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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**, **Zeiss Stemi 2000 stereomicroscope and Leica MZ6 stereomicroscope**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

    X Interview Statements are read by Authors outside. If the weather does not permit this, interview statements will be read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: ~**24**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Michael J. Galko** or **JoVE Voiceover Talent**: This protocol is significant because it allows the user to build a tool that can be used to precisely measure mechanical nociception responses in genetically tractable Drosophila larvae **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot outside, looking slightly off-camera OR Use script footage

REQUIRED:

1.2. **Roger Lopez-Bellido** or **JoVE Voiceover Talent**: The main advantage of this technique is that it uses simple materials to build custom tools that can be used to measure mechanical nociception from the sub-threshold to fully responsive range **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera OR Use 2.1., 2.4., and/or 2.6.

OPTIONAL:

1.3. **Michael J. Galko** or **JoVE Voiceover Talent**: These tools and methods can be used to measure baseline mechanical nociception and nociceptive sensitization following injury or disease **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera OR Use 4.3.2. Probe being applied

OPTIONAL:

1.4. **Roger Lopez-Bellido**: Probing larvae with custom Von Frey filaments takes practice. Users should be able to generate reproducible dose-response curves with control larvae before attempting an experiment **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera OR Use 4.4.1. Talent recording response

Protocol

2. Mechanical Probe Construction

2.1. To construct a mechanical probe, use a small wire cutter to cut each nitinol filament to the specified length perpendicular to its long axis **[1]**.

2.1.1. WIDE: Talent cutting filament

2.2. The filaments come in three different pre-set diameters **[1]**.

2.2.1. LAB MEDIA: Figure 1B

2.3. Examine the tip of the filaments under a stereomicroscope **[1]** to ensure that no sharp or irregular edges remain that could cause tissue damage to the larva skin and interfere with the calibration **[2]**.

2.3.1. Talent placing filaments under microscope
2.3.2. SCOPE: Shot of filament tip(s)

2.4. Then use a sharpening stone to manually smooth the edges of the probe until no sharp irregularities persist **[1]**.

2.4.1. Probe being sharpened *Videographer: Important step*

2.5. Next, use a hypodermic needle to poke a hole near the end of a wooden popsicle stick **[1]**.

2.5.1. Hole being made

2.6. Apply wood glue to a single nitinol filament **[1]** and insert the glue-coated filament into the needle slot **[2-TXT]**.

2.6.1. Glue being applied *Videographer: Important step*
2.6.2. Filament being inserted *Videographer: Important step* **TEXT: Allow glue to dry approximately 5 h**

2.7. When the glue has dried, press the probe against a scale until the probe bends to determine the maximum force that can be recorded in grams **[1-TXT]**.

2.7.1. Probe being pressed against scale, with scale readout visible in frame *Videographer: Important step*

2.8. Use the formula to convert the recorded mass to force in millinewtons **[1-TXT]**. Then divide the measured force **[2]** by the surface area of the filament tip **[3]** to convert the calculated force to kilopascals of pressure **[4]**.

2.8.1. BLACK TEXT WHITE BACKGROUND: f = ma
2.8.2. LAB MEDIA: Figure 1M*Video Editor: please emphasize force column*
2.8.3. LAB MEDIA: Figure 1M *Video Editor: please emphasize area column*
2.8.4. LAB MEDIA: Figure 1M *Video Editor: please emphasize pressure column*

2.9. Preparing multiple probes using filaments of different diameters and lengths will generate a full set spanning the responsive range for *Drosophila* larvae **[1-TXT]**.

2.9.1. Shot of range of probes OR LAB MEDIA: Figure 1N **TEXT: Check probes every 3-4 wks; Replace when pressure deviates ± 3% from original measure**

3. Preparation of Larvae

3.1. To prepare *Drosophila* larvae for an experiment, raise the larval progeny on standard food in a 25-degree Celsius incubator for about 96 hours **[1]**.

3.1.1. WIDE: Talent placing progeny into incubator

3.2. When the larvae reach the third instar stage, pour the soft fly food contents into a clean standard size Petri dish **[1]** and use forceps to gently sort the medium-sized, mid third instar larvae from the smaller, second and early third instar and larger, late or wandering third instar larvae **[2]**.

3.2.1. Talent pouring food contents into dish
3.2.2. Larvae being sorted *Video Editor: please emphasize smaller second/early third and larger late/wandering larvae when mentioned*

3.3. Then use the forceps to transfer 20-30 mid third instar larvae into a small Petri dish containing a small plug of fly food moistened with water at room temperature **[1]**.

3.3.1. Talent adding larvae to dish with fly food plug

4. Mechanical Nociception Assay

4.1. To perform a mechanical nociception assay, use forceps to place a mid-third instar larva onto a dark, thin, vinyl pad under a bright field stereomicroscope **[1-TXT]** and arrange optical fiber lights between the microscope objective lenses and the pad **[2]**.

4.1.1. WIDE: Talent placing larva onto pad *Videographer: Important step* **TEXT: Discard larvae that do not exhibit normal locomotion following pad transfer**
4.1.2. Talent arranging lights *Videographer: Important step*

4.2. Use a paper towel to wipe away any excess water surrounding the larva **[1]** and move the pad to orient the head and mouth of the larva toward the non-dominant hand of the researcher **[2]**.

