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TITLE:

An Improved Assay and Tools for Measuring Mechanical Nociception in *Drosophila* Larvae

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KEYWORDS:

neuroscience, *Drosophila,* larvae, mechanical probes, von Frey filaments, mechanical nociception, mechanical sensitization, mechanical allodynia, mechanical hyperalgesia, behavioral assay

SUMMARY:

The goal of this protocol is to show how to perform an improved assay for mechanical nociception in *Drosophila* larvae. We use the assay here to demonstrate that mechanical hypersensitivity (allodynia and hyperalgesia) exists in *Drosophila* larvae.

ABSTRACT:

Published assays for mechanical nociception in *Drosophila* have led to variable assessments of behavior. Here, we fabricated, for use with *Drosophila* larvae, customized metal nickel-titanium alloy (nitinol) filaments. These mechanical probes are similar to the von Frey filaments used in vertebrates to measure mechanical nociception. Here, we demonstrate how to make and calibrate these mechanical probes and how to generate a full behavioral dose-response from subthreshold (innocuous or non-noxious range) to suprathreshold (low to high noxious range) stimuli. To demonstrate the utility of the probes, we investigated tissue damage-induced hypersensitivity in *Drosophila* larvae. Mechanical allodynia (hypersensitivity to a normally innocuous mechanical stimulus) and hyperalgesia (exaggerated responsiveness to a noxious mechanical stimulus) have not yet been established in *Drosophila* larvae. Using mechanical probes that are normally innocuous or probes that typically elicit an aversive behavior, we found that *Drosophila* larvae develop mechanical hypersensitization (both allodynia and hyperalgesia) after tissue damage. Thus, the mechanical probes and assay that we illustrate here will likely be

important tools to dissect the fundamental molecular/genetic mechanisms of mechanical hypersensitivity.

INTRODUCTION:

Drosophila larvae exhibit a characteristic aversive rolling behavior when exposed to different noxious stimuli: thermal[1], mechanical[2], and chemical[3]. This behavior is clearly distinct from normal locomotion. Here we describe an improved mechanical assay that can be used to assess mechanical nociception and mechanical sensitization.

In a recent study, we fabricated von Frey-like filaments using nitinol wires[4]. Probes exerting different forces and pressures were made by varying the lengths and diameters of the nitinol wires forming each probe. Mechanical probes were calibrated and the measured force values (in millinewton, mN) were converted to pressure (kilopascal, kPa), based on the tip area of each probe[4]. Custom fabrication of mechanical probes allowed to us to generate subthreshold (≤200 kPa) to suprathreshold (225 kPa to 5318 kPa) pressures, which could, in principle, be beneficial for studying mechanical hypersensitivity. Using these improved mechanical von Frey-like filaments, we showed that pressure[4], as opposed to the previously examined force[2,5,6] correlates more consistently with aversive behavioral responsiveness in *Drosophila* larvae. The improved mechanical assay described here also helped to identify a conserved Vascular Endothelial Growth Factor (VEGF)-related receptor tyrosine kinase signaling a pathway that regulates mechanical nociception in flies and rats[4].

Mechanical allodynia and hyperalgesia, two modalities of hypersensitivity, are relatively understudied in *Drosophila* larvae, compared to the thermal (heat and cold) and chemical sensory modalities[3,7–10]. This is probably due to the lack of specific mechanical probes that span from innocuous stimuli to the high noxious range[2,5,6]. A normally innocuous stimulus that elicits the typical aversive rolling behavior after *Drosophila* larvae experience tissue damage[3,7] is referred to as allodynia. An exaggerated rolling response to a typically noxious stimulus is known as hyperalgesia[7]. Noxious stimuli are defined as those that elicit tissue damage and can activate nociceptors[11]. Noxious stimuli delivered to *Drosophila* larvae damage either the barrier epidermis, the peripheral nociceptive sensory neurons[3,4,7], or both.

In this article, we demonstrate how to custom fabricate and calibrate von Frey-like mechanical probes that are appropriate for *Drosophila* larvae. Further, we show how to use these probes to assay mechanical nociceptive responses in *Drosophila* larvae. Finally, we further demonstrate the utility of these probes by using them to demonstrate the presence of mechanical hypersensitivity, both allodynia and hyperalgesia, following tissue damage in *Drosophila* larvae (see **Representative Results**).

PROTOCOL:

1. Mechanical probe construction

1.1. Cut each nitinol filament (**Figure 1B**), perpendicular to its long axis, to the specified length (**Figure 1M–N**) using a small wire cutter (**Figure 1C**). The filaments come in three different pre-set diameters (**Figure 1B**).

NOTE: The lengths specified here are a guide to achieve the approximate pressures indicated, using a similar protocol for construction of the mount. Ultimately, regardless of the length of the filament cut, and the depth of the hole in the mount, the filaments must be measured/calibrated on a balance to obtain the exact force/pressure value.

1.2. Examine the tip of the filament under a stereomicroscope to ensure no sharp or irregular edges remain as these could cause tissue damage to the skin of the larvae and interfere with calibration.

1.3. Manually smoothen the sharp edges of the mechanical probe using a sharpening stone until no sharp irregularities persist (**Figure 1D**).

1.4. Make a hole towards the end of a wooden popsicle stick (**Figure 1E**) using a hypodermic needle (see **Table of Materials**). Insert the needle at least halfway through the height of the popsicle stick (**Figure 1E**). This creates a chamber for insertion of the nitinol filament.

1.5. Apply wood glue to a single nitinol filament (**Figure 1F**) and insert the glue-coated filament into the needle slot in a wooden popsicle stick (**Figure 1G**). Allow to dry for ~5 h.

1.6. Calibrate each mechanical probe by pressing it against a scale until the mechanical probe bends (**Figure 1H–L**). This is the point of maximum force that can be recorded in grams. Depending on the filament diameters (pre-set) and lengths (user-determined) a full range of forces and pressures can be generated.

1.7. Convert the mass recorded in step 1.6 to force in millinewton (mN) using the formula f = ma (Force is equal to mass multiplied by gravitational acceleration). f: force; m: mass; a: gravitational acceleration (9.8 m/s^2) (**Figure 1M**).

1.8. Finally, convert the calculated force to pressure (force/area) in kilopascal (kPa) by dividing the measured force by the surface area of the filament tip (**Figure 1M**). To calculate the area, convert the diameter of the different nitinol filaments from inches (0.04”, 0.06”, and 0.08”) to centimeters. Then, πr^2 (where, r = the nitinol filament radius) determines the area (see **Figure 1M**). Preparing multiple probes using filaments of different diameters and lengths will generate a full set spanning the responsive range for *Drosophila* larvae (sample set shown in **Figure 1N**).

NOTE: Check each mechanical probe at least every 3–4 weeks. When the pressure deviates by more than $\pm$ 3% from the original measure, a new mechanical probe must be fabricated.

2. Preparation of larvae

2.1. Raise control strain (*w*^{1118}) larval progeny or larvae containing the transgenes *ppk-Gal4>UAS-mCD8-GFP* (for visualizing damage to sensory neurons) on standard food in a 25 °C incubator. Typically, stocks are routinely maintained at 18 °C but both parents and larval offspring are reared at 25 °C on standard cornmeal food for experiments.

NOTE: Adult flies (five males and ten females, 1:2 ratio) are kept in the fly vials, to allow egg laying, for about 24 h. The time after egg laying (AEL) begins from when the adults are removed.

2.2. Collect the third instar larvae, after approximately 96 h of egg laying, by gently squirting tap water into the soft fly food containing the larvae. Wandering larvae that have left the food, or which have everted anterior or posterior spiracles, are too large/old for this assay. Second instar larvae (~ less than 4 mm in length) are too small.

2.3. Pour out the contents of the soft fly food into a clean standard size Petri dish (100 mm x 15 mm).

2.4. Using forceps, sort mid third instar, medium sized, larvae (see **Figure 2A**) from smaller (second instar and early third instar) or larger (late or wandering third instar) larvae. Gentle manipulation with forceps to avoid any tissue damage to the larvae is recommended.

NOTE: The transfer using forceps is based mostly on water tension and not by applying pressure to the larvae with the blades of the forceps. An alternative to the use of forceps for maneuvering larvae is soft paint brushes. With either tool, the user should practice transferring the animals, so as not to cause unintended tissue damage that could complicate behavioral measurements.

2.5. Transfer the mid third instar larvae, using forceps, into a small Petri dish (30 mm x 15 mm) containing a small plug of fly food moistened with water at room temperature. Keep the larvae in the small Petri dish until the experiments are performed, but not longer than 20 min.

NOTE: Generally, transferring 20–30 larvae to the food plug will give an adequate number for 20 min of behavioral assays.

3. Mechanical nociception assay

3.1. Place a mid-third instar larva (using forceps) onto a thin pad of black or dark vinyl under a bright field stereomicroscope. The dark color provides contrast that improves visualization of the larva. It is preferable to have a freely movable piece of dark vinyl because it allows the user to align the larva without touching or hurting it.

3.2. Put the optical fiber lights between the microscope objective lenses and the black or dark vinyl pad; this will allow adequate high contrast illumination for seeing the larva.

