 to be immobilized with glue^{15,20,21}, limiting experimental throughput and disallowing the
53 recovery of animals for screens or further experimentation. Moreover, gluing itself is likely to
54 affect neuronal or circuit response, and differences in the extent of gluing introduce additional
55 experimental variability.

56
57 Microfluidics has long been used as a “lab-on-a-chip” technology, allowing for well-
58 controlled and high-throughput experiments with small samples²². In addition to enabling precise
59 perturbations on the micron scale, microfluidic devices can easily be designed to work together
60 with optical microscopy, allowing for imaging of fluorescent probes such as calcium indicators.
61 For *C. elegans* experimentation particularly, microfluidics has been a widely adopted technology
62 due to the match in length scale and compatibility with fluid handling²³. Various devices have
63 been developed for delivering a variety of stimuli, including chemical cues and temperature
64 gradients, while simultaneously recording neuronal responses through calcium imaging²⁴⁻³³. In
65 contrast, there are currently no microfluidic devices capable of delivering mechanical stimuli to
66 *C. elegans*, or do so while recording neuronal activities in a controlled manner. In this work we
67 present a microfluidic platform for delivering robust and precise mechanical stimuli to *C.*
68 *elegans* by using pneumatically actuated structures. The device is fully automated, minimizing
69 human variability and improving experimental throughput; it is fully compatible with fluorescent
70 imaging of calcium dynamics of neurons, which enables mechanistic interrogations as well as

71 high-throughput genetic or drug screens. Furthermore, the mechanical stimulus module of the
72 device can be easily integrated with other microfluidic modalities, allowing for multimodal
73 stimulation for sensory integration studies. Here we demonstrate the design and utility of such a
74 system in the context of high-throughput screening, as well as interrogate circuit dynamics in
75 multimodal sensory behavior.

76

77 **Results**

78 Our microfluidic device is optimized to deliver precise and repeatable mechanical stimuli to
79 different anatomical regions of *C. elegans* (Fig. 1). After animals are loaded into an imaging
80 channel (where the animals are not immobilized but their movement is much reduced from freely
81 moving behavior), mechanical stimuli are delivered through two pairs of in-plane PDMS
82 membrane structures (Fig. 1a and Supplementary Fig. 1). The structures are pressure-actuated,
83 and when deflected, exert a mechanical stimulus on animals trapped in the imaging channel. The
84 deflection and deformation caused by these actuations are in similar ranges as conventional
85 approaches (Supplementary Fig. 2)^{20,21}. Two additional actuated structures act as loading and
86 imaging valves (Fig. 1a). This design retains animals in plane and relatively stationary but not
87 fully immobilized, thus allowing high-quality imaging of calcium transients in cell bodies and
88 subcellular processes (Fig. 1b). To automatically identify the fluorescently labeled neuron of
89 interest and extract quantitative calcium transients, we developed a neuron tracking algorithm
90 (Supplementary Fig. 3). The actuated structures are connected to a pressure source via
91 individually controlled off-chip solenoid valves, allowing for an automated and rapid “load-and-
92 image” routine (Fig. 1c). Additionally, the duration and pressure of stimuli can be easily
93 controlled, allowing for the study of a variety of behaviors upon mechanical stimuli such as
94 graded response, habituation, and arousal. Furthermore, this design can be easily adapted to
95 allow for sorting and imaging animals of various sizes.

96

97 To demonstrate the utility of the system, we examined the responses of the classic gentle
98 (AVM, ALMR/L, PVM, and PLMR/L) and harsh (PVD) touch receptor neurons¹⁹ (Fig. 1d). The
99 stimulus is traditionally delivered to moving worms by a metal pick¹⁹, or to immobilized worms
100 by a stiff probe^{15,20}. By imaging calcium transients in animals expressing the genetically encoded
101 calcium indicator (GECI) GCaMP6m³⁴ in these touch receptor neurons, we show that the same

102 device can deliver stimuli capable of exciting both the gentle and harsh touch neurons. Upon
103 delivery of either a 1- or 2-second stimulus, calcium levels in cell bodies as well as in neuronal
104 processes of both the gentle and harsh touch neurons rose as expected (Fig. 1e-m and
105 Supplementary Movie 1-4). Spatially, these responses were consistent with the individual
106 neurons' receptive fields as defined by anatomical and/or calcium imaging data^{5,18,20,35}. AVM
107 responded to anterior but not posterior stimuli (Fig. 1k). In contrast, PVD responded to both
108 anterior and posterior stimuli, as did PVM, with the responses to posterior stimuli being stronger
109 for both of these classes of neurons (Fig. 1l, m). These results demonstrate that the
110 mechanosensory chip delivers biologically relevant, spatially well-defined stimuli.

111
112 Because our system delivers mechanical stimuli by applying externally controlled pressure to
113 actuated structures, the stimuli can be regulated by the magnitude and duration of the applied
114 pressure (Fig. 2 and Supplementary Fig. 2). In the range of stimuli of relevance, the deformation
115 in the animal tissue is roughly linear to the actuation pressure (Supplementary Fig. 2). To
116 examine the effects of these two parameters, we applied anterior stimuli of varying levels of
117 pressure and durations, and measured calcium activity in AVM neurons (Fig. 2a-d and
118 Supplementary Fig. 4a, 5). Peak calcium transients were roughly proportional to the pressure
119 applied (Fig. 2a, c) and the stimulus duration (Fig. 2b, d and Supplementary Fig. 4, 5). We also
120 tested AVM's responses in the well-known *mec-4*/DEG/ENaC channel mutant (Fig. 2e). As
121 expected, the *mec-4* mutant gives negligible response and is insensitive to the magnitude of the
122 stimulation input in the gentle touch regime, but is responsive to harsh touch, perhaps even more
123 so than wild-type (Fig. 2e,f). Similarly, in the harsh touch regime, we presented posterior stimuli
124 of varying pressure and durations, and observed responses in PVD neurons (Fig. 2g, h). As
125 expected, compared to gentle touch neurons, PVD required higher pressure (55 psi) or longer
126 duration of stimulus (5s) at low pressure to elicit similar responses. Furthermore, PVD also
127 shows graded response to pressure and duration (Fig. 2g, h and Supplementary Fig. 4b).

128
129 Interestingly, in addition to response magnitude, the response rates of both the gentle touch
130 and the harsh touch neurons are also functions of the stimulation pressure and duration (Fig. 2i).
131 For AVM, stimuli with actuation pressure higher than 40 psi produce a response rate (fraction of
132 animals responding) of >90%, while below 30 psi the response is more stochastic (<20%) (Fig.

133 2i and Supplementary Fig. 4-6). Applying stimuli at lower actuation pressure also elicits a less
134 sustained response or small magnitude of response and shorter stimuli elicits less response.

135
136 Besides simple stimulation, our system can also be used to deliver repeated stimuli in order
137 to examine phenomena such as habituation and desensitization. Previous work has shown that
138 presenting repeated mechanical stimuli can cause habituation in mechanosensory neurons²⁰. To
139 ask whether this phenomenon can be recapitulated in our system, we delivered repeated stimuli
140 to animals using either short (1s) or long (3 min) inter-stimulus intervals (Fig. 2j-m and
141 Supplementary Fig. 7). When receiving repeated stimuli with short intervals, the neurons
142 exhibited an incremental increase in response magnitude up to the second stimuli, and then a
143 reduced response in later stimuli (Fig. 2j, l). In contrast, when using long inter-stimulus intervals,
144 the response magnitude was reduced after each stimulus (Fig. 2k, m). These results are consistent
145 with previous observations that habituation is dependent on inter-stimulus durations²⁰. Thus,
146 these experiments demonstrate how simple changes of operational parameters allow us to use the
147 same device for a wider repertoire of the device utility.

148
149 In contrast to gluing protocols, our system allows for automated imaging by streamlining the
150 handling of the worms; this in turn allows for high-throughput experiments that were not
151 practicable before. To demonstrate the ability to perform rapid screens, we examined the effect
152 of small molecules from an orphan ligand library on mechanosensation. We exposed animals to
153 the compounds in L4 stage, and imaged AVM activity when delivering an anterior stimulus to
154 adult worms (Fig. 3a). Figure 3b shows a typical response of wildtype animals without drug
155 perturbation: calcium traces typically reach a maximum value shortly after the end of stimulus,
156 and then slowly decline back to baseline levels. In order to examine how each drug affects
157 mechanosensation, we quantitatively compared three metrics (max $\Delta R/R_0$, delay time, and half-
158 life), as well as fraction of animals responding, between drug-treated animals and untreated
159 animals (Fig. 3c-h and Supplementary Fig. 8, 9). We imaged adult animals exposed to 13
160 different drugs and quantified the established parameters for the screen criteria (Supplementary
161 Table 1). While most of the drugs screened lowered the number of animals responding to
162 mechanical stimulus, interestingly, a few drugs slightly increased the response fraction (Fig. 3c).
163 We also analyzed differences in the metrics measure calcium dynamics for the drug treatment

164 conditions, and found that five of the drugs we tested significantly affected mechanosensation
165 response dynamics (Fig. 3d-f). Specifically, D-Alanine (#5) and D-Arginine (#8) significantly
166 attenuated the max $\Delta R/R_0$ while increasing the delay in the responses (Fig. 3d, e, h). In contrast,
167 D-Lysine (#4) only attenuated the max response (Fig. 3d, g). Two other drugs had effects only in
168 the decay-time of response (Fig. 3f-h). D-Isoleucine (#3) induced considerably smaller half-life
169 for the calcium transients to return to baseline, much faster than in untreated animals (Fig. 3f, g).
170 Lastly, β -Alanine (#6) significantly increased the half-life, and calcium transients decreased in a
171 slow linear gradient, instead of a typical exponential decay (Fig. 3f, h).

