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Using Looming Visual Stimuli to Evaluate Mouse Vision

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March 8, 2019

Aaron Berard, PhD
Science Editor
Journal of Visualized Experiments

Dear Dr. Berard,
I would like to resubmit a manuscript entitled, “Using Looming Visual Stimuli to Evaluate Mouse Vision.” for consideration for publication in Journal of Visualized Experiments (JoVE).

We have thoroughly revised the manuscript based on Editors and Reviewers’ insightful comments. We feel that the manuscript is substantially improved with full explanations of the each experimental step.

Thank you very much for your consideration. I look forward to hearing from you soon.

Sincerely,

Tomomi Ichinose

TITLE:

Using Looming Visual Stimuli to Evaluate Mouse Vision

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KEYWORDS:

mouse behavior, vision test, flight, freeze, motion track, fear.

SUMMARY:

To examine mouse vision, we conducted a looming test. Mice were placed in a large square arena with a monitor on its ceiling. The looming visual stimulus consistently evoked freezing or flight reactions in the mice.

ABSTRACT:

The visual system in the central nervous system processes diverse visual signals. Although the overall structure has been characterized from the retina through the lateral geniculate nucleus to the visual cortex, the system is complex. Cellular and molecular studies have been conducted to elucidate the mechanisms underpinning visual processing and, by extension, disease mechanisms. These studies may contribute to the development of artificial visual systems. To validate the results of these studies, behavioral vision testing is necessary. Here, we show that the looming stimulation experiment is a reliable mouse vision test that requires a relatively simple setup. The looming experiment was conducted in a large enclosure with a shelter in one corner and a computer monitor located on the ceiling. A CCD camera positioned next to the computer monitor served to observe mouse behavior. A mouse was placed in the enclosure for 10 minutes and allowed to acclimate to and explore the surroundings. Then, the monitor projected a program-derived looming stimulus 10 times. The mouse responded to the stimuli either by freezing or by fleeing to the hiding place. The mouse's behavior before and after the looming stimuli was recorded, and the video was analyzed using motion tracking software. The velocity of the mouse movement significantly changed after the looming stimuli. In contrast, no reaction was observed in blind mice. Our results demonstrate that the simple looming experiment is a reliable test of mouse vision.

INTRODUCTION:

The visual system starts at the retina, where visual signals are captured by photoreceptors, channeled to bipolar cells (2nd-order neurons), and finally passed to ganglion cells (3rd-order neurons). Retinal 2nd- and 3rd-order neurons are thought to form multiple neural pathways that convey particular aspects of visual signaling such as color, motion, or shape. These diverse visual features are relayed to the lateral geniculate nucleus and the visual cortex. In contrast, visual signals leading to eye movement are sent to the superior colliculus. Classically, two retino-cortical pathways have been identified: the magnocellular and the parvocellular pathways. These pathways encode moving and stationary objects, respectively, and their existence embodies the basic concept of parallel processing[1-6]. Recently, more than 15 types of bipolar cells[7-11] and ganglion cells[12-16] have been reported in the retina of many species, including the primate retina. These cells are distinguished not only by morphological aspects, but also by the expression of distinct markers and genes[8,10,17,18], suggesting that various features of visual signals are processed in parallel, which is more complicated than originally anticipated.

Cellular and molecular technologies have contributed to our understanding of visual processing and potential disease mechanisms that may arise from aberrant visual processing. Such an understanding may contribute to the development of artificial eyes. Although cellular examinations and analysis offer in-depth knowledge at a cellular level, a combination of behavioral experiments and cellular experiments would significantly augment our current understanding of minute visual processes. For example, Yoshida et al.[19] found that starburst amacrine cells are the key neurons for motion detection in the mouse retina. Following cellular experiments, they performed the optokinetic nystagmus (OKN) behavioral experiment to show that mutant mice in which starburst amacrine cells were dysfunctional did not respond to moving objects, thereby confirming their cellular investigations. In addition, Pearson et al.[20] conducted photoreceptor transplantation in the mouse retina to restore vision in diseased mice. They conducted not only cellular experiments, but also measured mouse behavior through the use of optomotor response recordings and water-maze tasks thus allowing Pearson et al. to verify that transplanted photoreceptors restored vision in the formerly blind mice. Taken together, behavioral experiments are strong tools to assess mouse vision.