3.3. Discard larvae that do not exhibit normal locomotion following transfer to the pad. These can interfere with the normal nociceptive behavioral response. For normal locomotion, see **Video 1**.

3.4. Wipe away, using a paper towel, any excess water surrounding the larva that might cause the larva to float on the vinyl pad.

3.5. Orient the larva by moving the dark vinyl pad. The head/mouth of the larva should point to the left if you are right-handed and vice versa if you are left-handed (**Figure 2A–B**).

3.6. Apply the chosen mechanical probe, typically for 1–2 s, onto the posterior dorsal side of the larva at approximately abdominal segment A8 (see **Figure 2B**), until the probe bends and elicits the previously measured amount of pressure (**Figure 2C**). It is important that the probe presses against the dorsal surface of the larva and compresses the larvae into the underlying pad at the point of probe contact.

NOTE: At the point of contact between the tip of the nitinol filament and the dorsal cuticle-epidermis, probes lower than 2,300 kPa, mainly bend without penetrating the cuticle and underlying tissues. Such probes seldom affect larval mortality[4]. At higher pressures (>5,000 kPa) the probes both bend and, occasionally, penetrate the cuticle and underlying tissues. Puncturing of the larvae impairs larval survival[4] and, if observed, these larvae are typically discarded from behavioral analysis.

3.7. Record the behavioral response for each larva. A positive nociceptive response (**Video 2**) is indicated if the larva shows a complete roll of 360˚ along the axis of its body within 3 s. Other responses (attempting to turn, fast crawling, and wiggling) are considered negative for the purposes of this assay.

NOTE: Larvae stimulated with a subthreshold mechanical stimulus (200 kPa) did not elicit the typical nociceptive or rolling response (**Video 3**). Some larvae did exhibit fast forward or light touch responses such as changes in the direction of movement.

3.8. Discard the larva and prepare the next one for assay, repeating steps 3.1 through 3.7.

3.9. Repeat steps 3.1–3.7 until the desired number of larvae is reached (three to six sets of n = 10 larvae were used here for each probe).

NOTE: When using lower pressure mechanical probes (174–462 kPa), the assay will take more time per larva. This is because the tip of longer filaments oscillates more, making it harder to poke the larva in the center of the A8 segment. Practice is necessary with these probes.

4. Confocal microscopy to assess neuronal morphology

4.1. Place a larva (of genotype *ppk-Gal4>UAS-mCD8-GFP* to label sensory neurons) previously stimulated with a nitinol filament into an etherization chamber inside a Coplin jar containing a 10 mL beaker carrying a cotton ball soaked with ~1 mL of diethyl ether. Let the larva sit in the chamber for ~5 min.

NOTE: A detailed protocol for etherization is provided in a previous study published by our group[12].

4.2. Rinse the larva gently from the etherization chamber into a small Petri dish.

4.3. Hav ready one microscope slide, two small coverslips (22 x 22 mm), and one long coverslip (22 x 54 mm) (see **Table of Materials**).

4.4. Add small drops of ether:oil solution (1:5 ratio of ethyl ether to halocarbon oil solution, see **Table of Materials**) to both ends of the slide, then place the small coverslips on top of the small droplets. This arrangement creates a small space gap where the larva can fit.

NOTE: Press the small coverslips against the microscope slide until it is difficult to slip.

4.5. Add some drops of ether:oil solution on the middle of the microscope slide and then place the larva, using forceps, onto the center of the microscope slide (between the small coverslips). Make sure that the anteroposterior axis of the larva is parallel to the short side of the slide and that the dorsal side is facing up.

4.6. Cover the larvae with the long coverslip placed on top of the larva and the two smaller coverslips.

NOTE: Generously press the long coverslip until the larva is almost flat.

4.7. Image segment A8 of the larva using a confocal microscope (see **Table of Materials**) using laser wavelength 488 (GFP).

NOTE: Image the larva immediately because the anesthetization via ether will fade quickly (~5–10 min) and the larva will wake up and move, which will complicate further imaging.

4.8. Capture Z-stack images at a resolution of 1024 x 1024 pixels using a 20x numerical aperture (NA) 0.7 dry objective lens at 1x zoom, step size of 1.5 µm.

5. Quantitation of tissue damage

5.1. Collect and convert the Z-series stack images, from section 4.8, into a single Z projection (a flattening of multiple images taken at different focal planes into a single composite image). This can be performed using commercially available software (e.g., Olympus Fluoview) or any equivalent open source platform, e.g., Fiji/Image J. Save the single Z projection in the TIFF format.

5.2. Open the image analysis program Fiji/ImageJ.

5.3. Click on **File**, from the menu bar, and select **Open** from the window that is displayed.

5.4. Select the stored single image projection, saved in the TIFF format, to be analyzed.

5.5. Click on **Edit**, from the menu bar, and select the **Invert** option from the window that is displayed.

5.6. Click on the **Image**, from the menu bar, then select **Adjust**, from the window that is displayed, and finally select the **Brightness/Contrast** option.

5.7. Select the **Freehand Shape** option from the tool bar to measure the area of the gap (if any).

5.8. Click on **Analyze**, from the menu bar, and select the **Measure** option. This will display the area of the gap or wound.

REPRESENTATIVE RESULTS:

We developed customized mechanical probes, using nitinol filaments (**Figure 1A,N**), to elicit mechanically-evoked behaviors and generated a full behavioral dose response curve using both innocuous and noxious mechanical probes of varying intensity (**Figure 2D**) demonstrating that these probes can be used to study baseline (in the absence of injury) mechanical nociception.

Our behavioral assay results determined that probes exerting pressures below 200 kPa (~1.57 mN) (**Figure 1M**), when applied to *Drosophila* larvae, do not provoke an aversive rolling response (**Figure 2D** and **Video 3**). As expected, these subthreshold or non-noxious mechanical probes (175 kPa or 200 kPa) did not elicit visible neuronal tissue damage (**Figure 2E**). Because they do not induce damage, such probes could be useful to assess mechanical allodynia (hypersensitivity to a normally non-noxious mechanical stimuli). Conversely, suprathreshold or noxious probes (from 462 kPa to 5,116 kPa), elicited an augmented behavioral response (**Figure 2D**) in a dose dependent manner—with the higher pressures eliciting stronger behavioral responses. As anticipated, suprathreshold mechanical pressure also induced dose-dependent tissue damage to the peripheral sensory neurons themselves (**Figure 2E**). The measured area of tissue damage (in μm^2 ± standard deviation) taken from four larvae for each group were: 2,051.03 ± 703.81 (462 kPa), 5,102.29 ± 1,004.67 (2,283 kPa), and 12,238.83 ± 3,724.11 (5,116 kPa). Thus, pressures greater than or equal to 462 kPa (~63 mN), which evoke an aversive rolling response (in 25% or more of the larvae) and cause visible neuronal tissue damage (**Figure 2E**), could be appropriate to study mechanical hyperalgesia (hypersensitivity to normally noxious mechanical stimuli). Nociceptive mechanical probes (≥462 kPa) always induce tissue damage (n = 10, evaluated qualitatively) but do not always provoke an aversive rolling response.

To evaluate mechanical hypersensitivity (allodynia and hyperalgesia), we used a well-established *Drosophila* larval model of nociceptive sensitization that uses ultraviolet light (UV) irradiation to

induce tissue damage[7,12]. This assay has helped to dissect the genetic and cellular mechanisms of thermal nociceptive hypersensitivity[8-10,13-15]. To determine whether UV treatment causes mechanical allodynia, mid third-instar control (*w*^{1118}) larvae were mock-irradiated or UV-irradiated (15–20 mJ/cm^2) (**Figure 3A**). Then, the larvae were tested behaviorally at 2 h, 4 h, 8 h, 16 h, and 24 h post-treatment with a normally subthreshold mechanical probe (200 kPa, 1.57 mN). Approximately 20% of larvae responded as early as 2 h after UV treatment while 50% responded at 4 h, compared to 6.6% and 8.3% mock UV-irradiated animals, respectively (**Figure 3B**). This indicates that UV-induced tissue damage causes mechanical allodynia at 4 h post-irradiation. At later time points (8 h, 16 h, and 24 h) the behavioral response of the UV-treated larvae was in the range of 16%–20% responders (average mean of n = 3–6 sets of 10 larvae each), slightly increased (but not statistically significant) compared to the mock-irradiated control group (in the range of 3%–6% of responders, average mean of n = 3–6 sets of 10 larvae each) (**Figure 3B**).