4.2.1. Water being wiped *Videographer: Important step*
4.2.2. Pad/larva being oriented *Videographer: Important step*

4.3. Select a mechanical probe **[1]** and apply the probe to the posterior dorsal side of the larva at approximately abdominal segment A8 for 1-2 seconds **[2]**, carefully compressing the larvae into the underlying pad at the point of probe contact until the probe bends and elicits the previously measured amount of pressure **[3]**.

4.3.1. Talent selecting probe *Videographer: Important step*
4.3.2. Probe being applied *Videographer: Important/difficult step*
4.3.3. LAB MEDIA: Video 3: 00:08-00:12 *Videographer: Important/difficult step*

4.4. A positive nociceptive response is indicated if the larva shows a complete corkscrew roll of 360 degrees along the axis of its body within 3 seconds **[2]**. Record the behavioral response for each larva **[1]**.

4.4.1. Talent at computer, recording response, with monitor visible in frame NOTE: Place 4.4.2 before 4.4.1.
4.4.2. LAB MEDIA: Video 2: 00:06-00:11

4.5. Then discard the tested larva **[1]** and prepare the next larva for the assay **[2-TXT]**.

4.5.1. Talent discarding larva
4.5.2. Talent placing larva onto pad **TEXT: Repeat 3-6 sets of 10 larvae/probe**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.4., 2.6., 2.7., 4.1.-4.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
4.3. User has to be patient coming onto the larva with the probe, especially for the lower pressure ones that can be a little wobbly.

Results

5. Results: Representative Mechanical Nociception and Hypersensitivity

5.1. These customized mechanical probes with nitinol filaments can be used to elicit mechanically evoked behaviors **[1]** and to generate a full behavioral dose response curve using both innocuous and noxious mechanical probes of varying intensity **[2]**.

5.1.1. LAB MEDIA: Figure 2D
5.1.2. LAB MEDIA: Figure 2D *Video Editor: please emphasize dose response curve*

5.2. As these behavioral assay results demonstrate, probes that exert pressures of 200 kilopascals or less **[1]** do not provoke an aversive rolling response in *Drosophila* larvae **[2]**.

5.2.1. LAB MEDIA: Figure 1M *Video Editor: please emphasize P1 and P2 probe rows*
5.2.2. LAB MEDIA: Video 3: 00:04-00:16

5.3. As expected, these subthreshold, or non-noxious, mechanical probes do not elicit visible neuronal tissue damage **[1]**.

5.3.1. LAB MEDIA: Figure 2E *Video Editor: please emphasize 200 kPa image*

5.4. Conversely, suprathreshold, or noxious, probes elicit an augmented behavioral response **[1]** as well as tissue damage to the peripheral sensory neurons themselves in a dose dependent manner **[2]**.

5.4.1. LAB MEDIA: Figure 2D *Video Editor: please emphasize data points from 462-5116*
5.4.2. LAB MEDIA: Figure 2E *Video Editor: please emphasize 462-5116 kPa images*

5.5. The probes can also be used to measure mechanical hypersensitivity after injury **[1]**.

5.5.1. LAB MEDIA: Figure 3B

5.6. Approximately 20% of larvae respond with aversive rolling as early as 2 hours after UV treatment **[1]**, while 50% respond at 4 hours **[2]** compared to mock UV-irradiated animals **[3]**. Because the probe used for this analysis was a normally subthreshold 200 kilopascal, this response was considered to be mechanical allodynia **[4]**.

5.6.1. LAB MEDIA: Figure 3B *Video Editor: please emphasize 2h UV data bar*
5.6.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize 4h UV data bar*

5.6.3. LAB MEDIA: Figure 3B *Video Editor: please emphasize 2 and 4 h mock data bars*
5.6.4. LAB MEDIA: Figure 3B

5.7. At later time points, the behavioral response of the UV-treated larvae is slightly increased but not statistically different than that of the mock-irradiated control group **[1]**.

5.7.1. LAB MEDIA: Figure 3B *Video Editor: please add/emphasize ns and brackets over 8, 16, and 24 h data bars*

5.8. Larvae probed with a 462-kilopascal probe at 4, 8, and 16 hours following UV treatment exhibit a significant increase in the aversive rolling response **[1]**, with 4 hours being the peak of the behavioral hypersensitivity **[2]**. As this stimulus was initially noxious, the response was considered to be mechanical hyperalgesia **[3]**.

5.8.1. LAB MEDIA: Figure 3C *Video Editor: please add/emphasize P= texts and brackets over 4, 8, and 16 h data bars*
5.8.2. LAB MEDIA: Figure 3C *Video Editor: please emphasize 4h UV data bar*
5.8.3. LAB MEDIA: Figure 3C

Conclusion

6. Conclusion Interview Statements

6.1. **Roger Lopez-Bellido** or **JoVE Voiceover Talent***:* The probe preparation and larva selection and compression are critical to the success of the procedure **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.4, 2.6., 2.7., 4.1-4.3.) OR Use any of 2.4, 2.6., 2.7., 4.1-4.3.

6.2. **Roger Lopez-Bellido** or **JoVE Voiceover Talent**: Using these probes, both the baseline nociception and mechanical nociceptive hypersensitivity can be measured following injury **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera OR Use 4.3.2. Probe being applied

6.3. **Michael J. Galko** or **JoVE Voiceover Talent**: Using these tools and assay, the molecular and genetic bases of mechanical nociception and nociceptive hypersensitivity are able to be measured in the genetically tractable *Drosophila* model **[1]**.

6.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera OR LAB MEDIA: Figure 2D