Multiple methods are available for measuring mouse vision. These methods have advantages and limitations. In vivo ERG provides information on whether the mouse retina, particularly photoreceptors and ON bipolar cells, appropriately responds to light stimuli. ERG can be tested either under scotopic or photopic conditions[21,22]. However, ERG requires anesthesia, which might affect the output measurement[23]. The optokinetic reflex (OKR) or optomotor response (OMR) is a robust method to assess contrast sensitivity and spatial resolution, both functional components of mouse vision. However, OKR requires surgery to attach a fixation device to the mouse skull[24]. OMR requires neither surgery nor mouse training; however, it requires training to allow an experimenter to subjectively detect subtle mouse head movements in response to a moving grating in an optic drum [25,26]. Pupil light reflex measures pupil constriction in response to light stimuli, which does not require anesthesia and exhibits objective and robust responses [19]. Although the pupil reflex simulates retinal light response in vivo, the reflex is mediated mainly by

the intrinsically photosensitive retinal ganglion cells (ipRGCs) [27]. Because ipRGCs represent a small minority of RGCs and do not serve as conventional image-forming ganglion cells, this measurement does not provide information pertaining to the majority of ganglion cells.

The looming light experiment has not previously been considered a major test for measuring mouse vision. However, it is also a robust and reliable vision test across various species, such as mouse[28,29], zebrafish[30], locust[31,32], and human[33-35]. Importantly, the looming experiment is one of only a few methods to test the image-forming pathway—it is not a reflex pathway—given the visual and the limbic systems in the central nervous system are involved in this circuit[36-38]. We have established a looming visual stimulus system and have demonstrated its ability to elicit motion detection in the mouse, which we use as a proxy to assess the intactness of the mouse visual system.

PROTOCOL:

All experiments and animal care were conducted in accordance with protocol approved by the Institutional Animal Care and Use Committees at Wayne State University (protocol no. 17-11-0399).

1. Preparation for the experiment

1.1. Build a rectangular open-lid enclosure to house the mouse during looming visual stimuli presentation. We constructed a 40 cm x 50 cm x 33 cm enclosure using aluminum framing and PVC panels (**Figure 1A,B**). Lay a sheet of paper to cover the entire floor of the enclosure to ensure easy cleanup between trials. Add an opaque shelter in a corner of the enclosure with an entrance facing the center of the arena for easy entrance and exit.

1.2. Set up a camera with a wide-angle lens for capturing the mouse's behavior. Secure the camera to a table-mounted stand adjacent to the enclosure. For best quality video capturing, use a camera frame rate of 60 FPS or higher.

1.3. Set up a computer monitor on top of the enclosure. Because the monitor could not be seen from the outside, a second monitor was prepared, which duplicated the images shown on the primary monitor.

1.4. Prepare a looming pattern for projection. One way to do this is to use the PsychToolbox3 within MatLab software to code for an expanding black circle (**Figure 1C**). Set the stimulus to begin at a visual angle of 2° and expand to 50° over 250 ms; these parameters determine stimulus speed (see **Figure 1D** for visual angle calculation). Set the code to repeat the stimulus 10 times with an interval of 1 s.

NOTE: The stimulus began each repetition immediately upon termination of the previous presentation. For further information on stimulus presentation, please refer to section 3.

1.5. Select mice of interest for the looming stimuli. Here, use 32 healthy-eyed mice of a C57 background, male and female, 4 to 14 weeks old. Also, use 3 blind mice (severe cataracts in both eyes) to assess whether the response to looming stimulus was truly a visually guided behavior. These blind mice had no pupillary light reflex and no optomotor response.