To investigate mechanical hyperalgesia, a suprathreshold pressure (462 kPa, 3.63 mN), that normally induces an aversive rolling response in ~20% of larvae (**Figure 2D**) and causes neuronal tissue damage (**Figure 2E**), was used. We applied the 462 kPa probe onto the dorsal side of larvae with or without UV-induced tissue damage (**Figure 3A**). We found that larvae probed at 4 h, 8 h, and 16 h following UV treatment showed a significant increase in the aversive rolling response, with 4 h being the peak of the behavioral hypersensitivity (~60% responsive); mock UV-irradiated animals showed an ~27% of aversive response (**Figure 3C**). Similar to mechanical allodynia, the behavioral response at 8 h, 16 h, and 24 h of UV-treated animals (in the range of 36%–42%) was statistically indistinguishable from the non-treated larvae (in the range of 20%–26%). Larvae at the late third instar stage did show a slight decrease of the baseline behavioral response when compared with the middle third instar stage. We hypothesize this could be either by the increased size of the larvae (**Figure 2A**) or the increased thickness of the cuticle covering the body. This fact could explain why at a later stage of development the UV treatment does not induce greater mechanical sensitization, as observed 4 h post UV treatment.

Taken together, our results indicate that *Drosophila* larvae develop both mechanical allodynia and mechanical hyperalgesia following UV-induced tissue damage. The peak time of mechanical allodynia and hyperalgesia is the same, 4 h after UV treatment; however, mechanical hyperalgesia has a more pronounced temporal tail as it returns to baseline more slowly compared to mechanical allodynia.

FIGURE AND TABLE LEGENDS:

Figure 1: Development of a Von Frey-like tool to evaluate mechanical nociception in *Drosophila* larvae. (**A**) Picture of a mechanical probe used to study mechanical nociception in *Drosophila* larvae. (**B**) Nitinol filaments of different diameters and the wire cutter used for probe assembly. Nitinol filaments and their relative diameters are shown to relative scale. (**C**) Picture of the diagonal wire cutter used to cut the nitinol filaments. (**D**) Smoothing the sharp edges of the cut nitinol filament with a sharpening stone. (**E**) Hypodermic needle used to make a hole into the wooden popsicle stick handle of the probe. The tip of the needle needs to reach at least half the

height of the handle stick for secure filament insertion. (**F–G**) Attachment of the nitinol filament by gluing into a wooden popsicle stick handle with insertion hole. (**H–L**) Calibration of mechanical probes by pressing them against a scale. (**M**) Values of force (in mN) and pressure (in kPa) generated by different mechanical probes. The length of each nitinol filament used to construct the probes (P1–P10; P: probe) is detailed in centimeters (cm). (**N**) A picture of a complete set of mechanical probes, ranging from 174 kPa to 5,116 kPa.

Figure 2: Mechanical nociception assay: Von Frey-like filaments generate a dose-response curve of aversive rolling behavior and cause tissue damage to sensory neurons. (**A**) Pictures of the different stages (second and third instar) of *Drosophila* larvae. Scale bar: 2 mm. (**B**) Cartoon of the dorsal view of the third instar *Drosophila* larvae. The red dot indicates the abdominal segment where the mechanical probe is applied. T: thoracic segment; A: abdominal segment. Other anatomical landmarks are labeled. (**C**) Cartoon of the assay: A mechanical probe is applied to the dorsal side of the larva until it bends against the surface below and is then held for 2 s. If the pressure is sufficiently high, this elicits an aversive rolling response upon release. (**D**) Behavioral dose response; each blue dot represents the percent of larvae that responded, with aversive rolling, to the mechanical stimulation within a set of 10 animals. Violin plot of the percent of aversive rolling behavior induced by different mechanical probes. kPa: kilopascals. Box plots represent median (green), whiskers (red) represent the 10th and 90th percentiles. (**E**) Tissue damage: Third instar larvae (of genotype *ppk-Gal4>UAS-mCD8-GFP*) to label nociceptive sensory neurons) were probed at dorsal segment A8 with the indicated pressures. Fluorescently labeled paired ddaC class IVs sensory neurons (across the dorsal midline) were then examined (see sections 4 and 5). White areas (red asterisks) represent gaps or tissue damage. Scale bar: 100 μm. In panel B, the larva is shown in the dorsal view, while in C it is the lateral view. Mechanical probes pressed against the dorsal cuticle-epidermis side of the larva produce a depression like-pocket at the point of contact of the tip of the probe and the surrounding areas. The solid black line curved toward the ventral side is the top of the pocket, while the dashed gray lateral line represents the lateral side and the bottom of the pocket.

Figure 3: Mechanical hypersensitivity after UV damage. (**A**) Schematic of the experimental design to test sensitization. Mid third instar were mock treated (non-UV) or UV irradiated. The mechanical nociception assay was then performed at different time points (2 h, 4 h, 8 h, 16 h, and 24 h) following mock treatment or irradiation. **(B)** Mechanical allodynia: The percentage of larvae exhibiting aversive rolling after probing with a normally subthreshold or non-noxious mechanical stimulus (200 kPa, 1.57 mN) at the indicated time points after mock-treatment or UV irradiation. (**C**) Mechanical hyperalgesia: The percentage of larvae exhibiting aversive rolling after probing with a normally suprathreshold or noxious mechanical stimulus (462 kPa, 3.63 mN) at the indicated time points after mock-treatment or UV irradiation. Error bars indicate mean +/- SEM. Two-tailed unpaired *t*-test was used for statistical analysis: *$p < 0.05$, **$p < 0.01$; ns: not significant. Each red dot, in panels B and C, represents the mean proportion of 10 larvae, n = 3–6 sets per timepoint/condition.

Video 1: Normal locomotion of *Drosophila* larvae.

Video 2: Noxious mechanical stimulation of *Drosophila* larvae.

Video 3: Subthreshold mechanical stimulation of *Drosophila* larvae.

DISCUSSION:

We modified an established mechanical assay[1,2,16] using customized mechanical probes fabricated from nitinol filaments. This metal alloy allows us to use smaller diameter filaments that are appropriate to the size of the *Drosophila* larvae. Fishing line-based monofilaments have dominated the field of fly mechanical nociception to date[2,5,6,16]. Our nitinol filaments maintain their shape and measured pressure for approximately ~3–5 months (in our experience). By varying the length and diameter of the nitinol filaments, the user can generate a wide range of pressures spanning from subthreshold to a nearly complete rolling response. In particular, making subthreshold probes is simpler with the smaller diameter nitinol filaments. Using these probes, we found that pressure, rather than force, elicits more consistent nocifensive behavioral responses[4]. We demonstrate here, using a well-established UV-induced nociceptive sensitization model[7,10,13], that these filaments are also a useful tool for studying mechanical hypersensitivity—allodynia and hyperalgesia.

Previous studies using mechanical probes fabricated from fishing line have led to a certain variability in behavioral responsiveness[2,6,16,17]. Several factors may account for this. First, because pressure is the important variable, the buffing of the filament tip so that it is rounded and does not have any sharp edges is critical. Second, reporting pressure values rather than only force is important for the reproducibility of the experiments, because different mechanical probes that generate similar forces can elicit disparate pressures[4]. Third, it is critical to apply only one mechanical stimulation per larva using noxious probes, because such probes produce a dose-dependent tissue damage at the epidermal[4] and sensory neuronal levels (**Figure 2E**). A second or subsequent noxious mechanical stimulus, after tissue damage has been induced, could conceivably impair the function of the affected peripheral sensory neurons and elicit an altered behavioral response. In another study, larvae stimulated twice with noxious mechanical probes mostly displayed an enhanced behavioral response[5], suggesting development of an acute mechanical sensitization (hyperalgesia), which might result from the tissue damage provoked by the first noxious mechanical stimulus. Conversely, other authors[6] reported a mixed (increased or decreased) behavioral response, indicating that the altered behavioral response could be due to damage/dysfunction of the neuronal tissue. Stimulating each larva only once eliminates possible variance in behavioral responses resulting either from sensitization or tissue damage. Fourth, we mechanically stimulated segment A8, which is more posterior than previous studies (preferred areas A3–A4)[2,5,16]. Probes between ~3,900 kPa and 5,300 kPa applied to either segment A2 or A8 did not show any behavioral differences[4]. In addition, A8, compared to A2–A4, is easier to stimulate with mechanical probes that generate lower pressures (<300 kPa) because the larva is thinner in this region and thus more easily compressed. Other studies showed that noxious mechanical stimulation of the posterior end of the larva (delivered by a rigid insect pin, held with forceps) mostly evoked forward locomotion, rather than an aversive or rolling response[18]. This different behavioral response could be due to differences in the properties of the used materials (bendable nitinol filament vs incompressible insect pin) or to different pressures delivered to the

larvae (the pressure value of the insect pin was not reported).