172

173 Another advantage of using microfluidics to deliver mechanosensory stimuli is that it is
174 compatible with other microfluidic components to provoke additional sensory responses, e.g.
175 chemosensation²⁵. *C. elegans* is a convenient system for studying multimodal sensory integration
176 *in vivo*; worms have distinct sensory modalities such as mechanosensation and chemosensation,
177 which allow them to find food sources and estimate danger. The difficulty to study sensory
178 integration thus far is that there has not been a convenient method to integrate mechanosensory
179 input with the existing tools, including microfluidic and optical methods^{23,25,36-38}. With our
180 mechanical stimulus device, incorporating chemosensory modules is readily attainable by simply
181 adding channels to deliver chemical stimuli (Supplementary Fig. 10); without mechanical
182 stimulation, the response of a chemosensory neuron to a chemical cue is as expected
183 (Supplementary Fig. 11). To demonstrate the utility of the system for multimodal sensory
184 integration, we focused on the response of the PVC command interneurons to both mechanical
185 and chemical stimuli. PVC interneurons are postsynaptic to both the posterior mechanoreceptor
186 neurons PLML/R, as well as the posterior chemosensory neurons PHBL/R, that have been shown
187 to respond to 0.1% SDS stimulus^{5,39-41} (Fig. 4a and Supplementary Fig. 12a). Using our device to
188 deliver multi-modal stimuli, we tested the ability of PVC to respond to stimuli within the same
189 mode and cross-modality. When a single sub-threshold stimulus in either modality is delivered
190 (i.e. 30s SDS stimulation to the tail, or 1s mechanical stimulation to the tail), PVC shows a low
191 probability of response (Fig. 4b, c). Compared to upstream sensory neurons, PVC also responds
192 with a lower magnitude and the response is more variable (Fig. 4b, c and Supplementary Fig.
193 12b, c). Perhaps not so surprisingly, PVC's response to subthreshold stimuli in the same
194 modality can be sensitized for subsequent stimulation (Supplementary Fig. 12d). More

195 interestingly, when pre-sensitized by cross-modality sub-threshold stimuli (i.e. chemical before
196 mechanical stimulus, or vice versa), PVC shows similar sensitized responses (Fig. 4d, e, and
197 Supplementary Movie 5, 6). This sensitization is seen both in terms of the magnitude of the
198 individual responses and the fraction of responding animals.

199

200

201 **Discussion**

202 For fundamental studies of mechanosensation, quantitative live imaging is necessary, and to
203 perform screens based on mechanosensory phenotypes requires large sample sizes. Our
204 microfluidic platform allows for studying mechanosensation in *C. elegans* quantitatively and
205 conveniently, allowing for the delivery of a variety of types of mechanical stimuli to live animals
206 while recording neuronal activity. Experimental preparation (mainly washing) can be
207 accomplished for a batch of animals, so the limiting step is imaging (tens of seconds to minutes
208 depending on the experiments). Experimental throughput using our streamlined microfluidic
209 system can be as high as ~100 trials per hour; it is also straightforward to automate and run these
210 systems in parallel to further improve throughput. In contrast, the conventional approach (gluing
211 worms and stimulating with a micro stylus and micromanipulator) generally yields
212 ~10 successful trials per day. The integration of hardware and software also allows for automated
213 operations of imaging, stimulation, and quantitative analysis, further reducing potential human
214 error and bias. This important improvement in throughput and standardization over conventional
215 methods allowed us to conduct a novel drug screen based on neuronal dynamics due to
216 mechanical stimuli. By using our system, we identified several candidates that strongly affect
217 dynamics in mechanosensory neurons in a variety of ways. One can envision genetic screens
218 performed in a similar manner to identify mechanosensory mutants. Many worm
219 mechanosensory modalities, such as harsh touch and nose touch, involve multiple partially-
220 redundant cell types, making behavioral assays ineffective for finding genes affecting these
221 processes. With simple integration of sorting mechanisms on chip^{36,42,43}, it will be possible to
222 conduct high-throughput forward screens for mutants affecting the responses of individual
223 neurons, using a GECI-based assay. The genes identified in such screens should provide insight
224 into the underlying mechanisms of mechanosensation, as well as find potential therapies for
225 sensory-loss conditions such as deafness.

226

227 Additionally, microfluidic incorporation of fluidic control can easily allow interrogation of
228 other sensory modalities (e.g. olfaction) in combination with mechanosensation. We have shown
229 that our platform is compatible with previous techniques for delivering chemical stimuli,
230 enabling for the interrogation of integration of multimodal stimuli in the interneurons. This
231 feature can greatly expand the repertoire of assay conditions to allow studies of sensory
232 integration, arousal, habituation, and sensitization. For example, it has been previously shown
233 that neural responses to sensory stimuli become more deterministic as information flows from
234 sensory neurons to interneurons; behavioral responses, however, correlate more strongly with
235 interneurons such as PVC^{33,44}. We show here that PVC's response can be modulated, by prior
236 sensory inputs, and that this modulation is cross-modal. This may point to an interesting
237 ecologically relevant strategy for animal behavior, such that the reliability of the escape response
238 depends both on the stimulus and on the current state of the circuit, as influenced by experience.

239

240 Because our platform employs a simple microfluidic device, it is easily adaptable to study
241 biological systems of various sizes. Scaling the devices to be smaller can allow studies of
242 mechanosensory neurons in worm larvae during development; scaling the devices larger can
243 allow studies of the mechanosensation circuit during aging in *C. elegans*, as well as neurons and
244 circuits in other model organisms such as zebrafish or fly larvae. Lastly, because the microfluidic
245 chip allows unhindered optical access, integrations of optogenetic methods^{37,38,45-49} can also be
246 straightforwardly carried out in this platform, thereby greatly expanding the repertoire of
247 biological problems to be studied.

248

249

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258 Resources and the International *C. elegans* Knockout Consortium.

259 **Methods:**

260 Strains

261 *C. elegans* were maintained under standard conditions and fed OP50 bacteria⁵⁰. The
262 following strains were used in this study:

263 AQ 3236 *ljIs142[mec-4::GCaMP6m::SL2TagRFP, unc-119] II; unc-119(ed3) III*

264 TV17924 *wyls5007[ser2prom3::GCaMP6, egl-17::mCherry] X*

265 CX10979 *kyEx2865[sra-6::GCaMP3, Pofm-1::GFP]*

266 GT243 *aEx2[pglr-1::GCaMP6(s), punc-122::GFP]*

267 RW1596 *stEx30[myo-3p::GFP + rol-6(su1006)]*

268 To construct AQ3236, we used a single-copy insertion vector containing a GCaMP6M transgene
269 codon-optimized for *C. elegans*, under the control of the *mec-4* promoter (a gift from Doug Kim
270 at HMMI Janelia Research Campus). Single-copy chromosomal integrations were obtained using
271 the MosTic procedure⁵¹. Unless otherwise specified, all worms imaged in this study are adults.

272

273 Chip Design and Fabrication

274 The device consists of worm inlet/outlet, imaging channel (50~60 μm deep), and four
275 sets of actuated PDMS membranes. Animals loosely fit in the channel, and are trapped (but not
276 held) in the imaging area by two sets of actuated members. The width of actuated PDMS
277 membrane is 150 μm , the distance between first and second sets of membrane is 200 μm and
278 second and third sets of membrane is 250 μm .

279 Since worms were not immobilized using drugs, animals' head or tail can move in the
280 imaging channel of the microfluidic chip. This movement sometimes blurs images. To reduce the
281 movement of head or tail part of worms, a three-step vertical tapering of the imaging channel
282 was used to restrict the out-of-plane movement. The thickness of first and second layers was 15
283 μm and third layer was 20 μm for the 50 μm deep imaging channel; these layers were created by
284 SU-8 2015 negative photoresist (MicroChem) using standard photolithographic techniques⁵².

285 To create the actuated PDMS structure to touch and trap worms, multi-layer soft
286 lithography process⁵³ was used. For the bottom flow layer of features, 23:1 PDMS was deposited
287 via spin coating to create a thin layer. For the top control layer, 10:1 PDMS was directly poured

288 onto a blank master, which does not have any features, to create a thick and mechanically rigid
289 handle layer. Both layers were then placed into a 90°C oven for 25-30 minutes until the control
290 layer PDMS was rigid but sticky. After they were manually aligned, additional 10:1 PDMS was
291 poured and cured for several hours to create a rigid handling layer for the device.

292

293

294 Calcium Imaging

295 All imaging experiments were performed on a Leica DMIRB inverted microscope using a
296 40x air objective (N.A. 0.75). Video sequences were captured using a Hamamatsu EM-CCD
297 camera with 100 ms exposure time. Simultaneous two-color imaging was performed using a
298 DV2 beamsplitter (Photometrics) containing a GFP/RFP filter set. Excitation light for fluorescent
299 imaging was delivered through a projector system previously developed³⁸. In experiments for the
300 measurement of mechanosensory neuronal responses, stimuli were delivered 10 s after recording
301 baseline activity of neurons. In experiment for the measurement of interneuronal recording,
302 stimuli were delivered 30 s after recording baseline activity of neurons. Videos were recorded for
303 60-180 s following stimulus delivery.

304

305

306 Data Analysis

307 Fluorescence intensities for each frame were extracted using customized neuron-tracking
308 Matlab scripts (Supplementary Fig. 7). In strains where both GCaMP6 and RFP are expressed,
309 the ratio between intensity values were computed ($R = \frac{I_{G_ROI}}{I_{R_ROI}}$) in order to minimize movement
310 artifacts. When only GCaMP was available, fluorescence values were computed by subtracting
311 background intensity ($F = I_{G_ROI} - I_{G_Back}$). GCaMP and RFP intensities were measured as the
312 mean pixel intensity of the 100 brightest pixels of a circular region of interest (ROI) of 10 pixel
313 radius. Background intensities were subtracted to adjust for variations in lighting conditions, and
314 were measured as the mean pixel intensity of an ROI in a background region (Supplementary
315 Fig. 7). Calcium traces were computed as the change in R or F from the baseline value ($\frac{\Delta R}{R_o} =$
316 $\frac{R-R_o}{R_o}$) or ($\frac{\Delta F}{F_o} = \frac{F-F_o}{F_o}$). Baseline values were computed as the mean R or F prior to stimulus
317 delivery.