2. Mouse acclimation

2.1. Place a mouse in the enclosure and let it freely explore its surroundings. If possible, try to minimize stress during animal transfer by using the back of your free hand as a resting place for the mouse instead of letting it hang without support. The mouse should find the entire enclosure to be safe and should discover the hiding place. Drop a few food pellets in the corner opposite the refuge to encourage the mouse to remain outside the refuge.

2.2. Allow the mouse to acclimate anywhere from 7 to 15 min[29,39]. We allowed 10 min of acclimation prior to stimulus onset. Furthermore, 10 min acclimation one day prior to the experiment may ease the mice.

3. Looming visual stimuli projection

3.1. Prior to inserting the mouse into the arena, make sure the stimulus code is ready to run to facilitate as few lighting changes as possible while the mouse is in the enclosure. Once the software is ready to run, gently place the mouse in the enclosure.

3.2. 10 seconds prior to the stimulation, start the video capturing.

3.3. Start the looming visual stimuli when the mouse is away from the shelter and moving freely in the open arena. Wait 10 seconds after the last stimulus presentation to terminate the recording.

3.3.1. Begin the stimulus presentation when the mouse is in the corner farthest from the refuge. However, when mice seem unwilling to explore the far corner, present the stimulus when the mouse is in a different corner of the arena. This does not appear to make a difference in animal behavioral response.

3.4. Transfer the mouse back to its original cage. Clean the enclosure for the next mouse by spraying the walls and refuge with 70% ethanol and wiping it down. Replace the paper floor liner if soiled and reposition the refuge to the same initial location if moved during animal transfer and enclosure cleaning.

4. Video analysis

4.1. Save the video clip for each mouse in .avi format without file compression in order to ensure no data loss during transfer to the analysis software.

NOTE: Lack of compression will lead to larger file size; therefore, use external hard drive for storage.

4.2. Use analysis software to track the animal's motion around the arena prior to, during, and after stimulus presentation. Use commercially available software (see **Table of Materials**) with a manual tracking ability to track the position of the mouse head in every video frame, which generated X and Y coordination every 1/60 ms. Other motion-tracking software includes FIJI (NIH)[40] and EthoVision (Noldus).

4.3. Calculate the velocity and the distance of the mouse from the refuge. If the image of the arena is distorted due to the video angle, correct the X and Y coordination prior to the calculation (**Figure 2**).

4.4. Compare the parameters before and after looming stimulus onset to determine how the mouse responded to the stimuli, whether by freezing, fleeing, or demonstrating no change in behavior[29]. Define freezing as episodes where speed was less than 20 mm/s for 0.5 s or longer. Define flight as episodes where speed increased to 400 mm/s or greater and ended with the mouse in the refuge. Definitions for freezing and flight were based on those set by Franceschi et al.[29]

REPRESENTATIVE RESULTS:

A mouse with healthy eyes was placed in the enclosure and allowed to acclimate for 10 min. The arena with the monitor on the ceiling was kept under mesopic light conditions (7×10^5 photons/μm^2/s). During the acclimation period, the mouse explored the space and found the opaque dome as a refuge. When the mouse moved away from the refuge, video capturing started, followed by initiation of the visual stimulus. In response to the looming stimulus, most mice ran into the dome (flight response), which was observed in 30 out of 31 mice (97%). Some of the mice exhibited freeze responses before they fled (19/31 mice, 61%). The looming stimulus reduced the light condition 1 log (6×10^5 photons/μm^2/s).

Captured video clips were analyzed using either a commercial analytics software with a manual tracking function (Image Pro Plus) or FIJI (NIH). Using the tracking feature, the mouse's position was identified in each frame of the video (60 frames/s) before, during, and after the looming stimuli. We analyzed the velocity changes over time as well as the distance to the shelter (**Figure 3**). When flight occurred, the velocity abruptly increased and the distance to the shelter reduced accordingly. In contrast, the velocity was near 0 mm/s when mice froze. The latency from the onset of the looming stimuli to flight ranged from 0.1 to 6.0 seconds (average 2.2 s, 30 mice). The range of maximum velocity for flight response was 500–3000 mm/s (30 mice).