The development of a mechanical nociception assay for *Drosophila* larvae has enabled the field to discover that different mechanical sensory ion channels and neural circuits mediate mechanical nociception[5,6,16,17]. However, the study of the mechanical hypersensitivity (allodynia and hyperalgesia) has lagged, compared to sensitization of the other sensory modalities—heat[7,8,10,13,14], cold[9], and chemical[3]. This lag may be due in part to the absence of suitable mechanical probes that can generate a full response range spanning subthreshold to suprathreshold pressures. Of particular importance, especially for assessing mechanical allodynia, are subthreshold probes that do not elicit an aversive rolling response from uninjured larvae. The significance of our improved mechanical probes is that they can be fabricated to span innocuous stimuli (subthreshold ~174 kPa–200 kPa) or the low to high noxious range (suprathreshold ~225 kPa to ~5,116 kPa). Here, we demonstrate using the nitinol von Frey-like filaments that *Drosophila* larvae develop both mechanical allodynia and mechanical hyperalgesia after UV irradiation. The mechanical sensitization shows some differences when compared to thermal sensitization. Both the onset and the peak of mechanical sensitization is earlier (~4 h) compared to thermal (heat) sensitization (~8 h for hyperalgesia and ~24 h for allodynia)[7]. In addition, the mechanical allodynia and hyperalgesia are concomitant (both peak at ~4 h). Furthermore, while heat sensitization (allodynia and hyperalgesia) resolves completely at later time points[7], mechanical hypersensitivity exhibited a long tail that remained slightly above baseline. Cold sensitization in *Drosophila* involves a switch in cold-evoked behaviors[9] and the emergence of new cold-evoked behaviors—a phenomenon that is not observed with mechanical stimulation. These differences in onset, duration, and observed behaviors suggest that each sensory modality may be controlled by different signaling pathways. Combining the sensitization assay described here with the powerful genetic tools available in *Drosophila* should allow a precise genetic dissection of the mechanical hypersensitivity (allodynia and hyperalgesia) observed.

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DISCLOSURES:

The authors have nothing to disclose.

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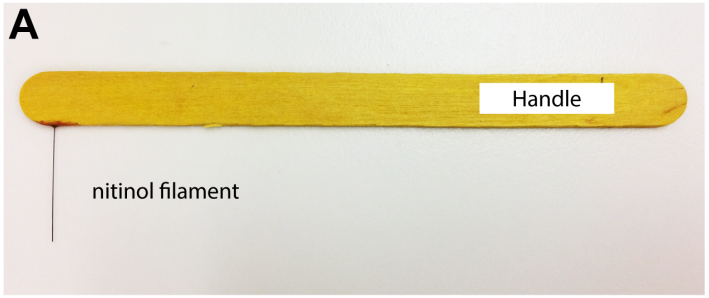
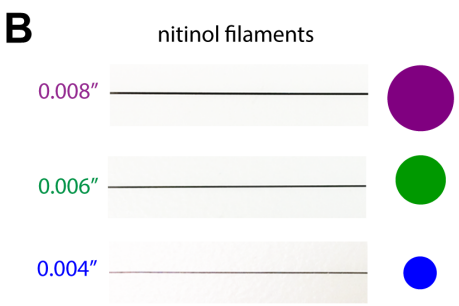
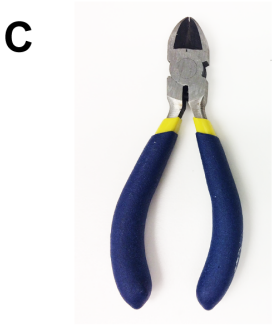
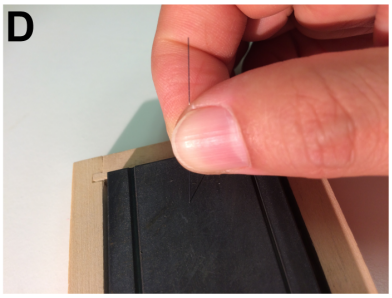
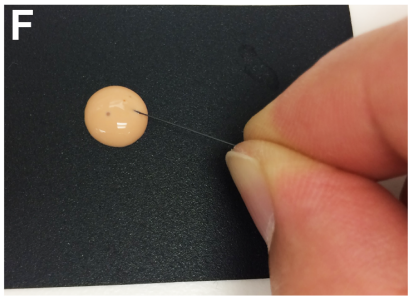
Figure 1

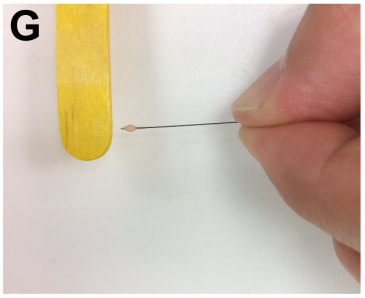












M

mechanical probes	length (cm)	mass (grams)	force (mN)	area (cm^2)	pressure (kPa)
P1	7.75	0.14	1.37	0.00007854	174
P2	6.60	0.16	1.57	0.00007854	200
P3	6.70	0.18	1.76	0.00007854	225
P4	5.35	0.24	2.35	0.00007854	299
P5	4.35	0.37	3.63	0.00007854	462
P6	3.65	0.51	5.00	0.00007854	636
P7	1.85	1.83	17.93	0.00007854	2283
P8	2.50	4.54	44.49	0.00017672	2518
P9	2.05	6.70	65.66	0.00017672	3716
P10	2.45	16.40	160.72	0.00031416	5116

N

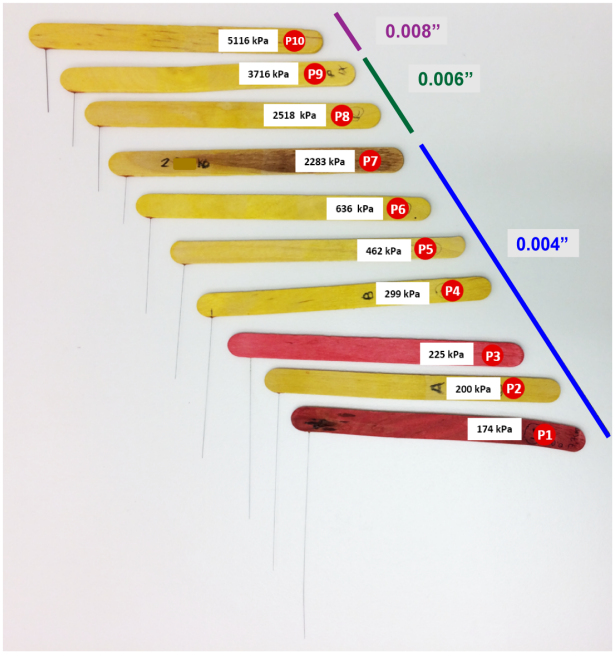
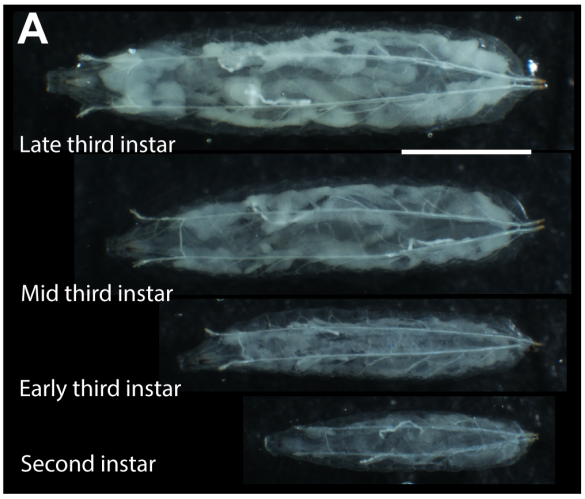
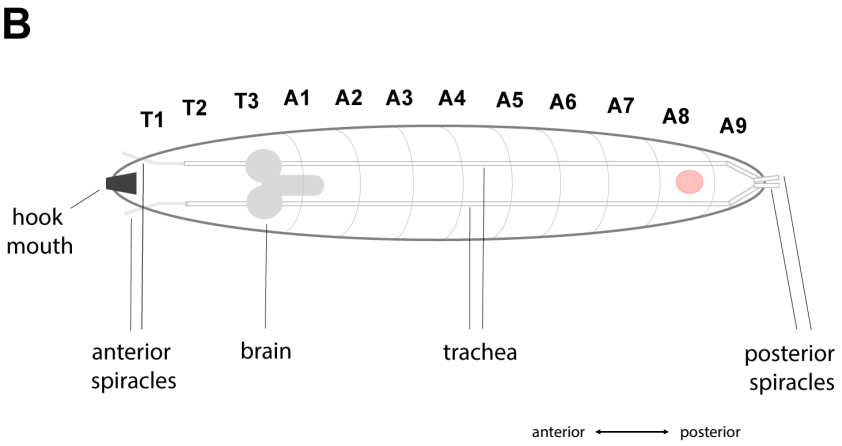
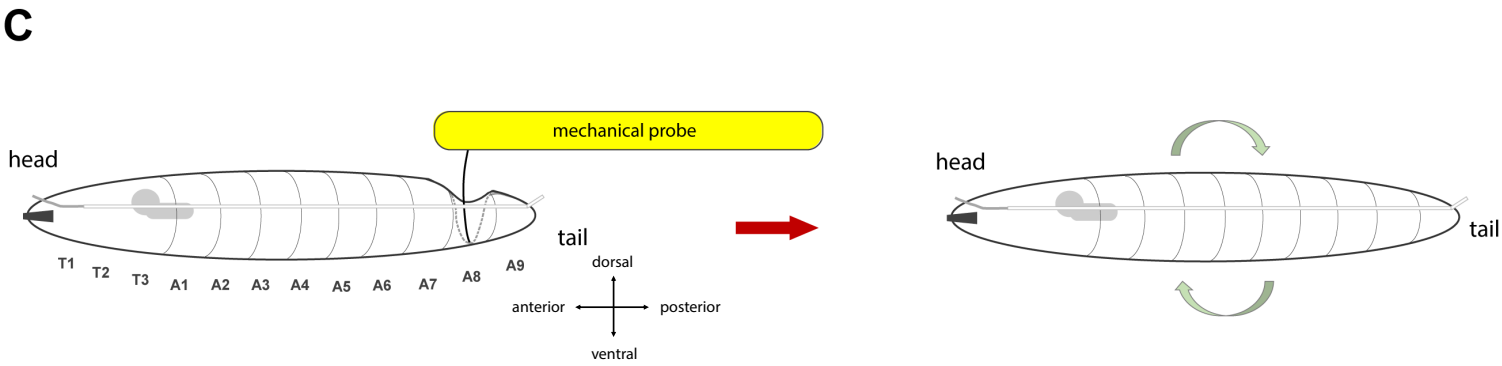


