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Light Spot-Based Assay for Analysis of Drosophila Larval Phototaxis

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Corresponding Author:	Zhefeng Gong Zhejiang University Hangzhou, Zhejiang Province CHINA
Corresponding Author's Institution:	Zhejiang University
Corresponding Author E-Mail:	zfgong@zju.edu.cn
Order of Authors:	Yixuan Sun
	Peipei Zhou
	Qianhui Zhao
	Zhefeng Gong
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Dear Editor,

On behalf my co-authors, I am submitting our revised manuscript “A light spot based assay for analysis of *Drosophila* larval phototaxis” to *the Journal of Visualized Experiments*. I hereby certify that this paper consists of details description of experimental procedures and is not under consideration for publication elsewhere.

We have revised the text according to suggestions from Editors and Reviewers. Additionally, we merge previous Figure 1 and Figure 2 into new Figure 1 to show the experimental setup. A simple cartoon demonstrating the parameters extracted from video are added to the time curves of the parameters in new Figure 2 (previously as Figure 3). And we added new Figure 3 to show data of larval groups.

The rebuttal to the Editorial and Reviewers are also attached with the manuscript.

We hope our paper be of interest to readers of your journal and meet the publishing criteria of *the Journal of Visualized Experiments*.

Thank you very much.

Sincerely yours,

Zhefeng Gong

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TITLE:

Light Spot-Based Assay for Analysis of *Drosophila* Larval Phototaxis

AUTHORS AND AFFILIATIONS:

Yixuan Sun[1], Peipei Zhou[1], Qianhui Zhao[1], Zhefeng Gong[1]

[1]Department of Neurobiology, School of Medicine, Zhejiang University, Hangzhou, China

Email addresses of co-authors:

Yixuan Sun (21718554@zju.edu.cn)
Peipei Zhou (zppconan@qq.com)
Qianhui Zhao (11818018@zju.edu.cn)

Corresponding author:

Zhefeng Gong (zfgong@zju.edu.cn)

KEYWORDS:

Drosophila, larva, phototaxis, light spot assay, video recording, visual system

SUMMARY:

This protocol introduces a light-spot assay to investigate *Drosophila* larval phototactic behavior. In this assay, a light spot is generated as light stimulation, and the process of larval light avoidance is recorded by an infrared light-based imaging system.

ABSTRACT:

The larvae of *Drosophila melanogaster* show obvious light-avoiding behavior during the foraging stage. *Drosophila* larval phototaxis can be used as a model to study animal avoidance behavior. This protocol introduces a light-spot assay to investigate larval phototactic behavior. The experimental set-up includes two main parts: a visual stimulation system that generates the light spot, and an infrared light-based imaging system that records the process of larval light avoidance. This assay allows tracking of the behavior of larva before entering, during encountering, and after leaving the light spot. Details of larval movement including deceleration, pause, head casting, and turning can be captured and analyzed using this method.

INTRODUCTION:

The larvae of *Drosophila* melanogaster show obvious light-avoiding behavior during the foraging stage. *Drosophila* larval phototaxis has been under investigation, especially in the past 50 years[1-8]. In recent years, despite the fact that 1) many neurons mediating larval light avoidance have been identified[4,5,9-12] and 2) the complete connectome of larval visual system at the resolution of synapses has been established[13], the neural mechanisms underlying larval phototaxis remain largely unclear.

A number of behavioral assays have been used in studying larval phototaxis. They can be largely divided into two classes: one involving spatial light gradients and the other involving temporal light gradients. For spatial light gradient assays, the arena is divided into equal number of sections in light and dark. The arena can be divided into light and dark halves[2,4] or light and dark quadrants[14,15], or can even be separated into alternate light and dark squares like on a checkerboard[7]. Usually, agar plates are used for spatial light gradient assay, but tubes that are divided into alternate light and dark sections can also be used[10,14].

In older version of assays, light illumination generally originates from below the larvae. However, illumination in newer versions largely originates from above, since larval eyes (e.g., the Bolwig's organs that are sensitive to low or medium light intensities[16]) are contained in the opaque cephalopharyngeal skeleton with openings towards the upper front. This makes larvae more sensitive to light from upper front directions than from below behind directions[7]. For temporal light gradient assays, the light intensity is spatially uniform in the arena, but the intensity changes over time. In addition to temporal square wave light (i.e., flashing on/off or strong/weak light[3,7]), temporally varying light that conforms to a linear ramp in intensity is also used[8] to measure the sensitivity of larvae to a temporally changing light stimulus.

A third type of phototaxis assay is the directional light scape navigation, which involves illumination from above at an angle of 45°[7]. Before the work of Kane et al.[7], only coarse parameters such as the number of larvae in light and dark regions, frequency of turning, and trail length were calculated in larval phototaxis assays. Since the work of this same group, with the analysis of high temporal resolution video record for larval phototaxis, detailed dynamics of larval movement during phototaxis (i.e., instant speeds of different parts of larval body, heading direction, turning angle and corresponding angular velocity) have been analyzed[7]. Thus, more details of larval phototaxis behavior have been able to be discovered. In these assays, larvae are tested in groups so that group effects are not excluded.

This protocol introduces a light-spot assay for the investigation of larval behavioral responses to individual light stimulation. The main experimental set-up consists of a visual stimulation system and infrared light-based imaging system. In the visual stimulation system, an LED light source generates a round 2 cm-diameter light spot on an agar plate, where the larva is tested. The light intensity can be adjusted using an LED driver. The imaging system includes an infrared camera that captures the behavior of the larva in addition to three 850 nm infrared LEDs that provide illumination for the camera. The lens of the camera is covered by an 850 nm band-pass filter to block light from the visual stimulation system from entering the camera, while the infrared light is allowed to enter the camera. Thus, interference of visual stimulation on imaging is prevented. In this assay, the behavioral details of the fast responses of individual larvae within a period including before, during, and after entering light are recorded and analyzed.

PROTOCOL:

1. Preparation of *Drosophila* larvae

1.1. Prepare standard medium consisting of boiled corn meal (73 g), agar (5.6 g), soybean meal (10 g), yeast (17.3 g), syrup (76 mL) and water (1000 mL).