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental system. (**A**) Schematic of the looming stimuli enclosure. A computer monitor (21") covers the ceiling. There is an opaque dome in one corner of the enclosure in which a mouse may take refuge. A video monitor with a wide-angle lens captures the mouse behavior. (**B**) Overall view of our entire setup. The secondary monitor duplicates the image showing on the

stimulus display. (**C**) Diagram of the looming stimulus. The looming stimulus begins at 2° (1.15cm) and holds at this size for 250 ms. It then expands to 50° (30.8cm) over the course of 250 ms and remains 50° for an additional 500 ms. This 1s sequence then repeats 9 more times prior to terminating. (**D**) Diagram of stimulus calculations. The height of the cage dictates the necessary start and end size (in centimeters) of the stimulus in order to produce a stimulus that expands from 2° to 50° when directly above the mouse.

Figure 2: Analysis calculations. Calculations to correct skew from wide-angle lens. Due to the placement of the camera, the floor of the arena appears as a trapezoid instead of a rectangle (left). Therefore, the X and Y coordinates of the mouse must be corrected to accurately analyze mouse position (mid). Using the geometry of congruent triangles, it is possible to find how much the x-coordinate must shift in order to correctly represent the mouse's movement in the 3-dimensional space (right).

Figure 3: Representative responses to looming stimuli. (**A**) An example of a mouse's movement tracked within the arena. A red circle shows the dome where the mouse fled to and stayed until the looming disappeared. 1 = mouse position starting point when video capturing started. 2 = movement prior to stimulus onset when the mouse explored the arena. 3 = looming stimulus started. The mouse dashed to the dome (shown by a red dashed line). 4 = the mouse stayed in the dome until and after termination of the stimulus. (**B**) Velocity changes as a function of time for this mouse. The dotted line indicates when the looming stimulation began. Stimulus duration is indicated by the yellow background. The full 10 second cycle is not shown here since the mice remained stationary in the refuge for the entire stimulus duration. (**C**) Distance from the dome over time for the same mouse in (**A**) and (**B**). (**D**) and (**E**) The velocity and distance to the dome for a mouse that exhibited the freeze reaction (freezing duration shown by the red double sided arrow) prior to flight. The velocity was reduced compared to the control (before looming). The distance to the dome did not change during this period.

DISCUSSION:

With the looming visual stimuli system, a majority (97%) of healthy eye-mice showed flight response. One of 29 mice did not show an obvious flight response. However, the mouse walked toward the dome and remained near it until looming disappeared, indicating that the mouse was at least cautious when the looming stimuli occurred. Therefore, the looming stimuli consistently elicited innate fear responses in healthy-eyed mice. On the other hand, three blind mice did not show any responses to the looming (preliminary results). Taken together, we demonstrate that looming experiments are a useful and consistent vision test for mice.

We set the speed of the looming stimuli at 192 degree/s. Franceschi et al.[29] examined the looming responses at varying speeds, 5 to 84 degree/s and observed freeze responses preferentially at lower speed levels. Yilmaz and Meister[28] observed flight responses at 35 to 350 degree/s; however, flight latency was longer at higher speeds. Therefore, to evoke consistent flight responses, looming should be at a speed of 50 degree/s or above. Looming stimuli can be generated easily even with standard presentation software. However, such software cannot create higher speeds of looming stimuli. We instead used MatLab and PsychToolbox3 to create

the visual stimuli at 192 degree/s.

We acclimated the mice for 10–15 min before the looming stimuli, which is the acclimation time previous researchers described [28,29,39]. We furthermore tested whether acclimation the day before changed the looming behavior. We placed the mice in the enclosure for 10 min without looming stimuli the day before the looming stimuli. This acclimation significantly shortened the flight latency ($p < 0.01$, $n = 7$ mice, data not shown). Although 10 min of acclimation on the day of looming consistently caused flight responses, 1 day prior acclimation decreased the latency of flight responses.