318

319 Drug screening

320 Worms were roughly synchronized by picking 20-25 L4 worms and allowing them to lay
321 eggs overnight before removing them from the plate. After two days at 20°C, tightly age-
322 synchronized populations of worms were obtained by washing adults and L1s off of these plates
323 and then washing newly hatched L1s from these plates after an hour interval. The 84 compounds
324 of the Screen-Well Orphan library (ENZO) were used for the drug screening. 20-30 tightly-
325 synchronized L4 worms were placed on a 48-well plate (Greiner Bio-One) with 0.5 ml OP50
326 bacteria (OD 5) for non-treated worms and both 0.495 ml OP50 bacteria and 0.005 ml (100 µM)
327 drugs for drug-treated worms. After 24 hours, worms were imaged. Among 84 compounds in the
328 library, we tested the effect of 13 compounds on AVM neuronal responses at three different ages
329 (from day 1 adult to day 3 adult). These compounds were chosen randomly from the orphan
330 ligand library.

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436
437

438 **Figure 1: Microfluidic platform can robustly deliver mechanical stimulus and allow**
439 **imaging of calcium responses in *C. elegans* mechanoreceptor neurons.**

440 **a)** The device employs multiple sets of actuated structures: valves to trap animals in a
441 reproducible position, and two sets of actuation valves used to deliver mechanical stimuli to the
442 anterior and posterior regions of the body.

443 **b)** Sample frames from an activated neuron show changes in fluorescence due to mechanical
444 stimulus. Because animals are not fully immobilized, and neurons of interest move during
445 recordings due to the mechanical stimulus and behavioral responses, a tracking algorithm
446 (Supplementary Fig. 3) was developed in order to automatically record the GCaMP and RFP
447 intensities of traces from individual trials.

448 **c)** Timeline of on-chip mechano-stimulation and functional imaging of neurons. The loading and
449 unloading of each worm requires only a few seconds. Each animal is given a waiting period to
450 acclimate to the environment before being stimulated and imaged. Each trial is performed by
451 recording video to track neuronal dynamic responses and applying mechanical stimuli.

452 **d)** Schematics of the mechanoreceptor neurons in this study: six gentle touch neurons -AVM,
453 ALML/R, PVM, and PLML/R - and harsh touch PVDL/R neurons.

454 **e - j)** Responses of the *C. elegans* gentle touch and harsh touch neurons to mechanical stimuli.
455 Average traces of GCaMP6 signals in **e)** ALM soma to 1s stimulus (n=16), **f)** ALM process to 2s
456 stimulus (n=7), **g)** PVD soma (n=9, 55psi), **h)** PVM soma (n=17), **i)** PLM soma (n=9), and **j)**
457 PVD process to 1s stimulus (n=5) at 45psi. Error bars represent SEM.

458 **k - m)** Gentle and harsh touch neurons exhibit reliable calcium responses when spatially resolved
459 stimulus was delivered to the appropriate regions of animals in our system. **k)** The activity of
460 AVM responses to 1s anterior but not posterior stimuli (anterior: n=10, posterior: n=5) at 45psi.

461 **l, m)** Gentle touch neuron, PVM, (2s stimulation, anterior: n=10, posterior: n=11) and harsh
462 touch neuron, PVD, (2s stimulation, anterior: n=9, posterior: n=3) respond to both anterior and
463 posterior stimuli at 45psi. Both neurons show the higher peak of neuronal responses to posterior
464 stimuli than anterior stimuli. Error bars represent SEM. Orange denotes anterior touch and green
465 denotes posterior touch.

466 For panels (e-m), arrow thickness indicates stimulation duration. 1s and 2s stimulations are
467 represented by thin and thick arrows, respectively.

468

469 **Figure 2: The microfluidic platform delivers mechanical stimuli emulating both gentle and**
470 **harsh touch by varying the magnitude of applied pressure and duration of the stimuli.**

471 **a - b)** Average traces of GCaMP6 signal in AVM neuron in response to diverse pressures and
472 stimulus durations. **a)** Applied 1s stimulation with diverse pressures (25 psi: n=11, 35 psi: n=25,
473 40 psi: n=8, 45 psi: n=27). **b)** Applied 40 psi stimulation with diverse stimulus durations (1s:
474 n=8, 2s: n=10, 5s: n=10).

475 **c - d)** Maximum responses of calcium transients correlate with **c)** the applied pressure (2s
476 stimulus, 35 to 45 psi) and **d)** the duration of stimuli (1 to 5s stimuli, 40 psi). Error bars represent
477 SEM.

478 **e)** Average calcium responses of *mec-4(e1611)* mutants in AVM neuron to diverse pressures
479 with 1s stimulus (25 psi: n=18, 35 psi: n=10, 40 psi: n=9, 45 psi: n=10).

480 **f)** Maximum responses of calcium responses of wild-type and *mec-4* mutant animals (Mann-
481 Whitney Test, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

482 **g - h)** Average traces of GCaMP6 signal in PVD neuron in response to diverse pressures and
483 stimulus durations. **g)** Applied 1s stimulation with diverse pressures (45 psi: n=9, 50 psi: n=6, 55
484 psi: n=9). **h)** Applied 45 psi stimulation with diverse stimulus durations (1s: n=9, 2s: n=4, 5s:
485 n=6).

486 **i)** Quantitative responses of AVM and PVD under different stimulation conditions. Both gentle
487 touch AVM neurons and harsh touch sensing PVD neurons can be stimulated using this platform
488 when using the right parameter regime. Each column refers to the applied pressure magnitude
489 and each row refers to the applied durations of stimulation. For each data point, the circle size
490 indicates the max response value from 0 to 3.0 and the rectangle size indicates response fraction
491 from 0 to 1. Response fraction is defined as the percentage of traces that show a max response
492 value of higher than 0.5.

493 **j-m)** Delivery of precisely repeated stimuli in AVM. **j, l)** When worms are exposed to 1s stimuli
494 with short inter-stimulus intervals (1s), the neurons exhibited an incremental increase in response
495 magnitude up to the second stimulus, and a reduced response in later stimuli (n=19). **k, m)** In
496 contrast, when exposed to 2s stimuli with long inter-stimulus intervals (3 min), the response
497 magnitude was reduced after each stimulus (n=10). Error bars represent SEM.

498

499 **Figure 3: The microfluidic platform enables screens to examine compounds that may affect**
500 **neuronal responses to mechanical stimuli.**

501 **a)** Experimental procedure for the drug screen performed. Synchronized L1 worms are grown in
502 NGM plates to the L4 stage and then deposited in a 48-well plate. Drug treated worms are
503 cultured with 0.5 ml OP50 *E. coli* bacteria (OD 5) and 100 μ M drugs. Control worms are cultured
504 with 0.5 ml OP50 *E. coli* bacteria (OD 5). Both groups of worms are incubated at 20°C for at
505 least 24h. Subsequently, AVM responses to 1s stimulus were measured on-chip.

506 **b)** Three metrics measured from individual calcium dynamic traces: maximum response, delay
507 time (time between the end of the stimulus and the arrival of maximum response), and half-life
508 (time it takes the response to decay to half of the maximum).

509 **c)** Fraction of animal responses upon compound treatment. Several compounds produced a
510 lowered fraction of responding animals, while a few slightly increased the response fraction.

511 **d - f)** Box plots show how compounds affect specific parameters of neuronal response upon
512 mechanical stimulation. Quantification of each response was normalized to that of the control
513 group from the same day (day 1 adult to day 3 adult). **d)** D-Lysine (#4), D-Alanine (#5), and D-
514 Arginine (#8) were shown to reduce maximum response, **e)** D-Alanine (#5) and D-Arginine (#8)
515 were shown to increase the time to peak, and **f)** D-Isoluecine (#3) was shown to decrease the
516 decay half-life. In contrast, β -Alanine (#6) was shown to increase the half-life. (Kruskal-Wallis
517 Test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

518 **g - h)** Average traces of GCaMP6 in AVM neuron for drug treated worms that cause significant
519 differences from untreated worms in responses to 1s mechanical stimulus. **g)** Day 1 adult worms
520 (Control Day 1: $n=53$, D-Isoleucine: $n=10$, D-Lysine: $n=10$), **h)** Day 2 adult worms (Control Day
521 2: $n=53$, D-Alanine: $n=15$, β -Alanine: $n=14$, D-Arginine: $n=13$). Error bar represent SEM.

522

523 **Figure 4: Sensitization of the PVC interneuron responses.**

524 **a)** Simplified circuit diagram showing three sensory neurons connecting PVC to forward
525 locomotion behavior (values and arrow thickness indicate number of synapses).

526 **b - c)** Responses of PVC interneuron to a single pulse of stimulation. Averages are plotted on the
527 top graph. Error bars represent SEM. Bottom graphs represent individual traces. **c)** PVC calcium
528 responses to 30s 0.1% SDS stimuli on tail (n=23). **d)** PVC calcium responses to 1s weak
529 mechanical stimuli at 20 psi on posterior region (n=18). In individual traces for outliers, if the
530 value of calcium transient is greater than 4 or less than 0, it would be equal to 4 or 0, respectively
531 (bottom).

532 **d - e)** Sensitized PVC interneuron responses. **e)** Applying 5s 0.1% SDS stimuli enhances the
533 responses of PVC interneuron to 1s weak mechanical stimuli at 20 psi (n=31). Averaged calcium
534 responses (top) and individual traces (bottom) **f)** Applying 1s weak mechanical stimuli at 20 psi
535 enhances the responses of PVC interneuron to 30s 0.1% SDS stimuli (n=24). Averaged calcium
536 responses (top) and individual traces (bottom). Error bars represent SEM (top). In individual
537 traces for outliers, if the value of calcium transient is greater than 4 or less than 0, it would be
538 equal to 4 or 0, respectively (bottom).