1.2. Raise all flies at 25 °C on standard medium in a room with a 12 h/12 h light/dark cycle.

2. Preparation of agar plates

2.1. Prepare 1.0% agar solution. Weigh 3 g of agar in a 500 mL beaker with a balance, then add 300 mL of distilled water. Place a foil paper over the breaker to prevent the water from evaporating. Heat the beaker in a microwave to boil.

2.2. Take out the beaker and stir well with a glass rod, then heat in a microwave oven to boil. Repeat until the liquid is completely transparent and fluid.

NOTE: The final hot agar solution should be free of air bubbles; otherwise, pouring the agar into the plate will result in uneven and dented surface of the agar plate, which will affect subsequent larval behavior testing. The concentration of the agar solution should not be too high or low. If the concentration is too high, the light diffuse reflection on the agar plate will be strong and smear the light/dark boundary. If the concentration is too low, the larvae will leave traces on the plate. Both of these errors will hinder video processing later in the protcol.

2.3. Slowly pour the hot agar solution into a Petri dish (diameter 15 cm) until the bottom is evenly covered with a layer of agar (~4 mm in thickness), and cool at room temperature (RT) until the agar solution is solidified.

2.4. Agar plate should be used when it is freshly prepared. If it is not, pour a layer of water onto the surface and store it in the refrigerator at 4 °C for later use.

3. Set-up of visual stimulation system

3.1. Select the LED light source: collimated LED blue light at 470 nm or warm white light.

NOTE: The light source can be replaced with LED light of any other wavelength. In this experiment, blue light LED is used as an example.

3.2. Roll a thick piece of aluminum foil or black cardboard (ensure opacity) to form an open cylinder of 12 cm in length with a diameter similar to that of the blue-light LED with a diameter of 3 cm. Let the top end of the cylinder cover the front end of the blue-light LED. Cover the bottom end with a black cardboard with a small round hole (0.5 cm diameter) in the center. This constitutes the light source system set-up.

3.3. Fix the prepared light source onto the iron frame with a clip, making sure the LED light projects down towards the desktop. Tilt the cylinder slightly. The angle between the cylinder plane and vertical plane is about 10° (see **Figure 1**). Connect the 470 nm blue LED light to the "LED1" plug of the high-power LED driver. Turn on the driver, turn the knob in the upper right corner of the driver to select channel **470 nm**, then click **LED**. Then, when the screen displays "√", a blue light spot will appear on the desktop.

NOTE: If the cylinder leaks light in addition to the small round hole, it is recommended to use black tape on the leaking parts to make sure that only the hole can pass light through.

3.4. Click **OK** and turn the knob to adjust the intensity of the light. Rotate the knob to a higher light intensity of 50 mA. Measure and record the spectrum of the light with a spectrometer.

3.5. Move the position of the light source up and down to adjust the diameter of the light spot to 2 cm. The desktop should be black for a better contrast effect.

3.6. Rotate the knob to choose the light intensity according to experimental needs. Use a compact power meter console with a standard photodiode power sensor to measure the maximal and minimal light power in the spot, record it, measure three times, and take the average value.

NOTE: It is recommended to use a photodiode power sensor to measure the light intensity for light of a specific wavelength and use a thermal power sensor to measure the light intensity for white light.

3.7. Calculate the light intensity in the light spot by dividing the measured light power by the area of the sensor.

NOTE: For example, if the measured light power in step 2.6 is 20 pW and area of the sensor is 0.81 mm^2, the light intensity is 24.69 pW/mm^2 (dividing 20 pW by 0.81 mm^2).

4. Set-up of the imaging system

4.1. Clamp a high-resolution web camera with an iron clip, at about 10 cm above the light spot on the desktop (**Figure 1**).

4.2. Adjust the orientation of the camera lens towards the desktop. Connect the camera to a computer through a USB interface.

4.3. Place an agar plate on the desktop right beneath the camera.

4.4. Open the "Amcap9.22" software on the computer with Windows 7, and the light spot will be automatically shown in the window of AMcap. Move the camera slightly left or right to

ensure that the light spot is near the center of the window. Ensure that the camera does not block the light path. The light spot should be complete and round.

NOTE: The software can be found at http://amcap.en.softonic.com/.

4.5. Fix an 850 nm ± 3 nm band-pass filter with a clip at 5–7 mm right below the camera.

NOTE: The diameter of the filter is about 2.5 cm, and the camera lens is less than 1 cm in diameter, so the filter can cover the visual field of the camera. With the filter below the camera, the light spot should not be seen in the window of AMcap.

4.6. Place three infrared-light-generating LEDs (central wavelength = 850 nm) evenly around the agar plate. Each LED should be about 5 cm away from the edge of the agar plate, and the lens face of the LED should be at a 70° downwards angle towards the agar plate. Connect the LEDs to the power through the AC-to-DC converter.

NOTE: It is better to fix the positions and angles of the infrared light LEDs to ensure consistency of the brightness of the field in various experimental trials and facilitate later video processing.

4.7. Put a black board between the computer and the device. Set down the brightness of the computer screen to prevent the computer screen light from affecting the experiment.

NOTE: Keep the environment dark when measuring wavelength or intensity of the light.

5. Setting parameters of imaging

5.1. On the menu of the AMcap software, choose **Options** | **Video Device** | **Capture format**, and set the pixel size of the captured video to 800 x 600 and frame rate to 60 fps.

5.2. Remove the filter from beneath the camera, put a ruler under the camera and adjust the camera focus to make the scale line clear and parallel to the width of the video field of view.

5.3. Click **Capture** | **Set up** | **Video capture** to select the save path, click **Start recording**, record the actual distance corresponding to 600 pixels, and calculate the ratio of each pixel to the actual distance.

6. Video recording of light avoidance behavior

6.1. Maintain a temperature of 25.5 °C through all experiments. Control room temperature with an air conditioner if required. Keep the humidity constantly at 60% with a humidifier.

6.2. Take a short video of the light spot position named “lightarea1”. Then, move the 850 nm ± 3 nm filter back to cover the camera lens.