There are some limitations for using looming stimuli as a vision test. First, it is hard to test one eye at a time. Unless suturing the one eye, both eyes are tested together. Second, the mechanisms of the behavioral looming response have not been fully established. In the retina, Yilmaz and Meister [28] suggested that ventral OFF-DSGCs (direction-selective ganglion cells), but not ON-DSGCs, convey the looming signals to cause responses. This conclusion arose from their results that mice responded to dark looming stimuli, but not to white looming. In the brain, Wei et al.[36] and Shang et al.[37] demonstrated that the pathways from the superior colliculus through the amygdala and the periaqueductal gray are responsible for the looming. However, more studies should be conducted to confirm these investigations.

Even though some limitations exist with respect to the looming experiment, the looming visual stimulus generates consistent and robust fear response in mice and should be a useful test of mouse vision which requires minimal training of the experimenter.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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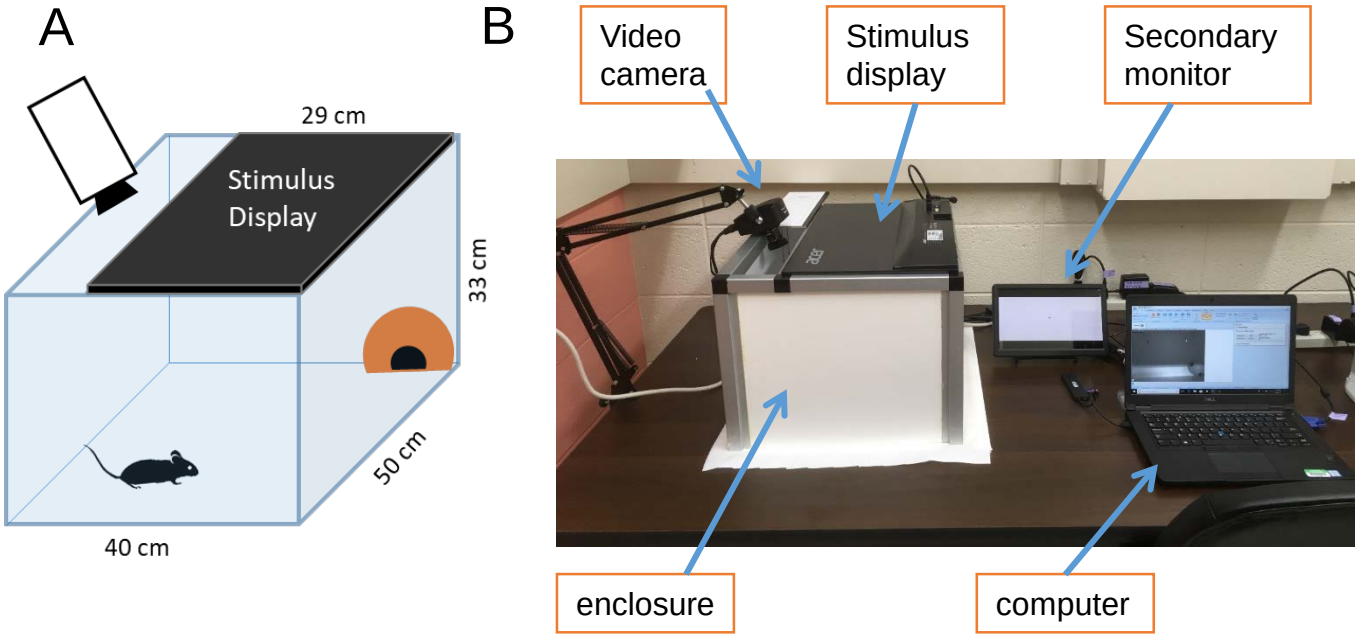
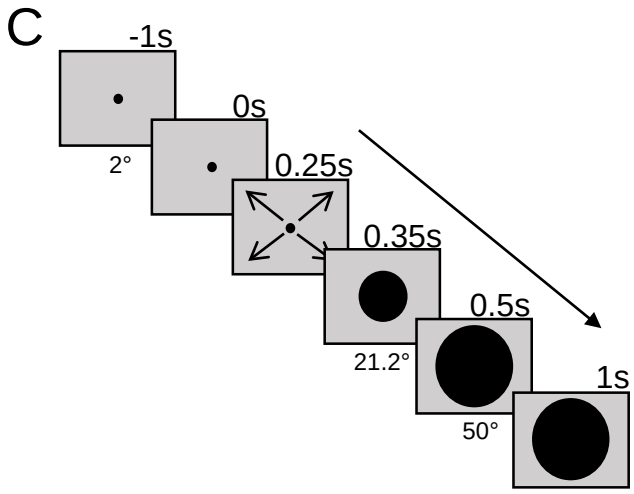
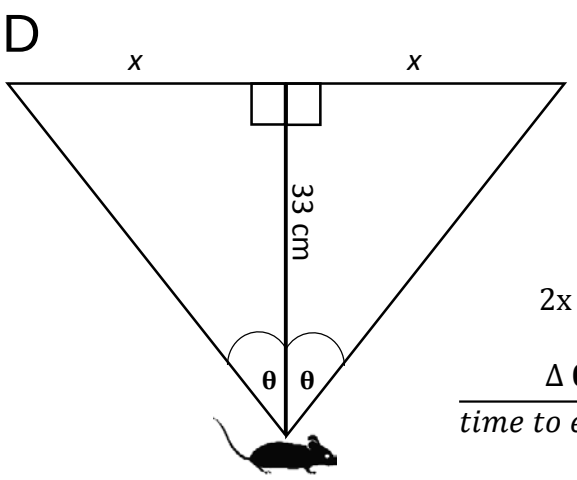