539 **f)** Quantified maximum responses of calcium transients to either chemical (left column) or
540 mechanical stimuli (right column). Data points in control groups represent maximum responses
541 to either single chemical or mechanical stimuli. Sensitization of PVC interneuron responses is
542 produced by applying prior weak mechanical stimuli at 20 psi or 5s 0.1% SDS chemical stimuli
543 (Mann-Whitney Test, * p<0.05, ** p<0.01).

544

545

546

547 **Supplementary Figure 1:** Overview of microfluidic device design and dimensions. The device
548 is composed of the channel for worms (50-60 μm deep and wide, which allow the animals to fit
549 loosely inside), two sets of actuated membrane, and two sets of trapping valve. The width of the
550 both of actuated PDMS membrane and trapping valve is 150 μm , the distance between first and
551 second sets of membrane is 200 μm and second and third sets of membrane is 250 μm .

552

553 **Supplementary Figure 2:** Displacement of the actuated membrane by applying pressure (n=4
554 worms). It is important to note that the measurements were taken by images from transgenic
555 worms expressing GFP along body-wall muscle (*stEx30[myo-3p::GFP + rol-6(su1006)]*). R-
556 square value is 0.9814.

557

558 **Supplementary Figure 3:** Neuron Tracking Algorithm. In order to extract fluorescence
559 intensities throughout recordings, a neuron tracking algorithm was developed. This was
560 necessary because worms are not fully immobilized in the device, and mechanical stimuli often
561 caused the neuron of interest to move within the field-of-view. **a)** Overall schematic of the
562 neuron tracking algorithm. For each frame i , raw images are processed through a blob filter
563 (Laplace of Gaussian filter) to improve contrast and facilitate segmentation. Blob filtered images
564 are segmented by applying an empirically determined threshold. The neuron of interest is
565 identified by the user in the first frame, and by distance to the neuron in the previous frame.
566 Lastly, once the neuron is detected for each frame, intensity values are extracted (Green ROI,
567 Red ROI, Green Background, and Red Background). **b)** Example of algorithm procedure.

568

569 **Supplementary Figure 4:** **a)** Average traces of GCaMP6 signal in AVM neuron in response to
570 diverse pressures and stimulus durations (**i-iii**: 35 psi, **iv-vi**: 40 psi, **vii-ix**: 45 psi / **i, iv, vii**: 1s
571 stimulus, **ii, v, viii**: 2s stimulus, **iii, vi, ix**: 5s stimulus, sample size **i**: n=25, **ii**: n=10, **iii**: n=8, **iv**:
572 n=8, **v**: n=10, **vi**: n=10, **vii**: n=27, **viii**: n=6, **ix**: n=10). Error bars represent SEM. **b)** Average
573 traces of GCaMP6 signal in PVD neuron in response to diverse pressures and stimulus durations
574 (**i-iii**: 45 psi, **iv-vi**: 50 psi, **vii-ix**: 55 psi / **i, iv, vii**: 1s stimulus, **ii, v, viii**: 2s stimulus, **iii, vi, ix**: 5s

575 stimulus, sample size **i**: n=9, **ii**: n=4, **iii**: n=6, **iv**: n=6, **v**: n=9, **vi**: n=10, **vii**: n=9, **viii**: n=10, **ix**:
576 n=10). Error bar represent SEM.

577

578 **Supplementary Figure 5:** AVM cell body responses to various stimuli with low pressures and
579 durations **a**) 30 psi and 1 s (n=4), **b**) 30 psi and 0.2 s (n=10), **c**) 15 psi and 0.2 s (n=5). AVM
580 response is reduced when using lower pressures (comparing a to Fig. 2a, and a to c). Response is
581 also attenuated when using shorter durations (comparing a to b). Error bars represent SEM.

582

583 **Supplementary Figure 6:** PLM cell body responses to various stimulus durations (1s: n=9, 2s:
584 n=4, 5s: n=4). Similar to those of AVM, maximum responses in PLM were proportional to the
585 stimulus duration.

586

587 **Supplementary Figure 7:** AVM cell body response to delivery of repeated stimuli with long
588 durations (5s, n=5). Similar to Figure 2l, traces showed incremental increases in the first few
589 stimuli, and showed a decreased response in later stimuli.

590

591 **Supplementary Figure 8:** Individual (gray) and average traces (blue) for AVM response in
592 untreated animals for different control groups for drug screen. **a**) Day 1 (n=53), **b**) Day 2 (n=53),
593 **c**) Day 3 (n=35) adult worms.

594

595 **Supplementary Figure 9:** Average traces for AVM response in drug-treated animals that do not
596 show a significant difference from the control groups. **a**) Day 1 adult worms (Control Day 1:
597 n=53, D-Glutamic acid: n=10, D-Serine: n=12), **b**) Day 2 adult worms (Control Day 2: n=53, γ -
598 D-Glutamylglycine: n=10, D-Asparagine: n=4), **c**) Day 3 adult worms (Control Day 3: n=35, 1,1'-
599 Ethylidene-bis(L-tryptophan): n=10, D-Cysteine: n=10, D-Glutamine: n=11, D-Histidine: n=13).

600

601 **Supplementary Figure 10:** Overview of microfluidic device for the delivery of multimodal
602 stimuli. The device is composed of a channel for worms (Inlet and Flush channel), two sets of

603 actuated membrane for mechanical stimuli (Touching valve 1,2), one set of trapping valve
604 (Loading valve), two inlets for chemical stimuli (Buffer and Stimulus), and outlet.

605

606 **Supplementary Figure 11:** Average traces of GCaMP3 signal in ASH neuron in response to 30s
607 0.1% SDS stimuli (n=13). Stimuli were delivered 30 s after recording baseline activity of
608 neurons. Error bars represent SEM.

609

610 **Supplementary Figure 12: a)** Neural wiring diagram showing five sensory neurons in a circuit
611 linking PVC to forward behavior, and the number of direct synapses between each pair of
612 neurons (arrow thickness indicates number of synapses). **b-c)** The activity of PVC responses to
613 localized strong mechanical stimuli: **b)** 1s anterior stimuli (n=5) and **c)** 1s posterior stimuli
614 (n=18) at 45psi. **d)** Applying prior 5s 0.1% SDS stimuli enhances the responses of PVC
615 interneuron to next 30s 0.1% SDS stimuli (n=13). **e)** The activity of PVC responses to buffer to
616 buffer changes (n=10). Error bars represents SEM.

617

618

619 **Supplementary Table 1:** The 13 compounds of the orphan library were used for the drug screen
620 (Fig. 3). Sample size is the total number of tested worms and if the value of maximum responses
621 is larger than 0.5, it is counted as a responding worm.

622

623

624 **Supplementary Movie 1:** Calcium dynamics of AVM neuron to 1s anterior stimulation at 45psi.
625 Stimulus was delivered 10s after recording baseline of neuronal activity. The transgenic animal
626 shown here expresses GCaMP6 and RFP in AVM neuron (*ljIs142[mec-*
627 *4::GCaMP6m::SL2TagRFP, unc-119] II; unc-119(ed3) III*). Left panel shows green
628 fluorescence from GCaMP6m (left) and red fluorescence from RFP (right) in false colors. White
629 boxes indicate location of AVM neuron and shows how algorithm tracks the neuron. Right graph
630 shows the quantitative calcium trace and red circle indicates the current time point of video.
631 Stimulus occurs at 10s (red dash line). 1x playback.

632

633 **Supplementary Movie 2:** Calcium dynamics of PLM neuron to 1s posterior stimulation at
634 45psi. Stimulus was delivered 10s after recording baseline of neuronal activity. The transgenic
635 animal shown here expresses GCaMP6 and RFP in PLM neuron (*ljIs142[mec-*
636 *4::GCaMP6m::SL2TagRFP, unc-119] II; unc-119(ed3) III*). Left panel shows green
637 fluorescence from GCaMP6m (left) and red fluorescence from RFP (right) in false colors. White
638 boxes indicate location of PLM neuron and shows how algorithm tracks the neuron. Right graph
639 shows the quantitative calcium trace and red circle indicates the current time point of video.
640 Stimulus occurs at 10s (red dash line). 1x playback.

641

642 **Supplementary Movie 3:** Calcium dynamics of PVM neuron to 1s posterior stimulation at
643 45psi. Stimulus was delivered 10s after recording baseline of neuronal activity. The transgenic
644 animal shown here expresses GCaMP6 and RFP in PVM neuron (*ljIs142[mec-*
645 *4::GCaMP6m::SL2TagRFP, unc-119] II; unc-119(ed3) III*). Left panel shows green
646 fluorescence from GCaMP6m (left) and red fluorescence from RFP (right) in false colors. White
647 boxes indicate location of PVM neuron and shows how algorithm tracks the neuron. Right graph
648 shows the quantitative calcium trace and red circle indicates the current time point of video.
649 Stimulus occurs at 10s (red dash line). 1x playback.

650

651 **Supplementary Movie 4:** Calcium dynamics of PVD neuron to 1s posterior stimulation at 45psi.
652 Stimulus was delivered 10s after recording baseline of neuronal activity. The transgenic animal
653 shown here expresses GCaMP6 in PVD neuron (*wyls5007[ser2prom3::GCaMP6, egl-*

654 *17::mCherry] X*). Left panel shows green fluorescence from GCaMP6 in false color. A white
655 box indicates location of PVD neuron and shows how algorithm tracks the neuron. Right graph
656 shows the quantitative calcium trace and red circle indicates the current time point of video.
657 Stimulus occurs at 10s (red dash line). 5x playback.

658

659 **Supplementary Movie 5:** Calcium dynamics of PVC interneuron. Applying 5s 0.1% SDS
660 chemical stimulus at 30s (red dash lines on the right panel) and then 1s weak mechanical stimuli
661 at 65s (blue dash lines on the right panel). The transgenic animal shown here expresses GCaMP6
662 in PVC interneuron (*aEx2[pglr-1::GCaMP6(s), punc-122::GFP]*). Left panel shows green
663 fluorescence from GCaMP6 in false color. A white box indicates location of PVC interneuron
664 and shows how algorithm tracks the neuron. Right graph shows the quantitative calcium trace
665 and red circle indicates the current time point of video. Stimulus occurs at 10s (red dash line). 5x
666 playback.