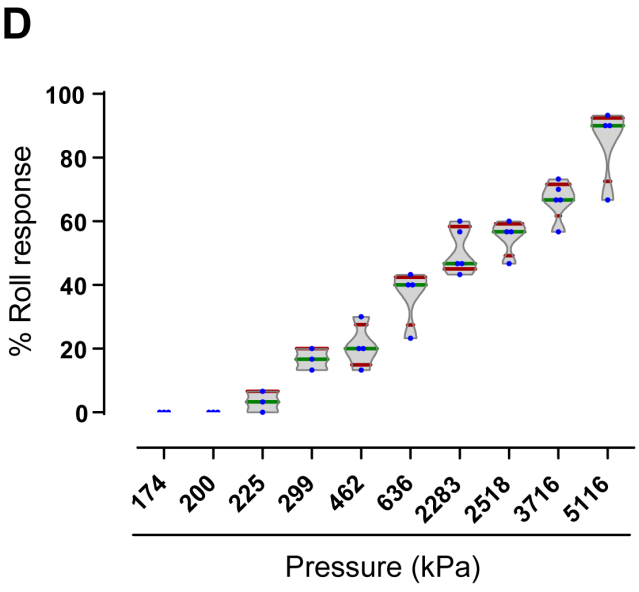
Figure 2

Click here to access/download;Figure;Figure 2artboards-01-2-2.pdf









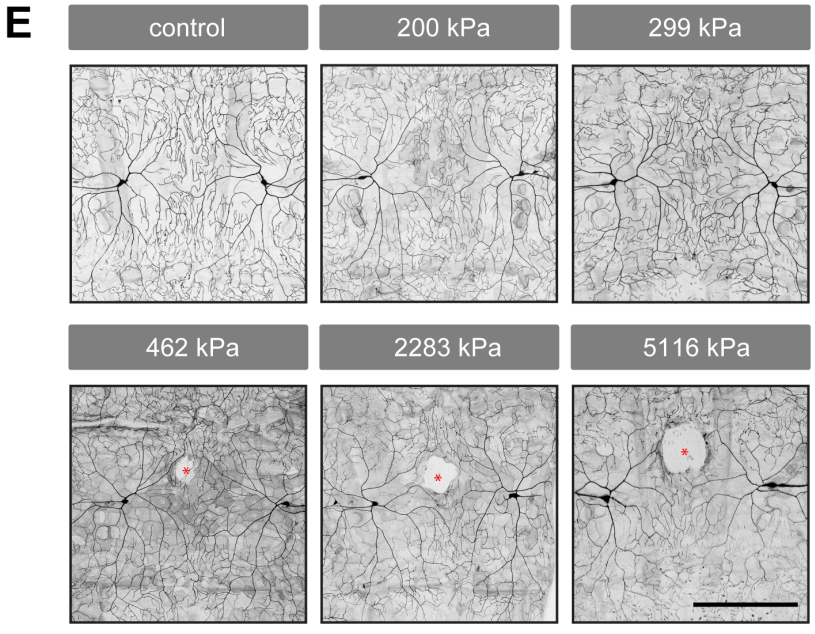
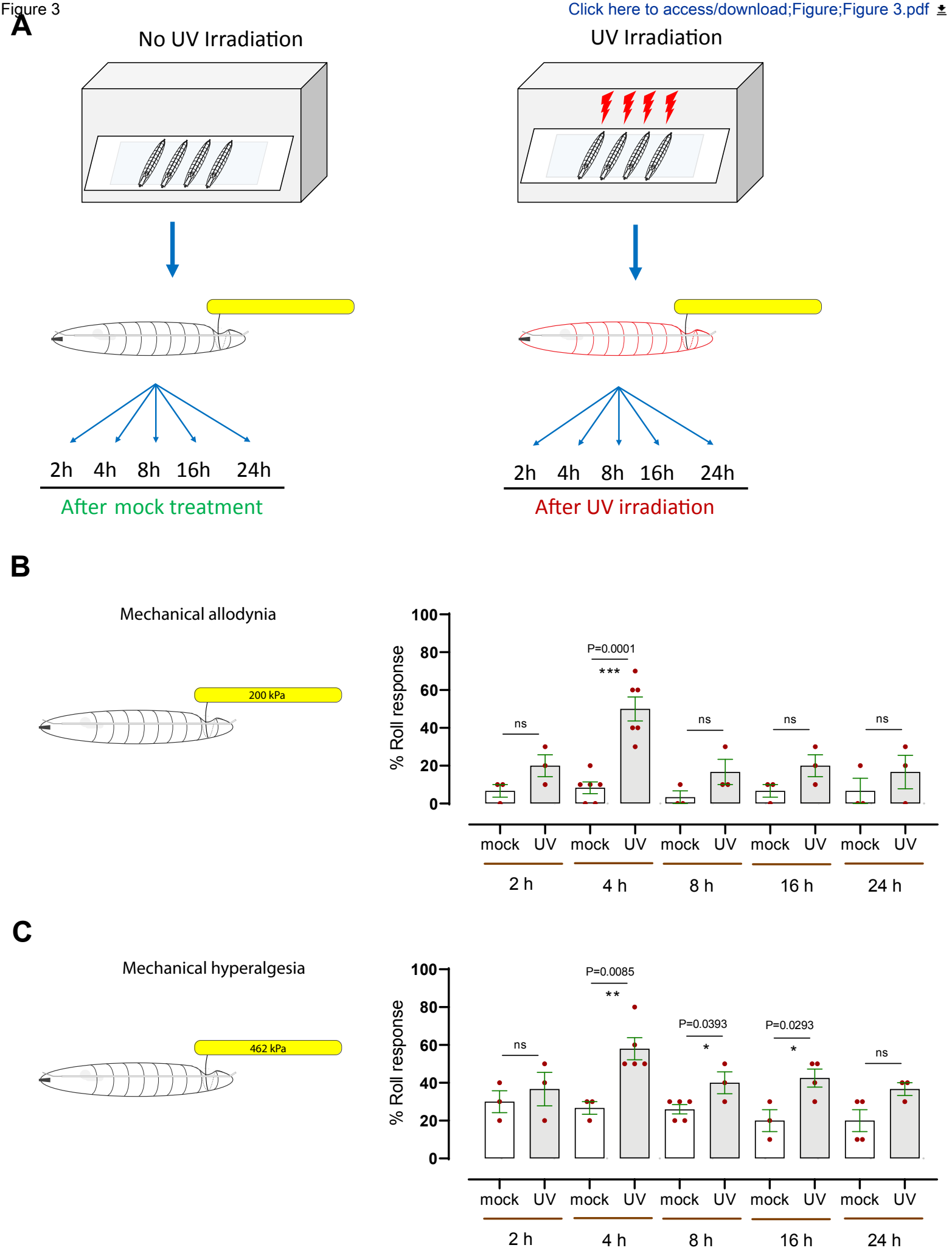


Figure 3



Video 1

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Video or Animated Figure

Video 1_regresolu.mp4

Video 2

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Video or Animated Figure

Video 2_regresol.mp4

Video 3

Click here to access/download

Video or Animated Figure

Video3_Subthreshold_short.mp4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Beaker	Fisher Scientific	02-540C	Beaker of 10 ml of capacity. Any similar container will do.
Black (Arkansas) bench stone	Dan's Whetstone	SKU: I200306B24b-HQ-BAB-622-C	Used to smoothe any irregularities of the nitinol wire tips. https://www.danswhetstone.com/product/special-extra-wide-black-bench-stone-6-x-2-1-2-x-1-2/
Confocal microscope	Olympus	FV1000	Any equivalent confocal microscope will do
Coplin Jar	Fisher Scientific	08-816	https://www.fishersci.com/shop/products/fisherbrand-glass-staining-dishes-10-slides-screw-cap/08816#?keyword=08-816
Diethyl ether	Fisher Scientific	E138-500	For anesthetizing larvae.