NOTE: When recording larval behavior, the camera lens is covered by the 850 nm ± 3 nm filter so that the light spot is not shown in the video. The light spot can be reconstructed in videos with larvae later with Matlab. Do not change the position of the camera, and avoid changing the ratio of each pixel to the actual distance measured in step 4.3.

6.3. Turn on a light (i.e., a room light) far away from the experimental device. Turn down the light as low as possible, as long as the larvae can be clearly seen with the eyes. Take the larvae out of the culture medium with a spoon, gently pick a third-instar larva, and wash it clean with distilled water. Be careful to wash larva one at a time to avoid interference from hunger. A single experiment requires at least 20 larvae.

6.4. Transfer the larva to the center of the agar plate placed beneath the camera during step 3.3. Gently remove excess water from the larva with a brush or use blotting paper to remove water from the larva to prevent reflection of light under the lens. Turn off the room light and allow the larva to acclimate for 2 min in the dark environment.

6.5. Turn on the LED light to generate infrared light, and gently brush the larva to the center of the plate. When the larva begins to crawl straight, rotate the plate to make the larva head towards the light spot. Make sure that it crawls straight from the start, or else it may not obtain access to the light spot.

6.6. Click **Capture** | **Set up** | **Video capture** to select the save path, then click **Start recording** to record. Allow the larva to crawl towards the light spot, enter the light spot, then leave the light spot until it is nearly out of the field of view. Click **Stop recording**. If the larva turns away from the light spot before getting close, directly click **Stop recording**.

6.7. Move the filter away from the camera. Take a short video of the position of the light spot named “lightarea2” and compare it to “lightarea1” to ensure that the light spot position is not changed. If an obvious position change is observed, discard the data.

7. Data analysis

7.1. Use SOS[17] to extract animal body contour and movement parameters from videos using image processing methods as previously described[17].

NOTE: Parameters including headSpeed (velocity of larval head), tailSpeed (velocity of larval tail), midSpeed (velocity of larval midpoint of skeleton line), and cmSpeed (velocity of larval centroid) were used to measure larval movement speed. Parameters including headTheta (the angle between the lines of head-midpoint and midpoint-tail) and headOmega (the changing speed of headTheta) were used to measure larval body bending and the angular speed of bending.

REPRESENTATIVE RESULTS:

According to the protocol, the light spot assay was used to investigate light avoidance behavior of third instar larva that were raised at 25 °C on standard medium in a room with a 12 h/12 h light/dark cycle. A single w^{1118} larva was tested using the light spot assay at 25.5 °C. The average light intensity of the light spot generated by a 460 nm LED was 0.59 μW/cm^2. The whole process of larval entering and exiting the light spot was recorded and analyzed using SOS software and custom written scripts[12,17]. Time curves of tail speed, body bending angle, and angular speed of body bending of a representative larva are shown in **Figure 2** and **Movie 1**.

To investigate the effects of octopaminergic neurons on larval light avoidance, third instar larvae with octopaminergic neurons inhibited by expressing tetanus toxin (*UAS-TNTG*) with a *Tdc2-Gal4* driver were tested with the light-spot assay. As shown in **Figure 3**, the size of the larval head cast (maximal body bending angle) was significantly reduced compared to the parental controls, indicating that the *Tdc2-Gal4* neurons are necessary for a normal larval light response.

FIGURE LEGENDS:

Figure 1: Experimental set-up. (**A**) Schematic representation of the set-up for the light spot-based larval fast phototaxis assay. The blue lines represent the paths of visible light used as visual stimulation, and the red lines represent the paths of infrared light. Arrows indicate the direction of the light. The 850 nm band-pass filter allows infrared light to pass, but it blocks visible light. (**B**) An image of the set-up for the light spot assay. It should be noted that the image was taken under light conditions for better visualization.

Figure 2: Quantitative description of the reaction of a larva when entering a light spot. (**A**) A diagram showing the parameters used in measuring larval body movement. The contour of a larva is shown in thin line. The thick line shows the skeleton of thinned larval body contour. The two ends and midpoint of skeleton line are assigned as positions of larval head, midpoint and tail. The angle between the line from the head to midpoint and the line from midpoint to tail is the body bending angle. The speed of the change of body bending angle over time is defined as angular velocity of larval head cast. Represented here are tail speed (**B**, tailspeed), head cast angular velocity (**C**, headomega), and body bending angle (**D**, headtheta) of a w^{1118} larva that enters and leaves a light spot. Green lines mark the timepoint that the larval head entered and left the light spot. The time window of a strong deceleration period is in yellow. Arrow heads point to deceleration periods and related peaks in head cast angular velocity and body bending angle. The behavioral process is shown in **Movie 1**. This figure has been modified from Gong et al.[12].

Figure 3: Inhibiting *Tdc2-Gal4* labeled neurons using tetanus toxin TNTG reduces the size of larval head cast in response to light spot entrance. **, $P < 0.01$, n = 81, 52, 92; Kruskal-Wallis test followed by post hoc Dunn's multiple comparison test was used. This figure has been modified from Gong et al.[12].

Movie 1: A *w*^{1118} larva enters and leaves a light spot in the light spot assay. Light spot with edge smoothed is in white. The track of larval head is shown. Corresponding curves of larval tailspeed, headtheta, and headomega are played simultaneously. This movie has been modified from Gong et al.[12].

DISCUSSION:

This protocol presents the light spot assay to test the ability of *Drosophila* larvae to escape from light. This assay allows tracking of the behavior of larvae before entering, during encountering, and after leaving a light spot. Details of larval movement can be captured and analyzed. The light spot assay is very simple and possesses strong practicability. The cost of the whole device is not high. In the experiment, LED light is used as the light source. It can be replaced with light sources of different wavelengths, if required. The light intensity can also be adjusted by the LED drive. The lowest light intensity in the spot can reach 1.80 pW/mm^2 (cold white light). Even at such a low light intensity, larvae still can sense the light and show light-avoiding behaviors[11].