667

668 **Supplementary Movie 6:** Calcium dynamics of PVC interneuron. Applying 1s weak mechanical
669 stimulus at 30s (blue dash lines on the right panel) and then 30s 0.1% SDS chemical stimulus at
670 60s (red dash lines on the right panel). The transgenic animal shown here expresses GCaMP6 in
671 PVC interneuron (*aEx2[pglr-1::GCaMP6(s), punc-122::GFP]*). Left panel shows green
672 fluorescence from GCaMP6 in false color. A white box indicates location of PVC interneuron
673 and shows how algorithm tracks the neuron. Right graph shows the quantitative calcium trace
674 and red circle indicates the current time point of video. Stimulus occurs at 10s (red dash line). 5x
675 playback.

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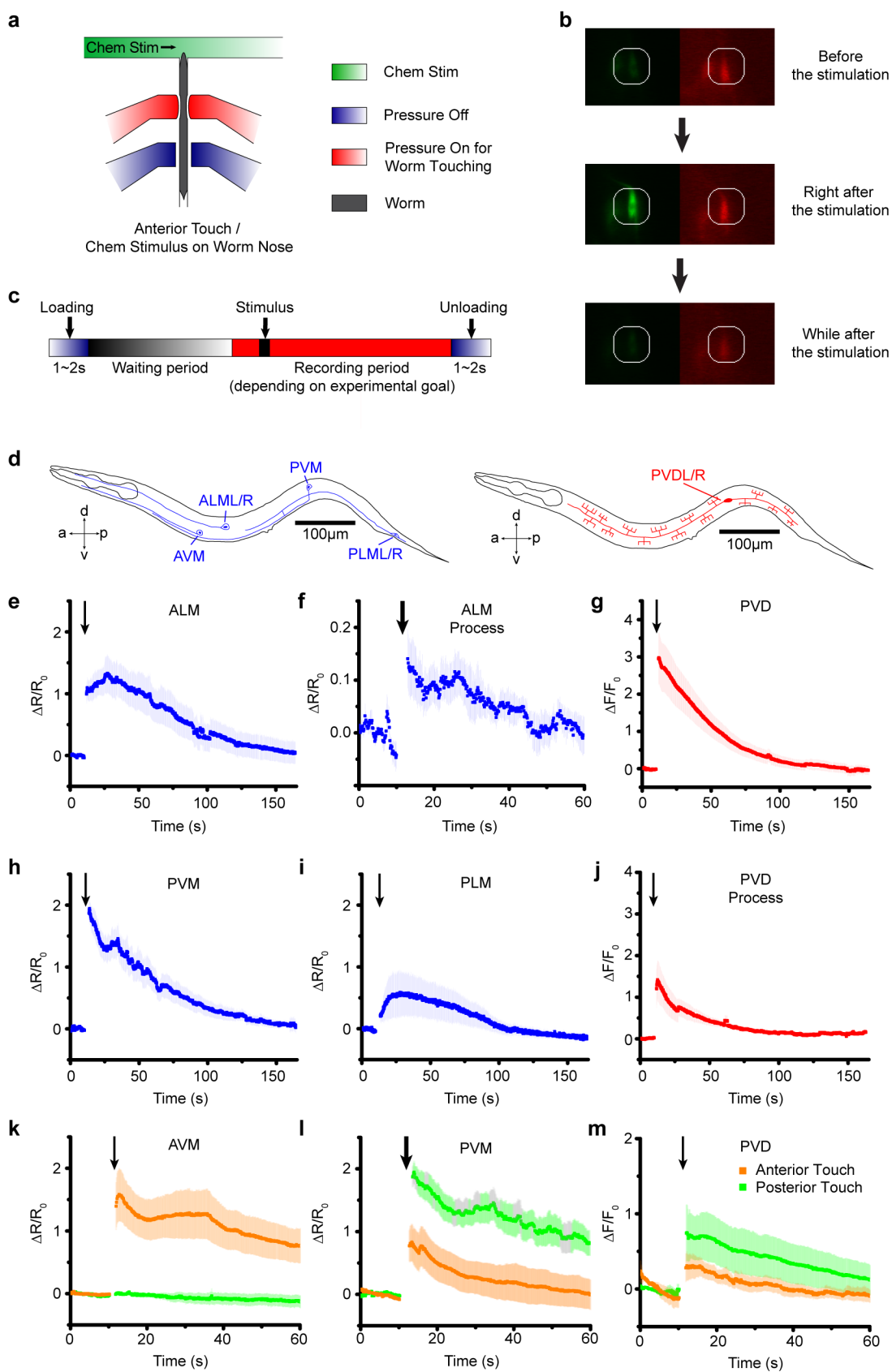
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678 **Methods References**