Etherization chamber			This is a homemade customized chamber. Please see details of its construction in our previous published paper[12]. The purpose of the etherization chamber is allow entry of diethyl ether fumes but prevent larval escape.
Fiber Optic Light Guide	Schott AG	A08575	Schott Dual Gooseneck 23 inch
Forceps	Fine Science Tool	FS-1670	For transferring larvae
Glue	Aleene's	N/A	Aleene's® Wood Glue, formerly called (Aleene's All-Purpose Wood Glue) https://www.aleenes.com/aleenes-wood-glue
Graspable holder	Loew Cornell	N/A	Loew-Cornell Simply Art Wood Colored Craft Sticks, 500 pieces.
Halocarbon oil 700	Sigma	H8898-100ML	
Hypodermic needle 30G 1/2"L	Fisher Scientific	NC1471286	BD Precisionglide® syringe needles, gauge 30, L 1/2 inches. Used to make a hole into the wooden holder for the nitinol wires

Large Petridish	Falcon	351007	60 mm x 10 mm Polystyrene Petridish
Microscope (Zeiss) Stemi 2000	Carl Zeiss, Inc.	NT55-605	Any equivalent microscope will do
Microscope Cover Glass 22x22	Fisher	12-545-B	
Microscope Cover Glass 22x40	Corning	2980-224	Tickness 1 1/2
Microscope Slides	Globe Scientific Inc.	1358Y	
Mini Diagonal Cutter	Fisher Scientific	S43981	For cutting nitinol filaments
Nitinol filaments, Diameters: 0.004”, 0.006”, 0.008”	Mailin Co	N/A	Fifteen pieces of each diameter of 12” length were ordered. https://malinco.com/
Piece of black vinyl	Office Depot	N/A	We use a small piece of vinyl cut from a binder. Dark color provides contrast. A small piece allows orientation of the larva
Small Petridish	Falcon	351008	35 mm x 10 mm Polystyrene Petridish
Spatula	Fisher Scientific	21-401-10	Double-Ended Micro-Tapered Stainless Steel Spatula. Used to place the food in the petri dish
Wipes	Fisher Scientific	06-666A	Kimpes KMTECH, Science Brand. Used to dry larvae of excess moisture.

w^{1118}	Bloomington Drosophila Stock Center	3605	Control strain for behavioral assays
ppk-Gal4>UAS-mCD8-GFP	Bloomington Drosophila Stock Center	8749	Strain for fluorescent labeling of class IV md neurons

Response to the Reviewers' comments

We are grateful that the reviewers in general were positive about our manuscript and thankful for their insightful and constructive comments. Below we address the reviewer comments/suggestions (their text in black bold). Our comments/responses to the reviewers are shown in red text and all new inserted changes/rearrangements in the manuscript text are shown in blue, along with directions as to where the new text can be found.

Reviewer #1:

Minor Concerns:
Title and abstract:
The only thing to say here is that the title makes one think this manuscript describes an "improved assay", yet it describes both a novel assay AND novel tools (customized filaments!) for testing mechanical nociception.

We use “improved” because others before us have used von Frey-like filaments, though not ones that span the full response range. Please see our revised title which incorporates the idea of improved tools:

An improved assay and tools for measuring mechanical nociception in *Drosophila* larvae

Materials / equipment needed:
In addition to the mention of their genotypes in the manuscript and Acknowledgements, I would find it useful to include the specific Bloomington Stock Center numbers for the specific fly stocks used in this work in the Material / Equipment list.

We have added the relevant stock numbers from Bloomington in the table of Materials section.

w^{1118}: Stock# 3605 (Bloomington Drosophila Stock Center)
Control strain for behavioral assays.

ppk-Gal4>UAS-mCD8-GFP: Stock # 8749 (Bloomington Drosophila Stock Center)
Strain for fluorescent labeling of class IV md neurons.

Clarity of steps in procedure and/or Missing steps:

Mechanical probes (#1): My comment here is that if one cuts a filament to an exactingly specific length such as 36.5 mm (Probe P6, Figure 1M), but then one makes the receiving hole in the popsicle stick with a needle, isn't what's important the actual length of the wire from its tip to where it inserts into the handle? So, for clarity, is the length shown in Figure 1M the original length of the cut wire, or the functional length once it is

mounted? I can't imagine the part of the wire imbedded in the wood glue contributes to its functional force delivery (?). Procedure #1 (step 1.1) implies it is the original cut length. If that's correct, we are fine. But if it's the functional length after mounting, this should be clarified.

The issue of the functional length is a little murky- it depends whether the pressure is registering from the end of the insertion hole or from the edge of the mount. We don't really know this for sure and what really matters in the end is the measured/calibrated pressure of the probe. The filament lengths should really be viewed as guides to achieve the approximate pressures listed here. In reality they are likely to be slightly variant in each case- why they need to be calibrated. Following the reviewer suggestion for clarification, we have added a note in the protocol #1, step 1.1, to clarify this. Please see page 3, lines 89-92:

NOTE: The lengths specified here are a guide to achieve the approximate pressures indicated, using a similar protocol for construction of the mount. Ultimately, regardless of the length of filament cut, and the depth of the hole in the mount, the filaments must be measured/calibrated on a balance to obtain the exact force/pressure value.

Larvae prep (#2): Most people reading this know what's going on here, but the phrase "4-5 days after egg lay" (step 2.2) is potentially cryptic. I'm assuming the adults are kept in the bottles for only a specific window (12 hours? 24 hours?) so one can indeed do "timed collections" of offspring. Adding that detail before step 2.2 will clarify for a total novice.

To clarify this point, we have added additional information in a Note of step 2.1, Please see page 4, lines 135-137, which now states:

NOTE: Adult flies (five males and ten females, 1:2 ratio) are kept in the fly vials, to allow egg laying, for about 24 hours. The time after egg laying (AEL) begins from when the adults are removed.

In addition, we have modified the step 2.2, please see page 4, lines 139-142, which now states:
2.2. Collect 3rd instar larvae, after approximately 96 hours of egg laying, by gently squirting tap water into the soft fly food containing the larvae. Wandering larvae that have left the food, or which have everted anterior or posterior spiracles, are too large/old for this assay. Second instar larvae (~ less than 4 mm in length) are too small.

Mechanical nociception assay (#3): The written descriptions here are clear. The only thing I can suggest is that in Figure 2B the larvae is shown in a dorsal view, while in 2C it is a lateral view. One can see this by how the brain is represented. For the utmost in clarity, perhaps note this in the Figure legend? The procedure clearly says to apply the probe dorsally (step 3.6).

To address this concern and make it easier for the readers to recognize the orientation of the animals in Figures, 2B and 2C, we have added the orientation of the cartoons in the figure 2 legend, panels 2B and 2C (please see page 9, line 357-358).

In panel B the larva is showed in a dorsal view, while in C it is a lateral view.

In addition, orientation lines (anterior: A, posterior: P, dorsal: D, and ventral: V) are displayed in each panel of Figure 2b and 2C. (Please see the new Figure 2).

Also, in Figure 2C it looks like the larvae has been skewered by the probe - am I seeing it right that the dark probe is shown going through the body down to the lower surface? Surely this might happen, but perhaps a clearer image is the probe stopping at the epidermis, bending but not penetrating the surface ("presses against the dorsal surface and compresses the larvae" [step 3.6])?

It is important to recall that larvae are filled with hemolymph- thus, they are compressible. When the mechanical probe is pressed against the dorsal cuticle-epidermis, it produces a depression like-pocket at the point of contact of the probe's tip and the surrounding tissue. The solid black line curved toward the ventral side is the top of the pocket, while the dashed grey lateral line shape represents the lateral side and the bottom of the pocket. Ultimately, the probe that contacts the dorsal surface of the larva presses against the surface underneath the ventral epidermis- the vinyl pad. Pressures less than 2300 kPa rarely elicit cuticle damage (measured by scab formation), but can produce epidermal (Lopez-Bellido et al., 2019, please see Figure 2) and neuronal tissue (Figure 2E) damage. Higher pressures (e.g., 5300 kPa) always cause cuticle, epidermal, and neuronal damage. We think that most of this damage is from compression of the tissues (not skewering) because it is rare for larvae to become stuck on the probes, even at the highest pressures. However, to address the reviewer´s concern and clarify step 3.6 in the manuscript we have added a note, please see page 5, lines 190-195, that states:

NOTE: At the point of contact between the tip of the nitinol filament and the dorsal cuticle-epidermis, probes lower than 2300 kPa, mainly bend without penetrating the cuticle and underlying tissues. Such probes seldom affect larval mortality (Lopez-Bellido et al., 2019). At higher pressures (>5000 kPa) the probes both bend and, occasionally, penetrate the cuticle and underlying tissues. Puncturing of the larvae impairs larval survival (Lopez-Bellido., et al., 2019) and if this is observed these larvae are typically discarded from behavioral analysis.

In addition, we have added information to figure legend 2. Please see page 9, line 358-362, that states:

Mechanical probes pressed against the dorsal cuticle-epidermis side of the larva produce a depression like-pocket at the point of contact of the tip of the probe and the

surrounding areas. The solid black line curved toward the ventral side is the top of the pocket, while the dashed grey lateral line represents the lateral side and the bottom of the pocket.

Confocal microscopy (#4): In step 4.1 I would change the word "poked". "Stimulated" perhaps? "Poked" might be interpreted as skewered / penetrated.