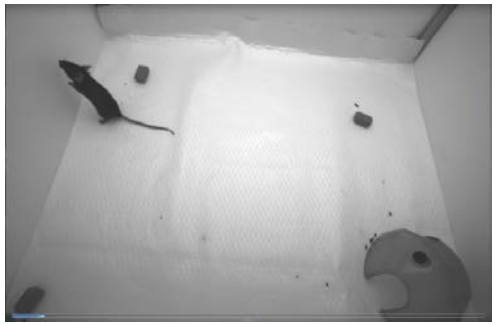
$$\tan(\boldsymbol{\theta}) = \frac{x}{33}$$

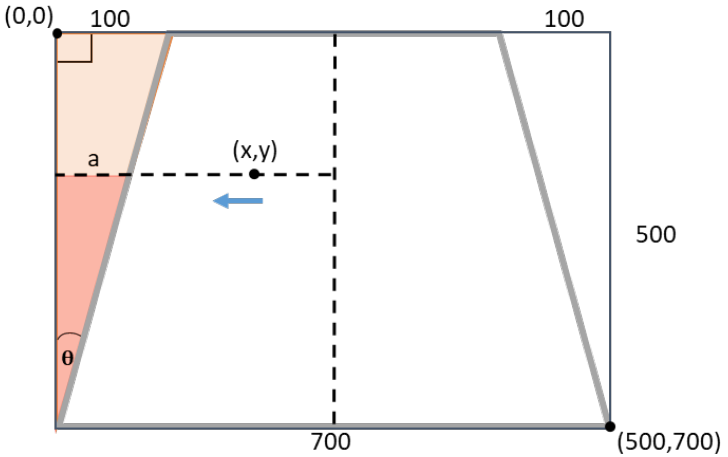
$$\mathrm{x} = \tan(\boldsymbol{\theta}) * 33$$

$$2\mathrm{x} = \text{diameter of stimulus}$$

$$\frac{\Delta\,\boldsymbol{\theta}}{time\ to\ expand} = \frac{50^0 - 2^0}{0.25s} = 192^0/s$$