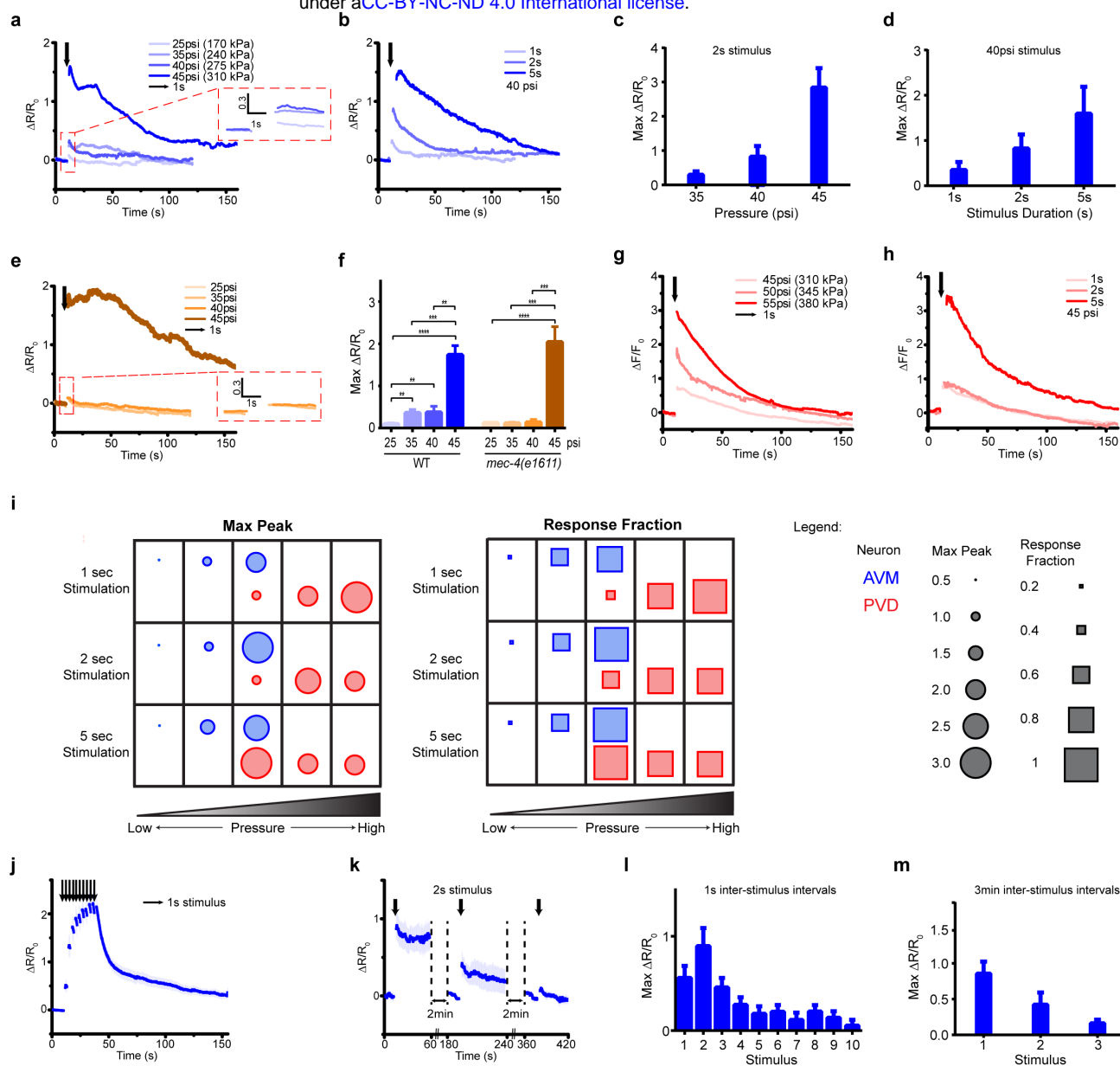
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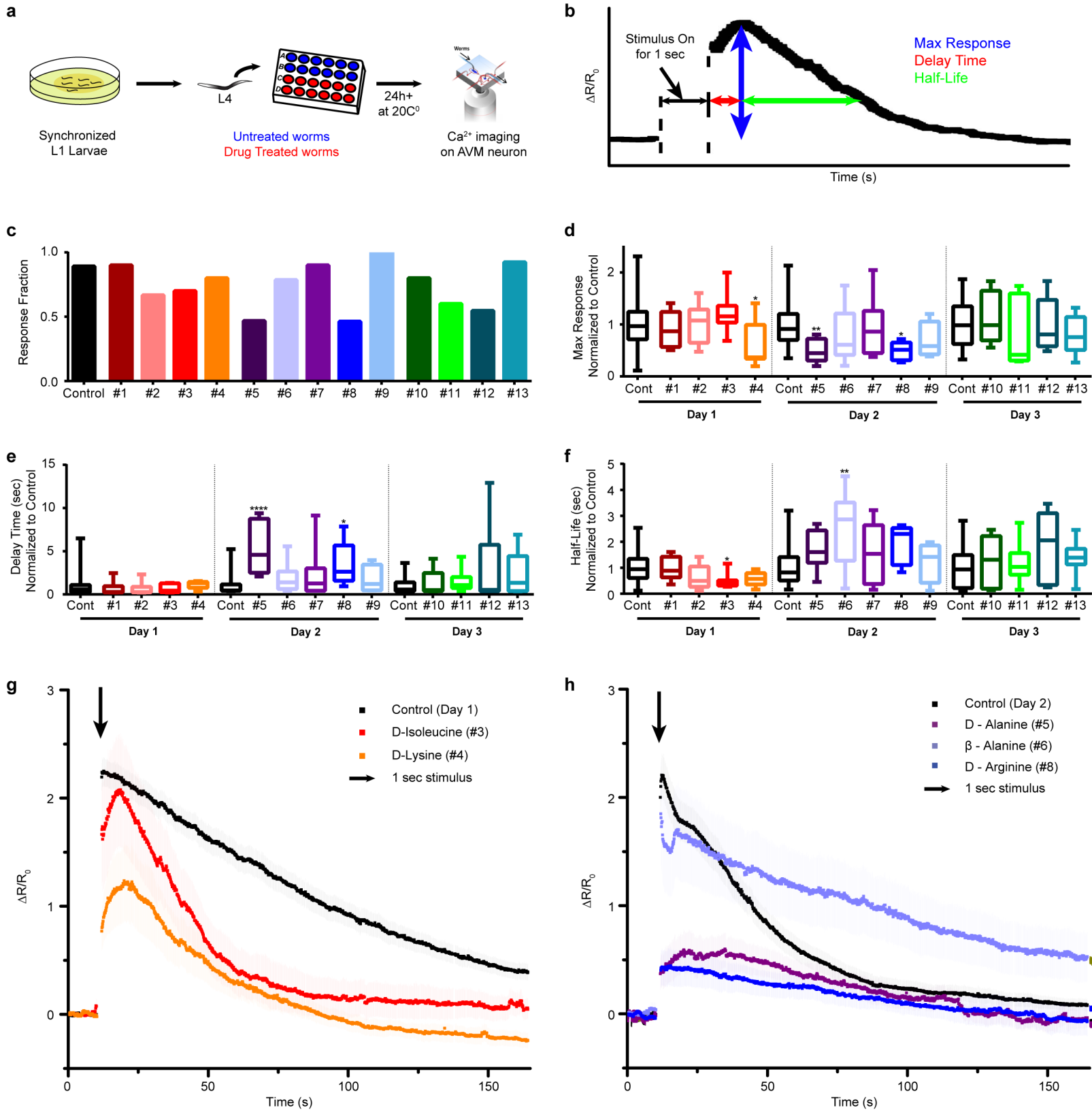
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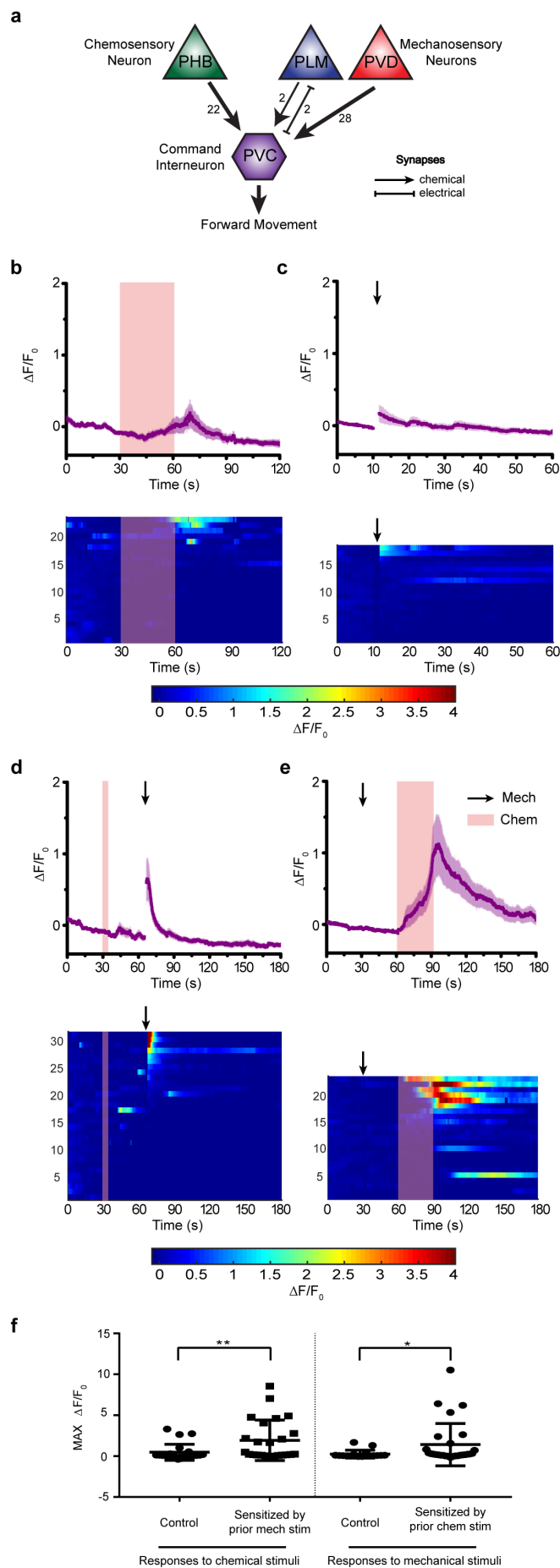


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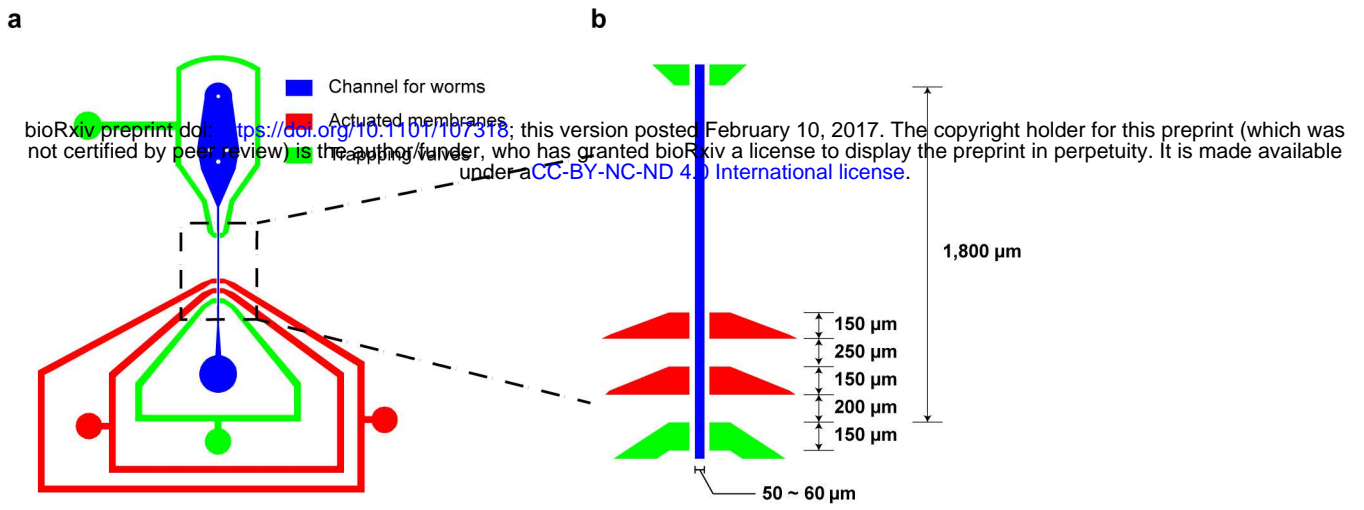


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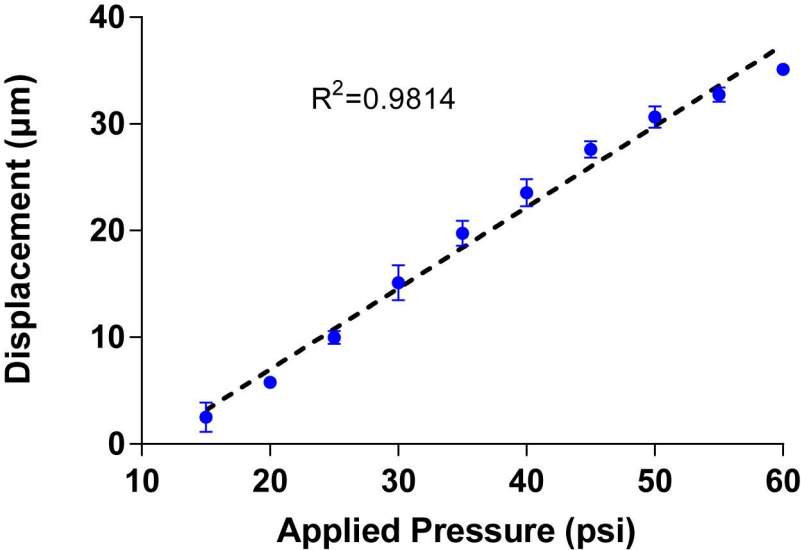