It should be noted that the concentration of the agar plate is controlled between 0.8% and 1.0%. If the concentration is too high, scattering of light on the agar plate can be serious, and the size of light spot recognized in the video is exaggerated. Therefore, the brightness of the spot should not be too high. Since larvae in a light spot are hardly visible, if visible light is used for illumination, it is necessary to use infrared light to illuminate the larva and add an 850 nm band-pass filter on the camera to prevent the light spot signals from entering the camera. The video of larval response to light spots can be synthesized later based on the larva-only and light-spot-only videos.

The light spot assay possesses three main virtues: 1) the process of larval light avoidance can be monitored and analyzed in detail; 2) larval light response is tested only once, so that the possible involvement of light adaptation can be excluded; and 3) possible effects on light response from other larvae can be excluded. One obvious disadvantage of this assay is that it is low throughput, since only one larva is tested at a time. Although this assay is used here mainly at low intensities of light[11,12], it can also apply to larval avoidance in strong light that can excite class IV DA neurons that tile the surface of body walls[16].

Our experimental device can also be used for optogenetics. The 850 nm band-pass filter can block the excitation light, as it does for the light spot signal, so that the camera can record larval behaviors before, during, and after red light stimulation clearly. Specifically, when 620 nm red light is used in combination with Chrimson for optogenetic stimulation, the low halves of infrared light LEDs need to be masked, and the direction of red light should be well-controlled to image the larvae clearly. Meanwhile, moderate levels of noisy signals originating from red light in the image can be used to judge the timing of on/off stimulation. In short, the light spot assay provides an addition method to monitor and analyze detailed spatial and temporal properties of the rapid larval light avoidance behavior.

ACKNOWLEDGEMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1-new.tif

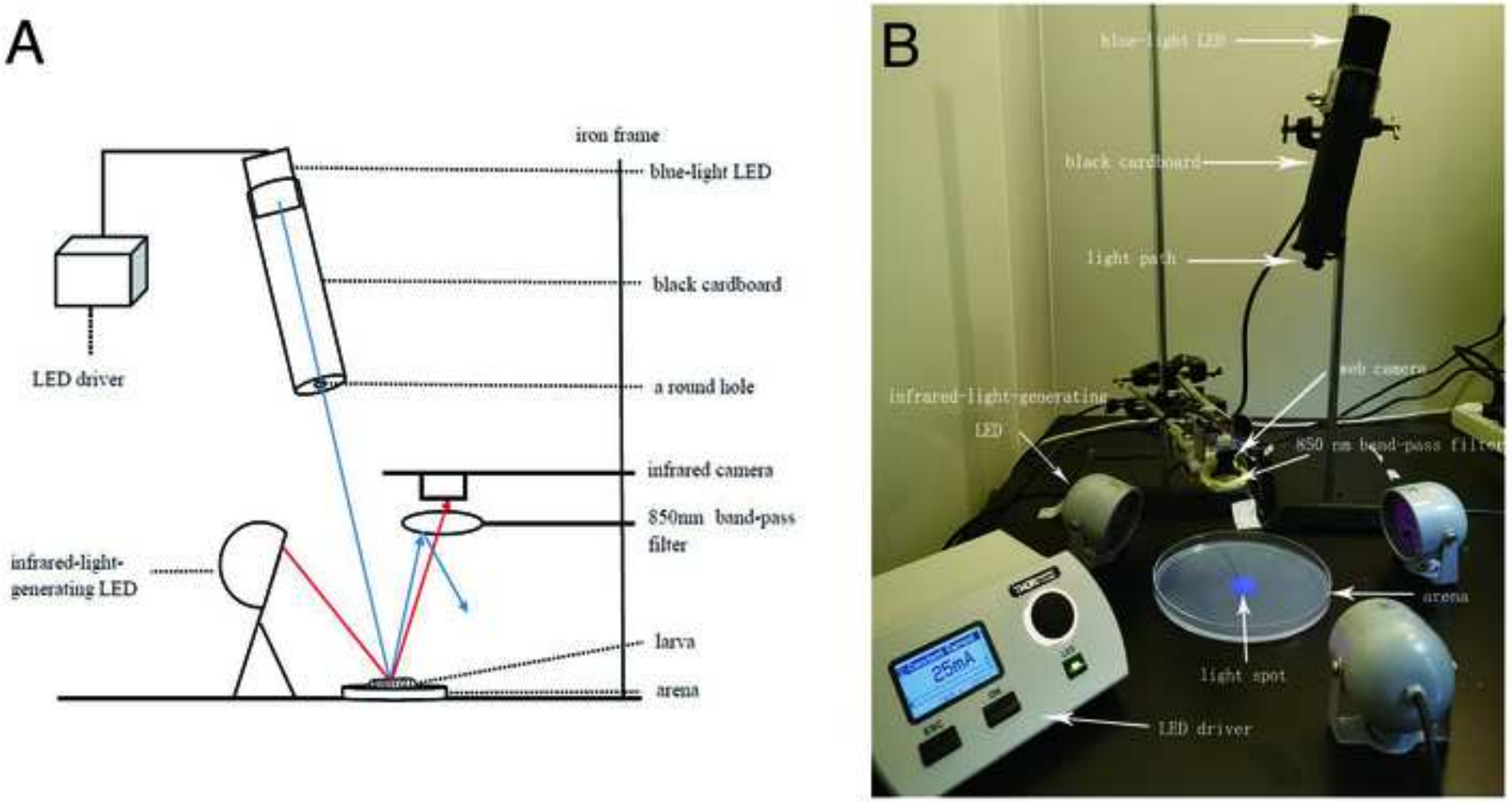

Figure 2

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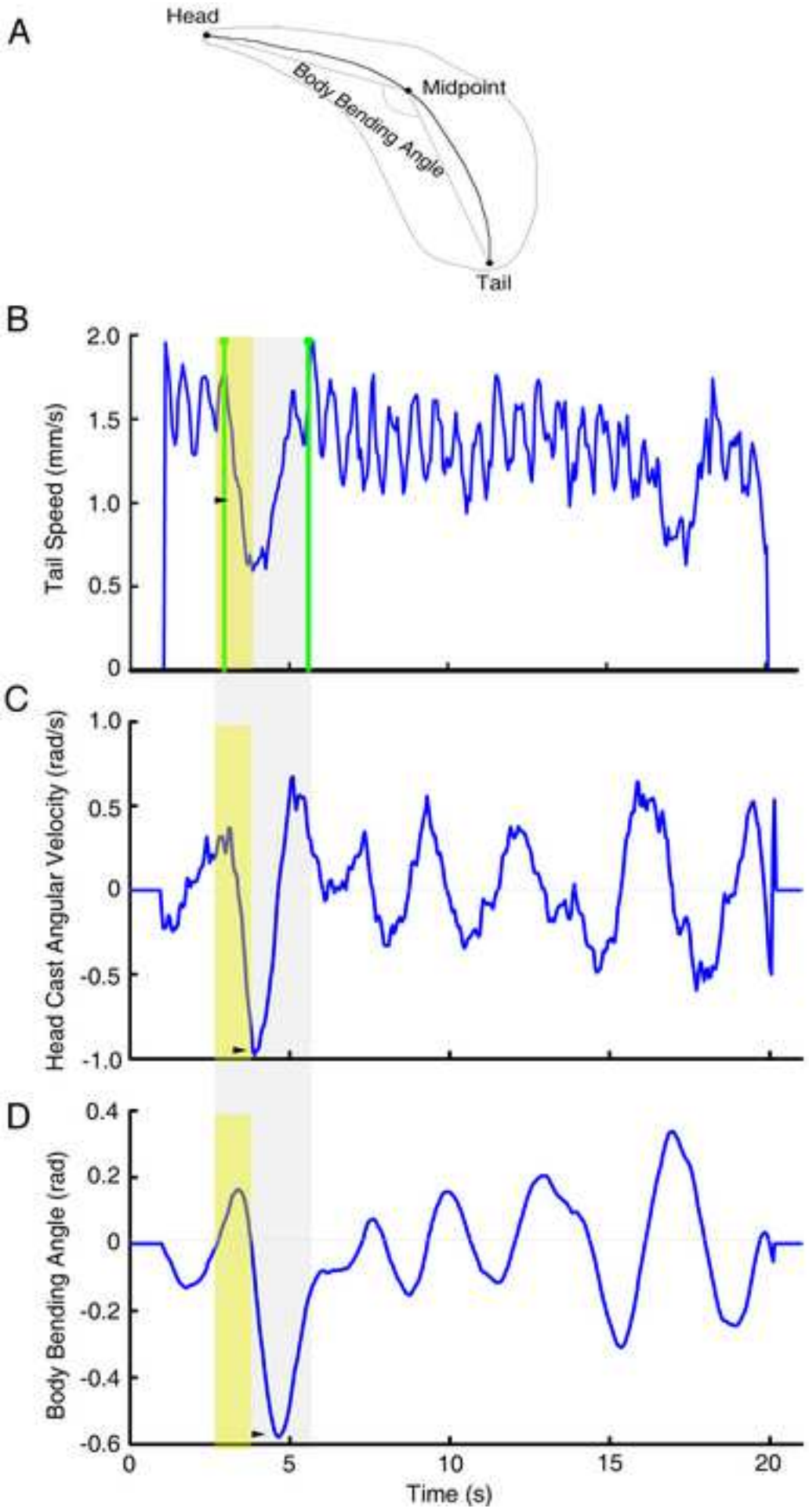

Figure 3

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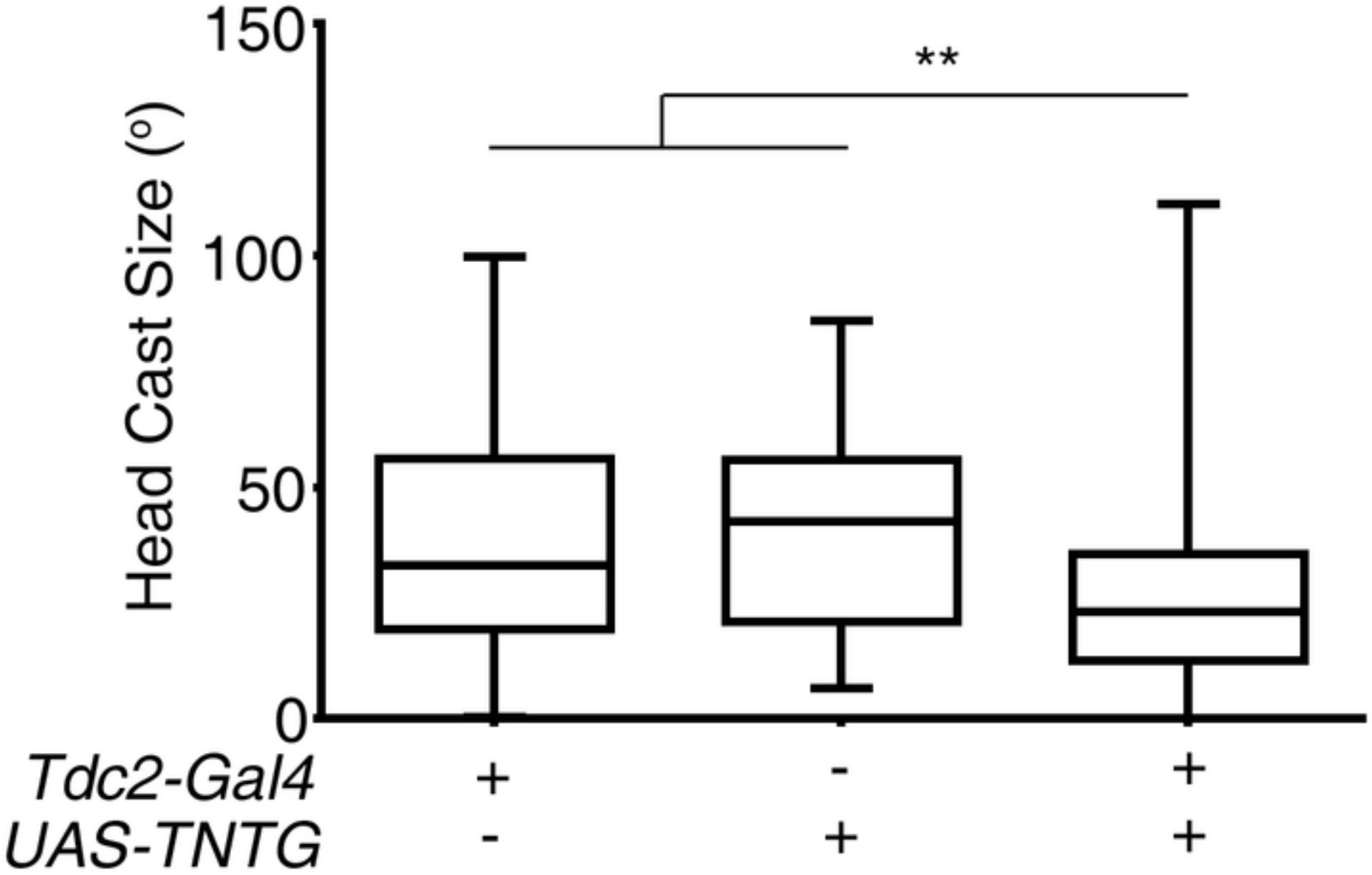