Figure 2





$$\tan(\theta) = \frac{100}{500} = \frac{a}{500 - y}$$

$$a = 100 - 0.2y$$

$$ratio\ (R) = \frac{350 - x}{350 - a}$$

$$new\ x = x - a * R$$

Figure 3

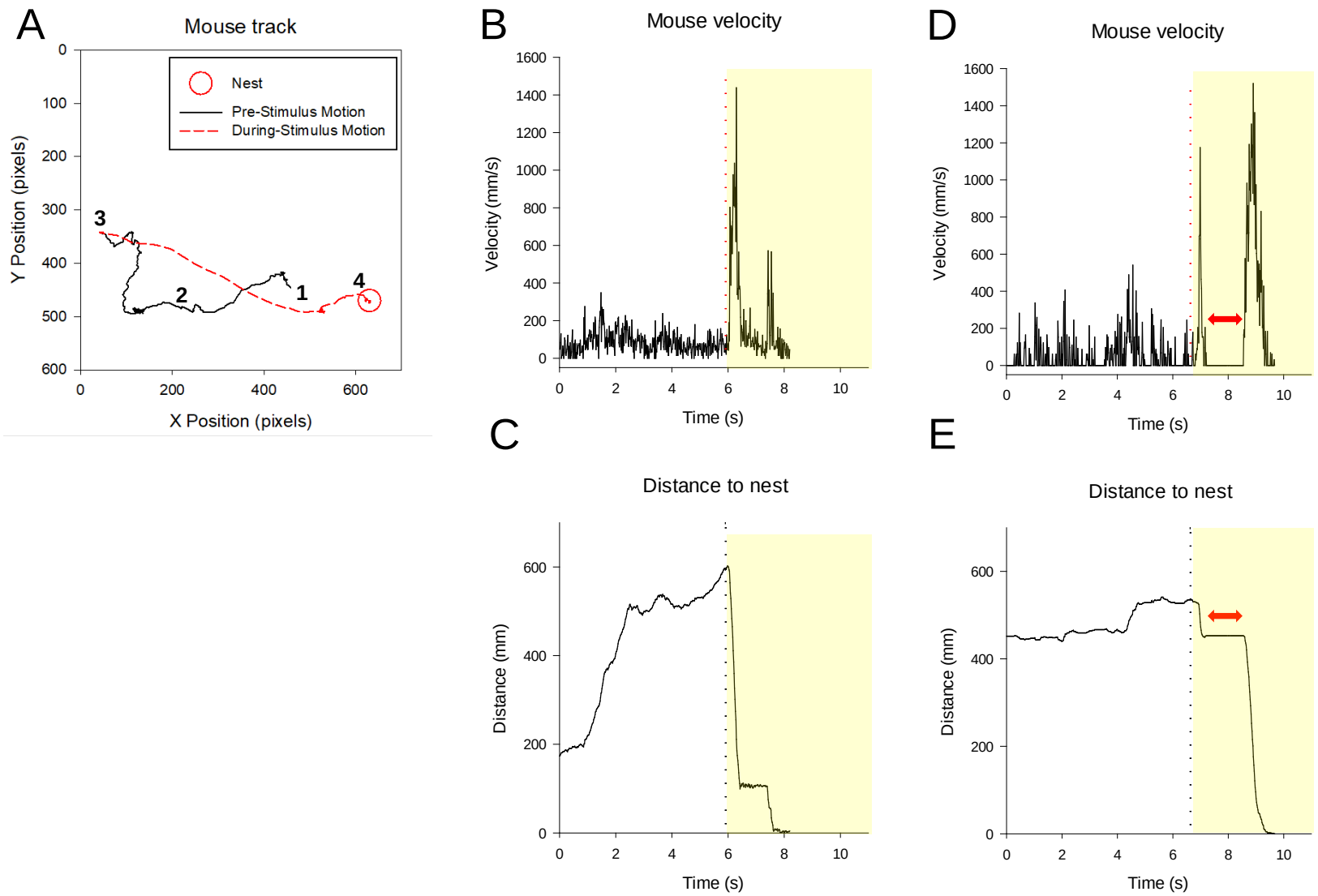


Table of Materials

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Material	Company
10.1" monitor (2° display)	Elecrow
14" Business Class Laptop 5490	Dell
20" x 50" Absorbant Liners	Fisher Scientific
21.5" monitor (1° display)	Acer
CCD Camera	Lumenera Corporation
Enclosure (alminum frames and PVC panels)	80/20 Inc.
Ethanol	Fisher Scientific
Excel Spreadsheet Software	Microsoft Office
Freearm	Amazon
ImagePro Premiere 3D	Media Cybernetics
Matlab software (Psychotoolbox 3)	MathWorks
SteamPix sorftware	Norpix
WD My Book External Hard Drive	Western Digital
Wide angle lens	Navitar