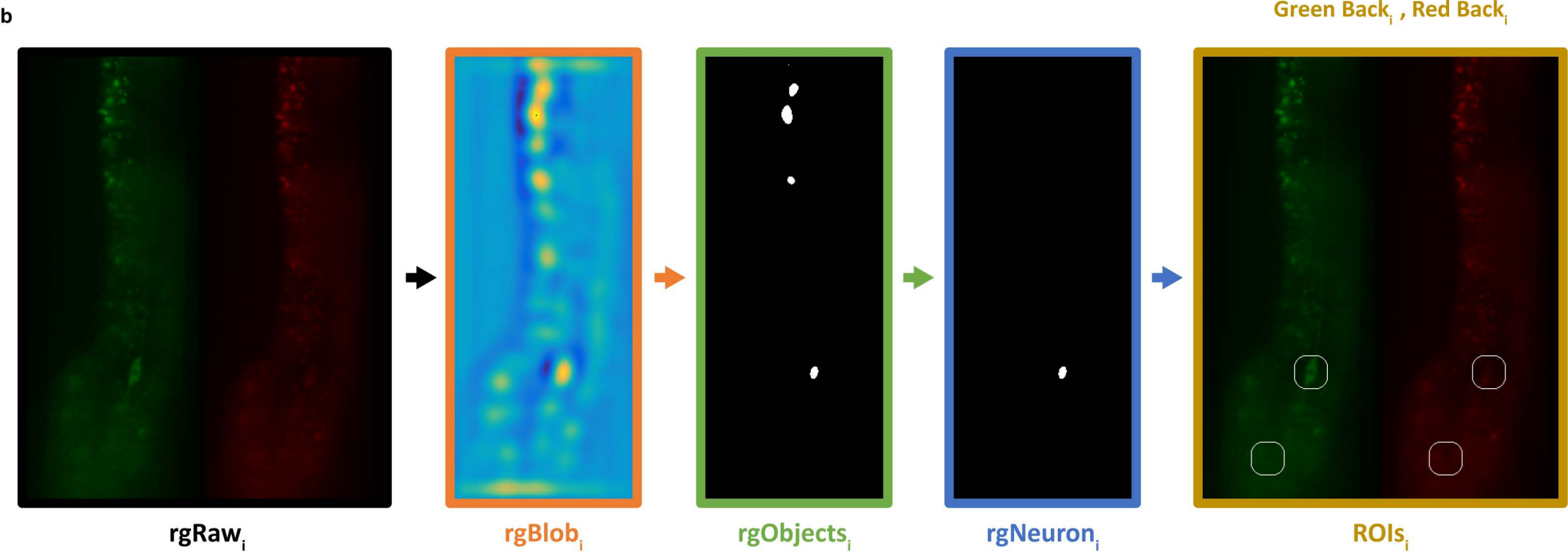
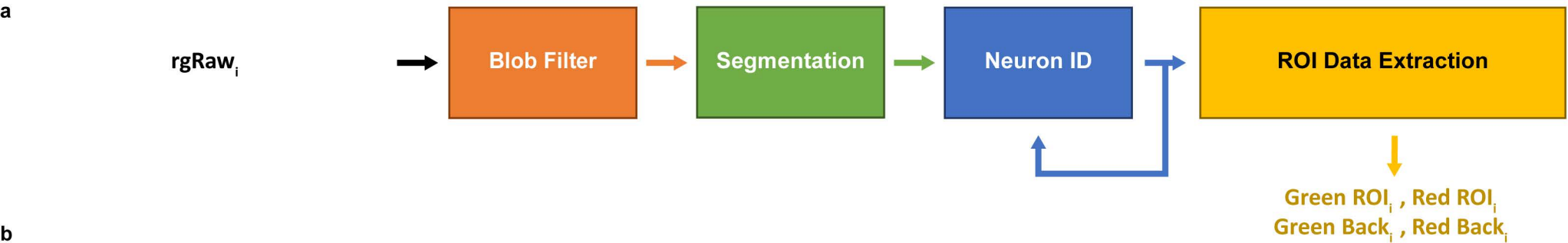


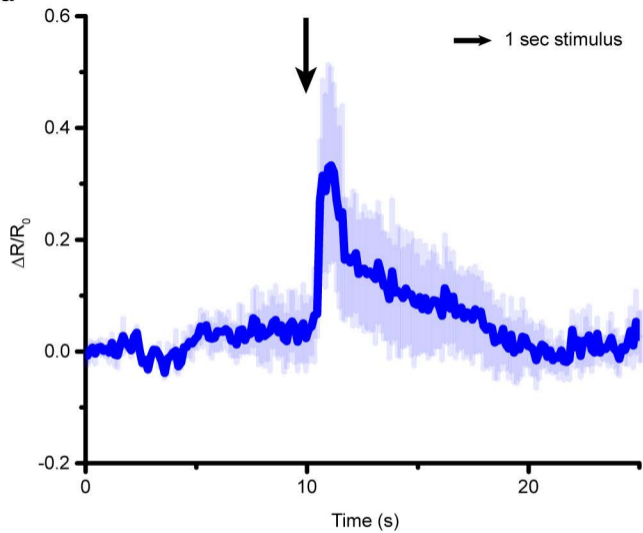
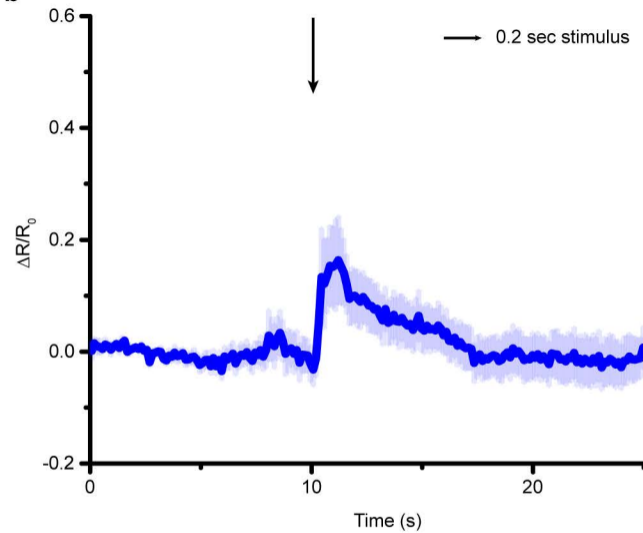
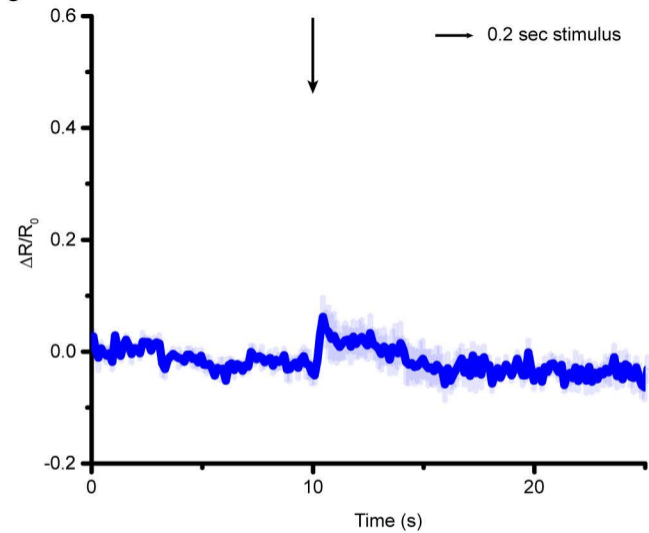
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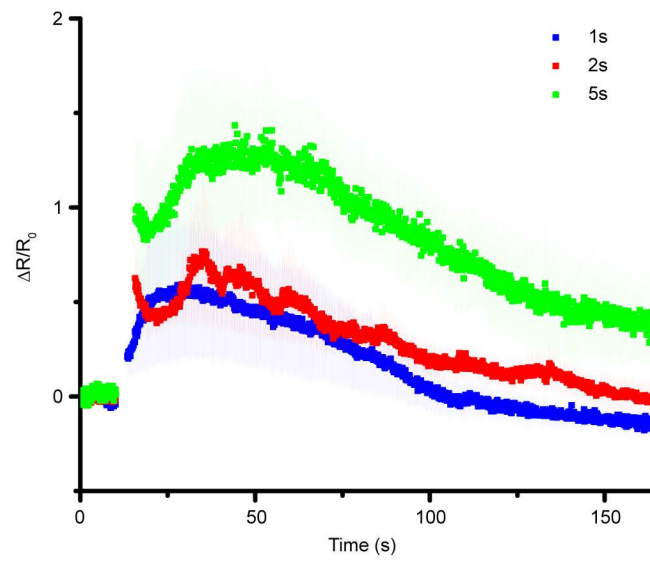


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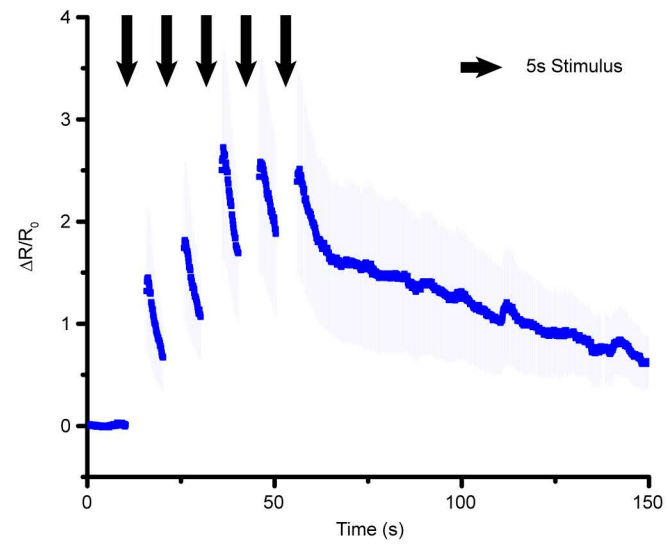