Please see page 6, line 217 that we have changed as suggested by the reviewer.

"4.1. Place a larva (of genotype *ppk-Gal4>UAS-mCD8-GFP* to label sensory neurons) previously stimulated with a nitinol filament…"

In step 4.5 it is noted that the A/P axis should be parallel to the slide's short end. But I would think it equally important to note the orientation of the D/V axis. Since you are stimulating on the dorsal side, perhaps simply noting "dorsal up" (or down depending on how you are doing the confocal slices) would be enough.

Following the reviewer´s suggestion, we have added an additional information to step 4.5. Please see page 6, lines 231-232:

4.5. "… Make sure the anteroposterior axis of the larva is parallel to the short side of the slide and the dorsal side is facing up."

In Figure 2E we are seemingly looking at paired ddaC class IVs across the dorsal midline (?). If that indeed the case, that information should be in Figure 2's legend!

Yes, these are the paired ddaC neurons. Please see the changes in the new Figure legend 2E, page 9, lines 355-356:

Fluorescently labeled paired ddaC class IVs sensory neurons (across the dorsal midline) were then examined (see protocol sections 4 and 5).

Quantification of damage (#5): Nothing to add.

Reasonable results:
The experimental data demonstrating the use of their novel, calibrated probes to elicit allodynic (to 200 kPa stimulation) as well as hyperalgesic (to 460 kPa stimulation) responses is clear and convincing. The mock-UV-treated larvae are the appropriate control. However, I do object to the description in both assays of "not returning to the normal baseline range" after 8 hours (allodynic, lines 270, 271) or after 24 hours (hyperalgesic, line 280). If there is not a statistical difference detected, I'd think one cannot then make note of differences between treatments!

We thank to the reviewer for this important note. To address the reviewer´s suggestion, we have deleted the sentence of the allodynia data (page 6, lines 270, 271 from the former manuscript). In addition, we have reworded the sentence of the hyperalgesia section. Please see the changes at page 8, lines 311-313, that states:

Similar to mechanical allodynia, the behavioral response at 8, 16, and 24 hours of UV-treated animals (range of 36-42 %) was statistically indistinguishable from the non-treated larvae (range 20-26%).

Reviewer #2:

Major Concerns:
The only significant suggestion that I have is that I think the protocol would be improved by including a third movie showing the behavioral response to sub-threshold stimuli (e.g. 200 kPa). This movie could make clear to viewers the difference between the rolling behavior that is the nociceptive response and behaviors that are induced by sub-threshold touch.

To address the reviewer´s main concern we have added a third Movie showing the behavioral response of the larvae to the sub-threshold stimulus (200 kPa). Please see the description of the Movie 3 as a note of the step 3.7, page 5 lines 202-204:

Note: larvae stimulated with a subthreshold mechanical stimulus (200 kPa) did not elicit the typical nociceptive or rolling response (Movie 3). Some larvae did exhibit fast forward or light touch responses such as changes in the direction of movement.

Minor Concerns:
I also have several suggestions to improve the clarity of the text specifically about word choices and including more information about how data are displayed. I have included suggestions below, labeled by section and line numbers.

Abstract
Line 35: I think investigated rather than "investigate" is the proper tense for this verb.

Thank you for catching this. We have changed it (please see line 35).

Introduction
Lines 56 & 57: The authors used probes that applied pressures below 200 kPa I think that "(<200 kPa)" is clearer in this sentence.

We have added the "**less than or equal to" symbol (≤)** Please see the abstract at page 2, line 56, of the new manuscript.

Custom fabrication of mechanical probes allowed to us to generate subthreshold (≤ 200 kPa)…

Line 61: I think that this sentence would be clearer if you defined VEGF. Is it an acronym for Vascular Endothelial Growth Factor?

Following this suggestion, we have defined the abbreviation on page 2, lines 61-62, which now reads:

The improved mechanical assay described here also helped to identify a conserved Vascular Endothelial Growth Factor (VEGF)-related receptor….

Protocol
Lines 171-174 - I would like to see the response to a subthreshold stimulus included as a third movie.

See above. We have made a third Movie, showing the behavioral response of a larva to the sub-threshold stimulus (200 kPa). Please see the description of the Movie 3 as a note of the step 3.7, page 5 lines 202-204 states as:

Note: larvae stimulated with a subthreshold mechanical stimulus (200 kPa) did not elicit the typical nociceptive or rolling response (Movie 3). Some larvae did exhibit fast forward locomotion but others light touch responses such as changes in the direction of movement.

Lines 207 & 208 - This note could be improved by including an average time it takes for a larva to emerge from anesthetization.
We have added this. Please see page 6 lines 238-239, that now states:

Note: Image the larva immediately because the anesthetization via ether will fade quickly (~ 5-10 minutes) and the larva will wake up and move, which will complicate further imaging.

Representative Results
Lines 242-244: I think that it is clearer to state that your behavioral assay results demonstrate that probes that exert pressures below 200 kPa do not induce aversive responses in Drosophila larvae.

We have changed the wording of page 7, line 272, as noted below. In addition, we have added a call for the movie 3 in page 7, line 274

Our behavioral assay results determined that probes exerting pressures below 200 kPa (~ 1.57 mN) (Figure 1M), when applied to *Drosophila* larvae, do not provoke an aversive rolling response (Figure 2D and Movie 3).

Lines 251-253: The reported results are not as clear as possible. First, the authors should make clear whether the reported measured areas of tissue damage are averages for several individuals and if so how many larvae were examined. Second, the authors should clarify whether probes that induce responses in only a portion of the larvae (for example 462 kPa, figure 2D & 2E) always induce tissue damage or whether tissue damage is only seen in larvae that respond to the stimulus.

To make it clearer for the readers we have added the number of larvae used to measure the tissue damage. Please see page 7, line 282. In addition, we have clarified that "nociceptive mechanical probes that always induce tissue damage (for example ≥ 462 kPa; n=10, evaluated qualitatively) do not always provoke an aversive rolling response" (please see pages 7-8, lines 287-288).

The measured area of tissue damage (in μm^2 ± standard deviation) taken from 4 larvae for each group were: 2051.03 ± 703.81 (462 kPa), 5102.29 ± 1004.67 (2283 kPa), and 12238.83 ± 3724.11 (5116 kPa). Thus, pressures greater than or equal to 462 kPa (~ 63 mN), which evoke an aversive rolling response (in 25% or more of the larvae) and cause visible neuronal tissue damage (Figure 2E), could be appropriate to study mechanical hyperalgesia (hypersensitivity to normally noxious mechanical stimuli). Nociceptive mechanical probes (≥ 462 kPa) always induce tissue damage (n=10, evaluated qualitatively) but do not always provoke an aversive rolling response.

Line 266 & 271: There is no explanation in the figure legend for figure 3B for what the red circles specify. Do they represent the percentage of animals that respond for a set of ten animals as the results in figure 2D? This absence of information makes comparing the results difficult and the information should be included in the figure legend for figure 3. The authors should also make clear if they are reporting average values in the text.

We thank to the reviewer for catching this undefined symbol in the Figure legend 3. To address this, we have defined the meaning of the red dots. Pease see it in the Figure legend 3 of the manuscript, page 9, lines 374-375:

Each red dot, in panel B and C, represents the mean proportion of 10 larvae, n = 3-6 sets per timepoint/condition.

In addition, we have added additional information in the results section to clarify the number of animals used and how the mean is reported. Please see page 8, lines 300-301, and page 8, lines 302-303, respectively:

This indicates that UV-induced tissue damage causes mechanical allodynia at 4 hours post-irradiation. At later time points (8, 16, and 24 hours) the behavioral response of the UV-treated larvae was in the range of 16-20 % responders (average mean of n = 3-6 sets of 10

larvae each), slightly increased (but not statistically significant) compared to the mock-irradiated control group (in the range of 3-6 % of responders, average mean of n = 3-6 sets of 10 larvae each) (Figure 3B).

Line 278: The authors should explain if the red circles shown in Figure 3C represent the results for ten animals as the results in figure 2D.

We thank to the reviewer for catching this undefined symbol in the Figure legend 3C. We have now defined the meaning of the red dots. Please see it in the Figure legend 3 of the manuscript, page 9, lines 374-375:

Each red dot, in panel B and C, represents the mean proportion of 10 larvae, n = 3-6 sets per timepoint/condition.

Figure Legends
Figure 2
Line 311: I think it would be clearer to state that each blue dot represents the percentage of larvae that responded to the probe in a set of 10 animals.

We thank to the reviewer for catching this. We have now defined the meaning of the blue dots. Pease see it in the Figure legend 3 of the manuscript, page 9, lines 349-350:

Behavioral dose response, each blue dot represents the percent of larvae that responded, with aversive rolling, to the mechanical stimulation within a set of 10 animals. Violin plot of the percent of aversive rolling behavior…

Figure 3
Lines 322-329: The description of the results shown in figure 3 B and 3C would be clearer if the authors stated what the red dots in the figures represented.

See related comment above. We have now defined this.