Video or Animated Figure

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Video or Animated Figure
Movie 1.mp4

Click here to access/download;Table of Materials;60235_Table_of_Materials-2.xlsx

Name of Material/Equipment	Company	Catalog Number
850 nm ± 3 nm infrared-light-generating LED	Thorlabs, USA	PM100A
AC to DC converter	Thorlabs, USA	S120VC
band-pass filter	Thorlabs, USA	DC2100
Collimated LED blue light	ELP, China	USBFHD01M

Compact power meter console	Ocean Optics, USA	USB2000+(RAD)
High-Power LED Driver	Minhongshi, China	MHS-48XY

high-resolution web camera	Thorlabs, USA	MWWHL4
LED Warm White	Mega-9, China	BP850/22K
Spectrometer	Noel Danjou	Amcap9.22
Standard photodiode power sensor	Super Dragon, China	YGY-122000

Thermal power sensor	Thorlabs, USA	M470L3-C1
Thermal power sensor	Thorlabs, USA	S401C

Co mments/Description

Compatible Sensors: Photodiode and Thermal
Optical Power Rangea: 100 pW to 200 W
Available Sensor Wavelength Rangea: 185 nm-25 µm Display Refresh Rate: 20 Hz
Bandwidtha: DC-100 kHz
Photodiode Sensor Rangeb: 50 nA-5 mA
Thermopile Sensor Rangeb: 1 mV-1 V
Aperture Size: Ø9.5 mm
Wavelength Range: 200-1100 nm
Power Range: 50 nW-50 mW
Detector Type: Si Photodiode (UV Extended)
Linearity: ±0.5%
Measurement Uncertaintyc: ±3% (440-980 nm), ±5% (280-439 nm), ±7% (200-279 nm, 981-1100 nm)
LED Current Range: 0-2 A
LED Current Resolution: 1 mA
LED Current Accuracy: ±20 mA
LED Forward Voltage: 24 V
Modulation Frequency Range: 0-100 kHz Sine Wave
Modulation: Arbitrary
Max. Resolution: 1920X1080
F6.0 mm
Sensor: 1/2.7" CMOS OV2710

Dimensions: 89.1 mm x 63.3 mm x 34.4 mm
Weight: 190 g
Detector: Sony ILX511B (2048-element linear silicon CCD array)
Wavelength range: 200-850 nm
Integration time: 1 ms – 65 seconds (20 seconds typical)
Dynamic range: 8.5 x 10^7 (system); 1300:1 for a single acquisition
Signal-to-noise ratio: 250:1 (full signal)
Dark noise: 50 RMS counts
Grating: 2 (250 – 800 nm)
Slit: SLIT-50
Detector collection lens: L2
Order-sorting: OFLV-200-850
Optical resolution: ~2.0 nm FWHM
Stray light: <0.05% at 600 nm; <0.10% at 435 nm
Fiber optic connector: SMA 905 to 0.22 numerical aperture single-strand fiber

Working voltage: DC12V
Central wavelength: 850nm

Color: Warm White
Correlated Color Temperature: 3000 K
Test Current for Typical LED Power: 1000 mA
Maximum Current (CW): 1000 mA
Bandwidth (FWHM): N/A
Electrical Power: 3000 mW
Viewing Angle (Full Angle): 120˚
Emitter Size: 1 mm x 1 mm
Typical Lifetime: >50 000 h
Operating Temperature (Non-Condensing): 0 to 40 °C
Storage Temperature: -40 to 70 °C
Risk Groupa: RG1 – Low Risk Group

Ø25.4(+0~-0.1) mm
Bandwidth: 22±3nm
Peak transmittance:80%
Central wavelength: 850nm±3nm

AMCap is a still and video capture application with advanced preview and recording features. It is a Desktop application designed for computers running Windows 7 SP1 or later. Most Video-for-Windowsand DirectShow-compatible devices are supported whether they are cheap webcams or advanced video capture cards.

Input: AC 100-240V~50/60Hz 0.8A
Output: DC 12V 2A

Color: Blue
Nominal Wavelengtha: 470 nm
Bandwidth (FWHM): 25 nm
Maximum Current (CW): 1000 mA
Forward Voltage: 3.2 V
Electrical Power (Max): 3200 mW
Emitter Size: 1 mm x 1 mm
Typical Lifetime: 100 000 h
Operating Temperature (Non-Condensing): 0 to 40 °C
Storage Temperature: -40 to 70 °C
Risk Groupb: RG2 – Moderate Risk Group
Wavelength range: 190 nm-20 µm
Optical power range:10 µW-1 W(3 Wb)
Input aperture size: Ø10 mm
Active detector area: 10 mm x 10 mm
Max optical power density: 500 W/cm^2 (Avg.)
Linearity: ±0.5%

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Title of Article: A Light-Spot based assay for analysis of Drosophila larval phototaxis

Author(s): Yixuan Sun, Peipei Zhou, Guanhui Zhao and Zhefeng Gong

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CORRESPONDING AUTHOR

Name:	Zhefeng Gong
Department:	Department of Neurobiology, School of Medicine
Institution:	Zhejiang University
Title:	Professor
Signature:	Zhefeng Gong
Date:	2019. 4. 28

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Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi.