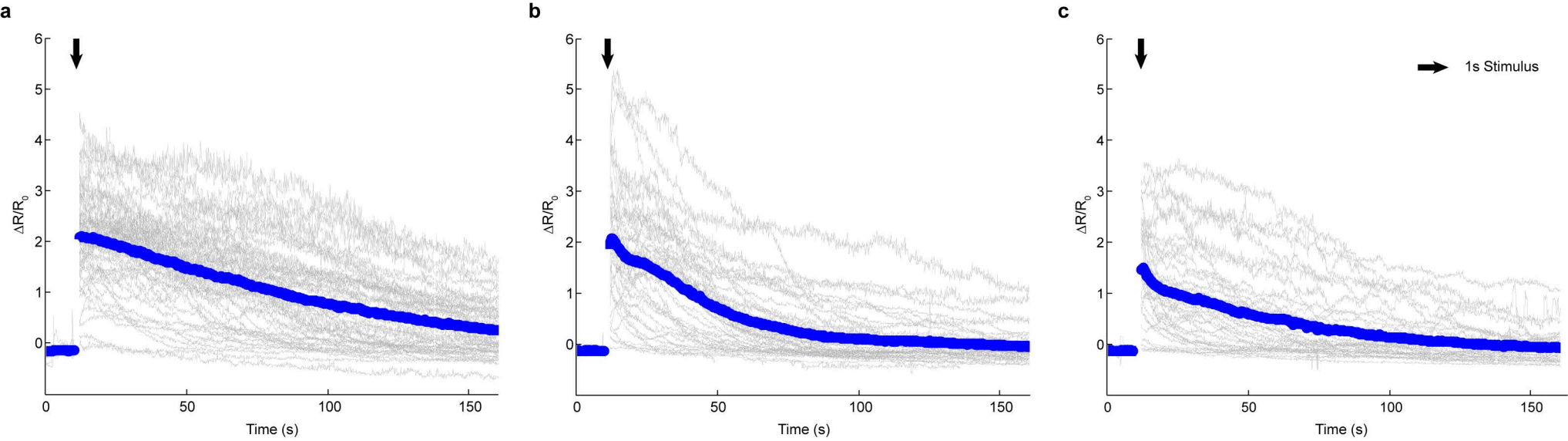
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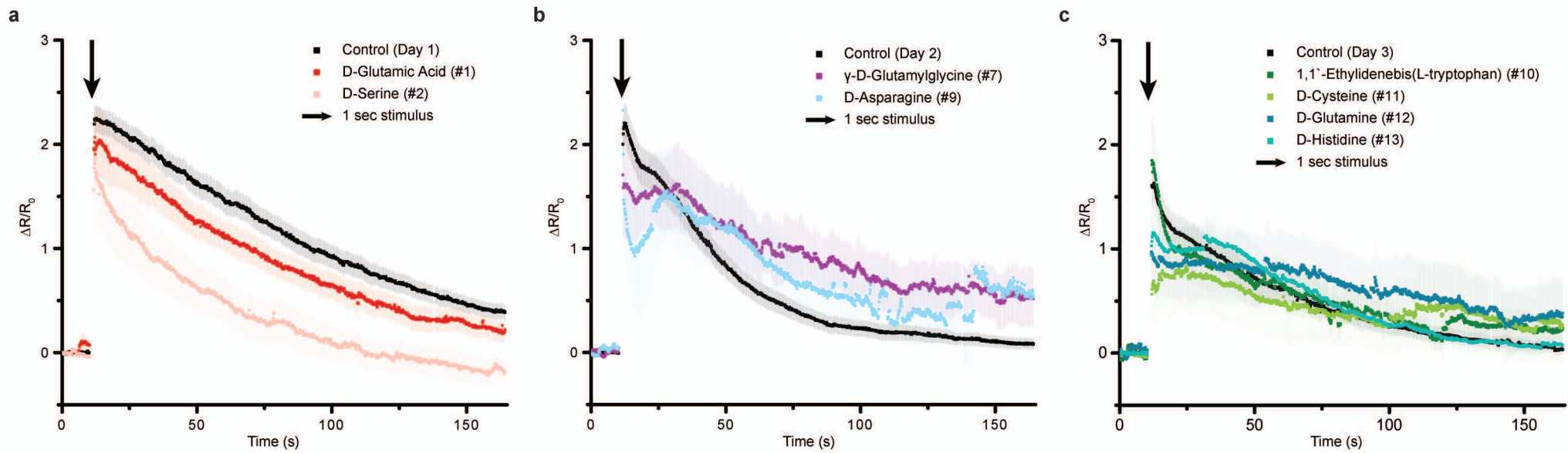


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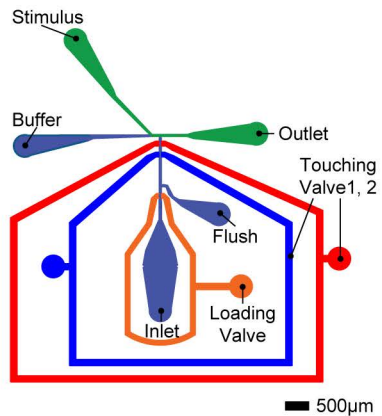


Cho et al., Supplementary Figure 7.

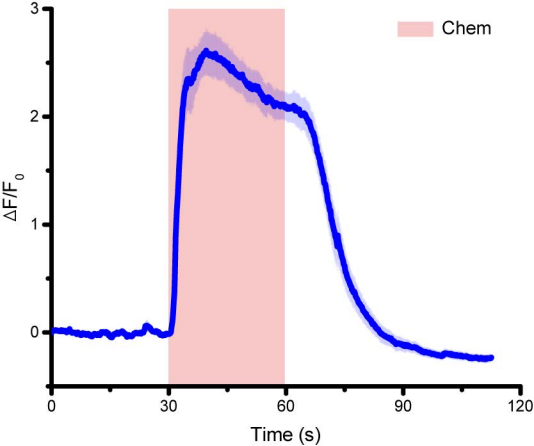




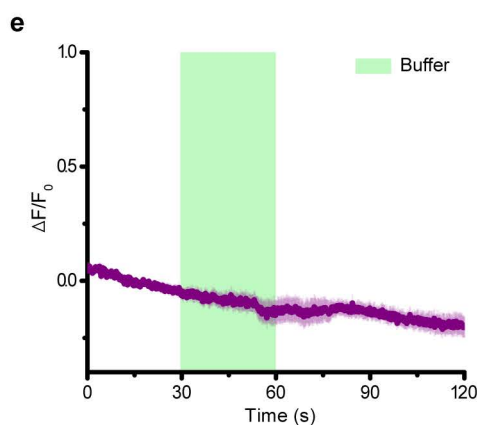
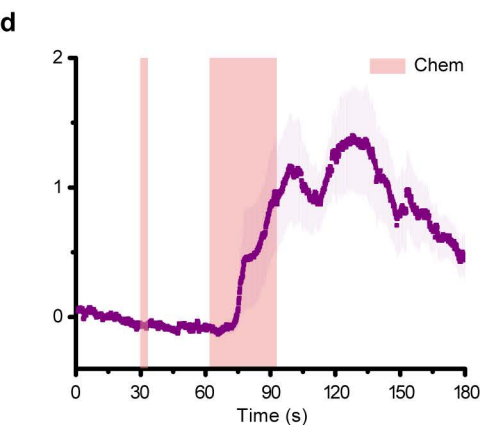
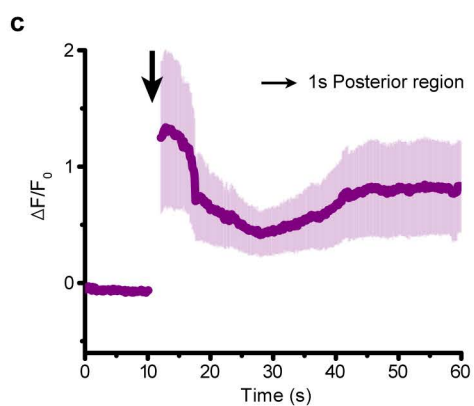
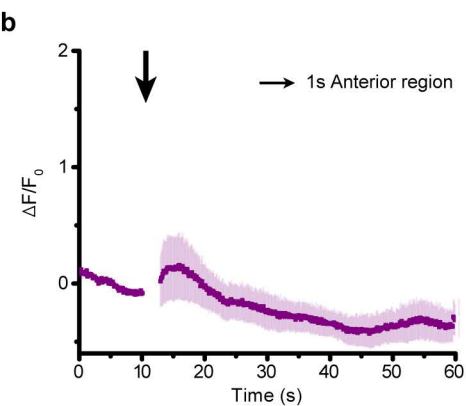
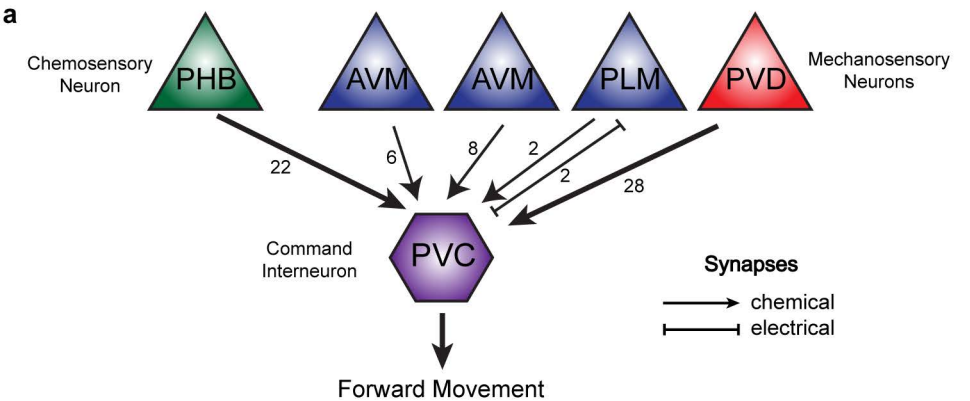
Cho et al., Supplementary Figure 9.



Cho et al., Supplementary Figure 10.



Cho et al., Supplementary Figure 11.



Number	Name	Rationale	Sample size	Number of responding worms
1	D-Glutamic acid	Putative endogenous ligand	10	9
2	D-Serine	Putative endogenous ligand	12	8
3	D-Isoleucine	D-Amino acid	10	7
4	D-Lysine	D-Amino acid	10	8
5	D-Alanine	D-Amino acid	15	7
6	β -Alanine	Endogenous	14	11
7	γ -D-Glutamylglycine	D-Amino acid	10	9
8	D-Arginine	D-Amino acid	13	6
9	D-Asparagine	D-Amino acid	4	4
10	1,1'-Ethylidene-bis(L-tryptophan)	Bioactive tryptophan derivative	10	8
11	D-Cysteine	D-Amino acid	10	6
12	D-Glutamine	D-Amino acid	11	6
13	D-Histidine	D-Amino acid	13	12

Cho et al., Supplementary Table 1.