Discussion
Line 343-345: This sentence is awkward. Perhaps it is clearer to state that "pressure, rather than force, elicits more consistent nocifensive behavioral responses."

We have made this change on page 10, lines 394-395:

Using these probes we found that pressure, rather than force, elicits more consistent nocifensive behavioral responses[4].

Reviewer #3:

Minor Concerns. Line 119: The authors describe periodic recalibration of the probe every 3-4 weeks. Do the authors have data about what kind of deviation can be expected from the probe over time? How much deviation can reasonably be tolerated?

We don't have any specific data of the deviation of the pressure for each mechanical probe. We mostly use the middle range probe 2346 kPa (Lopez-Bellido et al., 2019) for behavioral experiments. Every time we noticed a change of the pressure ± 3 % from the originally measured pressure we decided to make a new mechanical probe. To make it clear we have amended the original note of step 1.8 to clarify. Please find the changes in page 3, lines 125-126, which now state:

NOTE: Check each mechanical probe at least every 3-4 weeks. When the pressure deviates by more than ± 3 % from that originally measure, a new mechanical probe must be fabricated.

Line 128: The authors describe collection of mid-3rd instar larvae for their assays. Some previous work has used wandering 3rd instar larvae for mechanical nociception experiments (2). It also seems that the UV sensitization experiments conducted by the authors would end up with a variety of larval stages being tested, as all of the larvae are UV-injured at mid-3rd instar stage and then tested between 2 and 24 hours later. Can the authors comment on baseline differences in mechanical nociception between mid- and late-3rd instar stages?

To address the reviewer comment, we have added an additional information in the result section, please see page 8, lines 313-318, which states:

Larvae at the late third instar stage did show a slight decrease of the baseline behavioral response when compared with the middle third instar stage. We hypothesize this could be either by the increased size of the larvae (Figure 2A) or the increased thickness of cuticle covering the body. This fact could explain why at later stage of development the UV-treatment does not induce greater mechanical sensitization, as observed 4 hours post UV-treatment.

Line 212: The authors describe a confocal analysis of tissue damage that is applicable to a specific software package that is not universally used. Would it be possible to include a one- or two-sentence explanation of what each step is meant to accomplish (where not obvious). This would make the analysis more reproducible for other software packages.

We thank to the reviewer for this suggestion. We have added a brief explanation of the main idea of converting Z-series stack images. Please see pages 6-7, lines 244-247:

5.1. Collect and convert the Z-series stack images, from section 4 .8, into a single Z projection (a flattening of multiple images taken at different focal planes into a single composite image). This can be performed using Olympus Fluoview software or any

equivalent such as open source platform Fiji/Image J. Save the single Z projection as a TIFF format.

In addition, we have added an additional information for the image processing in step 5.4. Please see page 7, line 251, which states:

5.4 Select the stored single image projection, saved as TIFF format, to be analyzed.

Line 328: The authors describe using a t test to analyze nociception data. However, the data are presented as being proportional. Are the larvae assayed in smaller groups, and then mean proportions are calculated? This may just need some clarification.

We appreciate the suggestion for a better explanation of the statistical analysis. We have rearranged the original statement. Please see page 9, lines 374-375, which states:
Each red dot, in panel B and C, represents the mean proportion of 10 larvae, n = 3-6 sets per timepoint/condition.

Reviewer #4:

Major Concerns:
none

Minor Concerns:
1. The authors describe that the stimulus should be applied to abdominal segment 8, which is different from previous assays developed using monofilaments (typically mid-abdominal segments are stimulated her, e.g. Hwang et al. Curr. Biol. 2007, Hu et al. Nat. Neurosci. 2017). Moreover, a previous study suggested that noxious touch stimulation at the posterior end of larvae mostly induced forward locomotion rather than rolling (Takagi et al., Neuron 2017). Maybe the authors can discuss these differences, which might be intrinsic to the filaments used or the overall method assessing the behavioral responses.

To address the reviewer's comment, we have added additional information in the discussion section. A new reference was also added to the new manuscript, this is Takagi et al., 2017, represented by the number 18 as superscript. Please see pages 10-11, line 415-425, which states:

Fourth, we mechanically stimulated segment A8, which is more posterior than previous studies (preferred areas A3-A4) (Zhong et al., 2010, Hu et al., 2017, Hwang et al., 2007). Probes between ~3900 kPa and 5300 kPa applied to either segment A2 or A8 did not show any behavioral differences (Lopez-Bellido et al., 2019). In addition, A8 , compared to A2-A4, is easier to stimulate with mechanical probes that generate lower pressures (<300 kPa) because the larva is thinner in this region and thus more easily compressed. Other studies showed that noxious mechanical stimulation of the posterior end of the larva

(delivered by a rigid insect pin, held with forceps) mostly evoked forward locomotion, rather than an aversive or rolling response (Takagi et al., 2017). This different behavioral response could be due to differences in the properties of the used materials (bendable nitinol filament vs incompressible insect pin) or to different pressures delivered to the larvae (the pressure value of the insect pin was not reported).

2. The authors state that their filaments allow more defined and reproducible results than previously used monofilaments. While I do not necessarily want to argue with this statement, it would be useful to compare how monofilaments differ in terms of stimulation accuracy and noxious stimulus-induced injury.

Different groups using fishing line monofilaments have reported different behavioral responses using similar amount of force (Zhong et al., 2010, Hwang et al., 2007, Hu et al., 2017, Kim et al, 2012). These differences could be due to several reasons and below we detail some of them. Forces/pressures with monofilaments and with nitinol filaments result when the filament bends. In our hands, mounting the filaments perpendicular to the handle (rather than parallel) is easier for the user and gives more reproducible behavioral results. In particular with nitinol filaments of lower diameter, the user can more reproducibly deliver the force/pressure. In addition, the diameter of fishing lines monofilaments used in the previous studies are wider than the nitinol filaments described here (0.009 in= 0.23 mm vs 0.004 in= 0.10 mm). Thus, the fishing line monofilaments are expected to stimulate a larger area of the epidermis and underlying neuronal cells. Fishing line monofilaments are thus likely to produce more epidermal/neuronal tissue damage in comparison to nitinol-based filaments (though there is no data on this as yet). A side by side comparison of nitinol filaments vs fishing monofilaments would help to clarify how they differ in stimulation accuracy and noxious stimulus-induced injury. We have not yet done this but we can attest that it is easier to create a set of filaments that span the full response range (especially the low end of the range, as here) using the nitinol filaments available in three different diameters.

3. The protocol states that mid-3rd instar larvae should be used. However, the described staging in 2.2. is very broad and it might not be trivial for beginners to identify the correct larvae. A more precise description of animal selection and/or staging would be useful, particularly as noxious responses change during larval development.

We have modified step 2.2. Please see page 4, lines 139-142, which states:

2.2. Collect 3rd instar larvae, after approximately 96 hours of egg laying, by gently squirting tap water into the soft fly food containing the larvae. Wandering larvae that have left the food, or which have everted anterior or posterior spiracles, are too large/old for this assay. Second instar larvae (~ less than 4 mm in length) are too small.

4. Using forceps as stated in the protocol might injure the larvae, so I would recommend using soft brushes for transferring animals during the experiment.

We understand the reviewer´s concern but in our experience and with practice the use of forceps does not induce any injury to the larvae if handle appropriately. We do recommend that the user practices maneuvering the larvae with forceps, before performing real experiments. Following the reviewer´s concern we have added a note, in protocol #2, step 2.4. please see page 4, lines 151-155, the note states:

NOTE: The transfer using forceps is based on mostly on water tension and not by applying pressure to the larvae with the forceps blades. An alternative to the use of forceps for maneuvering larvae is soft paint brushes. With either tool, the user should practice transferring the animals, so as not to cause unintended tissue damage that could complicate behavioral measurements.

5. The behavioral response to a specific force/pressure likely also depends on the substrate, e.g. vinyl tape used here. Have the authors explored other substrates including previously used agar plates?

We agree that different underlying surfaces might affect the behavioral response and also the tissue damage. However, we have not tried agar plates for our experiments. It would be useful to compare different surfaces side by side with respect to the behavioral response of the larvae but we have not yet investigated this.

6. The authors state that it is important to apply only one stimulus per animal, as otherwise the induced tissue damage might result in reduced responses. Previous work has however shown stronger responses to a 2nd subsequent mechanical stimulus suggesting acute sensitization of behavioral responses (Hu et al. Nat. Neurosci. 2017). It would be good to either test this with the new probes and/or to balance the discussion in this regard.

Following the reviewer´s comment we have balanced our discussion. Please see page 10, lines 408-415, which now state:

In another study, larvae stimulated twice with noxious mechanical probes mostly displayed an enhanced behavioral response (Hu et al., 2017), suggesting development of an acute mechanical sensitization (hyperalgesia), which might result from the tissue damage provoked by the first noxious mechanical stimulus. Conversely, other authors (Kim et al., 2012) reported a mixed (increased or decreased) behavioral response, indicating that the altered behavioral response could be due to damage/dysfunction of the neuronal tissue. Stimulating each larva only once eliminates possible variance in behavioral responses resulting either from sensitization or tissue damage.