==========

We have adjusted all images to be 300dpi.

==========

Additionally, please upload tables as .xlsx files. Your revision is due by Jun 11, 2019. To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article. Best,Peer Review,Peer ReviewJoVE617.674.1888Follow us: Facebook | Twitter | LinkedInAbout JoVE_____

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==========

Thanks for your comments. We have looked through the whole manuscript and ensure that there are no spelling or grammar issues.

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====== ==========

We have looked through the whole manuscript and modified all units using SI abbreviations.

====== ==========

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===== ==========

We have removed all commercial language from the manuscript.

===== ==========

6. Please include single line spacing between each numbered step or note in the protocol.

======= ==========

We have added single line spacing between each numbered step and note.

======= ==========

7. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

===== ==========

We appreciate your suggestion. We have revised the Protocol text to avoid the use of personal pronouns.

===== ==========

8. Please use the active/imperative voice and complete sentences throughout the protocol.

We have checked whole manuscript and make sure we use the active/imperative voice and complete sentences throughout the protocol.

9. Lines 200-211: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We should explain here that here the numbers are not steps but parameters obtained from the software SOS. In order to avoid misunderstanding, letters are used instead of numbers. We use "a, b, c, d, e and f" to show the parameters separately.

10. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

We have highlighted protocol text in yellow which can be featured in the video.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

We have followed your instruction throughout the manuscript.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We make sure that the highlights include all relevant details.

13. Figure 1 and Figure 2: Please include a space between the number and its unit (850 nm).

====== ==========

We have added a space between "850" and "nm".

====== ==========

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===== ==========

We have uploaded Movie 1 as "video or animated figure" to our Editorial Manager account in form of .mp4.

===== ==========

15. References: Please do not abbreviate journal titles; use full journal name.

===== ==========

We have changed journal title into full name.

===== ==========

Reviewers' comments:

Reviewer #1:Manuscript Summary:Drosophila larvae shows robust negative phototaxis at the foraging stage. Considering their relatively simple nervous system and the accumulated knowledge about the relevant genetics and neuroanatomy, light-avoiding behavior of Drosophila larvae is an excellent model to investigate the detailed molecular, cellular, and systems mechanisms underlying regulation of behavior by taxis. In this study, Sun et al. described a new protocol to quantitatively analyze larval light-avoiding behavior. This is a nice addition to the currently available protocol for larval negative phototaxis because it is simple and effective, and the assay system can be easily set up at a low cost in any lab. For the benefit of the readers, however, several points need clarifying.

Major Concerns:

In Fig. 3 legend, clarify the definition of "the time point that larva entered and left the light spot". Does "entered" mean that any larval body part is inside of the light spot? Does "left" mean that the entire body is outside of the light spot"? The authors may only care the anterior part of the body where larval eyes, the Bolwig organ, is located. The authors mentioned the Bolwig organ as a light sensor. However, they failed to point out that the larval class IV dendritic arborization neurons respond to ultraviolet, violet and blue light, and are major mediators of light avoidance (Xiang et al. Nature. 2010, 468: 921-926). Since the class IV dendrites

cover the entire larval body, it is important to monitor how the larval body is exposed to light when they are displaying particular behaviors in response to light.
==========

We thank the reviewer for the very important remind.

We used larval head to indicate larval entering and leaving light spot. In the processing of larval image, the larval is thinned into a skeleton line of head-midpoint-tail. After thinning, the point of larval head is actually a little posterior to the actual anterior tip of larval body, so that the point of larval head is close to the actual position of larval Bolwig's Organs.

For the issue of involvement of class IV DA neurons in light avoidance, we can adjust light intensity to below the threshold of class IV DA neuron excitation. Nevertheless, even if high intensity light is used and class IV DA neurons are involved, the light spot assay can still be used. We have explained this in the manuscript in Introduction and Discussion.

==========

Minor Concerns:

Line 76: The described method may lead to agar concentrations higher than 1.0% due to water evaporation during heating. The authors mentioned that the assay requires the appropriate agar concentration. Why don't you adjust the volume to 300 ml with hot water after agar is completely dissolved?

========== ==========

In the experiment, we put a foil paper over the beaker to keep the water from evaporating to ensure that the concentration of agar are about 1.0%, and we add this detail in the manuscript in Line 80-81. The concentration should not be too high, but a little more than 1% is acceptable.

It is a nice idea to add hot water after agar is completely boiled. We will try. However, it may not be easy to make sure that agar solution concentration is completely even after adding hot water.

========== ==========

Line 127: Windows 7 is outdated.

==========

"AMcap" can also work on Windows 10. Users can choose the operating system according to their own needs. In our experiments, we used Windows 7.

==========

Line 181: Describe how to remove excess water from the larva.

========= ==========

We use a brush to gently brush the water off the larvae or a blotting paper to suck the water off the larvae. We add this detail in Line 179-180. The goal of the step is to keep the larva from reflecting light under the lens.

======== ==========

Line 208-210 & Fig. 3: Present simple schematic diagrams in Fig. 3 insets to show exactly what is measured (e.g., which angle is "body Bending Angle" and which movements correspond to "Head Cast Angular Velocity"?).Fig. 3, top panel, "the deceleration periods are labeled in magenta": How were the deceleration periods determined? For example, tail speed is significantly reduced at the end of the Tail Speed graph in Fig. 3 (top panel), but the corresponding part was not labeled in magenta. Why?

==========

We thank the reviewer for the suggestions. In Figure 3 (now as Figure 2), we have put a cartoon to demonstrate the key parameters measured. See Figure 2A.

We mentioned deceleration because the Figure 3 (now as Figure 2) was directly from the cited paper (Gong et al., 2019) which dealt with the stop and turn behaviour in Drosophila larva. We have removed the deceleration related marks in the figure and revise the figure legends since emphasizing on deceleration is not the central topic of this paper.

==========

Fig. 3, legend, Line 323: What is DOT?

==========

We have removed this because it was from the cited paper (Gong et al., 2019). DOT is the short for Deceleration-HeadOmage-HeadTheta, which was defined based on changes in tailspeed, headomega (angular velocity of body bending angle) and headtheta (body bending angle). We used DOT to judge a stop-and-turn behaviour.

==========

Reviewer #2:Manuscript Summary:This manuscript details an assay that can be used to measure light-avoidance behavior in Drosophila larvae on a single-animal level using an automated tracking system. The authors make a good case that larval light-avoidance remains relatively uncharacterized on a molecular/cellular/circuit level. The authors suggest that one hurdle preventing this sort of characterization is the relatively coarse nature of previous analyses, which focus on macro-scale parameters (location, velocity, etc.) of groups of animals. The behavioral assay described here provides an opportunity to analyze the component parts of the light avoidance response (head casting angle, turn angle, etc.) in single animals. The authors describe the construction of the light-spot assay apparatus, execution of the behavioral assay, and data acquisition with generally strong detail, however, additional detail is required to explain appropriate data analysis, experimental design, and interpretation. Should these detail be included and some minor corrections be undertaken, this manuscript should be suitable for publication.

Major Concerns:

1. The authors have given largely appropriate detail to construction of the behavioral apparatus, assay execution, and data acquisition, but this is where the protocol ends. I think that for this protocol to be useful for a laboratory that is trying to establish this assay, details about experimental design, sample size, and statistics are absolutely required. For example, the behavioral traces in Figure 3 are excellent examples of single-animal data, but which features of these behavior traces can be extracted for statistical analysis (and how is this done)? What sort of sample size is required for robust analysis? Which statistical tests are appropriate for analyzing these data? This should all be explained in the protocol. It would also be appropriate to include a figure showing group-level data.

==========

We thank the reviewer for the valuable suggestions.
We have added the description of the features of the behaviour traces in new Figure 2 (as Figure 2A). Also we have added group data in Figure 3 in which blocking octopaminergic motor neurons undermines light induced larval turning angle. The details of statistic for analyzing the data are added in the legend of Figure 3 in Line 344-345.

==========

2. The manuscript would be strengthened by including data from a non-control genotype. The power of this assay is its ability to detect defects in light-avoidance, but the authors do not describe what this kind of defect would look like. Including representative data showing a light-avoidance defect would be extremely useful for a laboratory setting up this assay.

=== ==========

We have added new Figure 3 to describe the effect of blocking octopaminergic neurons on light induced larval turning angle. Light induced larval turning angle in the experimental group and the control groups are shown. This data is directly from Figure 4c in Gong et al., 2019.

=== ==========

3. The description of the LED tube construction (lines 98-102) was somewhat confusing. I suggest revising this text for clarity and possibly including a schematic diagram of this construction.

======= ==========

We have revised the text, hoping to improve clarity.
We indicated the position of the black cardboard in Figure 1. The LED and the tin foil are separately drawn in Figure 1 for better understanding of the internal structure.

In line 105, we changed "LED light" into "blue-light LED" to keep it consistent with Figure 1.

======= ==========

Minor Concerns:

1. The manuscript is largely clearly written, but there are some passages with grammar and usage problems. I suggest that additional copy-editing is needed. For instance, the sentence in lines 33-36 is somewhat confusing grammatically and contains overly informal language (e.g. "a bunch of neurons").

========== ==========

We thank the reviewer for the pointing out the problems. We have rewritten the sentences.

========== ==========

2. In the introduction, the authors seem to suggest that one of the major advances of their experimental method is that it captures single-animal data, whereas previous analyses capture data at group level. If this is the argument being made, the authors should describe this more clearly. In general, it would be useful to have a clearer description of how this new assay is an advance over previous methods.

========== ==========

We did compared with previous analyses in Introduction, but we did not mean that single animal data is the advantage of our assay. We have rephrased the sentence in Introduction to avoid misunderstanding. The advantage of single animal assay is that interaction between individuals can be avoided. Actually we pointed out in Discussion that the low throughput is a disadvantage. We mentioned these also in Discussion, in line 250-251.

========== ==========

3. The authors explain light avoidance in terms of photoreceptors in Bolwig's organ, which seems to be the most relevant mechanism for the behavior being studied. However, the multidendritic neurons can also mediate light-avoidance behavior (see Xiang et al. 2010). Is this relevant to this assay? Even if it isn't, I would state this explicitly in order to avoid confusion.

==========

We thank the reviewer for the suggestion.
For the possible involvement of MD neurons in light avoidance, we can only use light of low intensities that are below the threshold of class IV DA neuron excitation. We have explained this in the manuscript in Introduction and Discussion.

==========

4. It would be useful to include a representative example of the intensity calculation described in lines 118-119.

======== ==

We have added an example in Line 119-121 "For example, the measured light power in step 2.6 is 20 pw, the area of sensor is 0.81 mm², we can calculate the light intensity by dividing 20 pw by 0.81 mm² is 24.69 pw/mm²".

======= ==================================

5. I would include a reference for the AMcap software (line 127) - this could be a scholarly reference or a permanent url for the software. I would also suggest including a version number for the software to ensure consistency.

==========

Thanks for the very nice suggestion! We can use AMcap9.22 which can be freely loaded from the site http://amcap.en.softonic.com/. We added this detail in Line 131.

==========

6. Some additional detail may be needed about use of the SOS package (or citation of a published protocol).

===== ==========

We have added citation for the use of SOS in line 199-200.

===== ==========

7. Lines 249-250 suggest some instability in the AMcap software. Can this be described in a bit more detail?

==========

The stability of AMcap may depend on the computer. We have not figured out the reason. Actually it can work well for long time on other computers with win7 or win10. Generally 2 min is enough for one larval light avoidance test.

To avoid confusion, we have removed this part.

==========

8. The optogenetic methods mentioned in the discussion (lines 251-256) seem like they should be included in the protocol itself, as they describe some technical considerations that are important for implementation.

==========

In this article, we introduced our light-spot assay mainly for investigation of larval behavioral responses to light stimulation. In the discussion, we

mentioned that this assay is also suitalbe for optogenetics, but this is not the focus of this article's. Rather, we added more details about application of optogenetics in this assay in line 257-261.

==========

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Author Signature
Zhefeng Gong

Address & E-mail address
Zhejiang University, School of Medicine
zfgong@zju.edu.cn

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