Cat. No.
Elecrow 10.1 Inch Raspberry Pi 1920x1080p Resolution Display
84 / rcrc961481-4860836
AL2050
Acer R221Q bid 21.5-inch IPS Full HD Display
Infiniyy3S-1UR
4x cat.#9010, 4x cat.#9005, 1x cat.#9000, 5x cat.#65-2616
22-032-601

version 9.3
Matlab R2018b 64-bit (9.5.0.944444)
StreamPix 7 64-bit Single Camera
WDBBGB0080HBK hard drive 8 TB USB 3.0
NMV-5M23

Comments/Description

works well to protect floor of arena, could use any type of liner

excellent for behavioral studies due to high fps rate (60 fps)
excellent, used quick build tab to find PVC, joints, and frame

user friendly and widespread knowledge of Microsoft Office software
used to mount camera to the table, could use any mountable extendable arm
good program, could use some updating with the automated tracking feature
excellent software to generate pattern stimuli of any conditions
works well, a few problems with frame dropping but good customer service
necessary if using .avi files with no compression codec due to large size of files
excellent and necessary to capture entire arena

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Author(s):	Christina C. Koehler, Leo M. Hall, Chase B. Hellmer, Tomomi Ichinose

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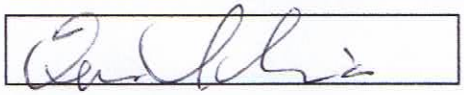
Signature:	
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The authors thank the editor and the reviewers for their insightful comments on our manuscript. We have thoroughly revised the manuscript based on their criticisms and added detailed descriptions to clarify the protocol.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have corrected all spelling and grammar errors to the best of our knowledge.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

We have fixed them throughout the manuscript.

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For example: Quick frame, 80/20 Inc, Image Pro Plus,

We have eliminated words that would be considered commercial language. This information has been updated in the Table of Materials and Reagents. However, we still used commercial language software programs such as Matlab and Image Pro Plus (Protocol 1.4 and 4.2). Reviewers requested a more detailed explanation of the software, thereby necessitating the use of commercial names. Please advise if we still need to eliminate these software names, and if so, how we can discuss without using software names.

Protocol:

1. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement has been added prior to the protocol.

2. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

The authors have included more detail in the protocol steps upon the recommendation of the editor. These revisions can be found throughout the entirety of the protocol section, taking special care to answer the "how" question to the best of our ability.

Specific Protocol steps:

1. I'm not sure what 'set the speed of looming…' means, exactly.

The phrasing above has been removed from the manuscript and revised with more detailed explanation (Protocol 1.4). Furthermore, we added Figure 1C and 1D to support the explanation.

2. 1.5: What age/sex/strain of mice do you use?

We described in Protocol 1.5. We used C57 background mice of male and female, and 4-14 weeks old.

3. Please provide more details regarding the use of analysis software or a citation.

We have included more information about its use in our experiment (Protocol 4.2).

Figures:
1. Please remove 'Figure 2' and 'Figure 2' from the Figures themselves.

We removed the titles "Figure 1" and "Figure 2" from their respective figures.

References:
1. Please do not abbreviate journal titles.

We did make sure that titles are not abbreviated.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
The authors have ensured that the Table of Materials includes all equipment and reagents used in the experiment.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors describe a simple setup to measure the looming behavior in mice. The behavior is robust and reliable and a good test of mouse vision.
The Introduction is strong and convincingly details the advantages and disadvantages of other tests of mouse vision currently used in the field to assess retinal and visual behavior. A few minor comments on other aspects of the manuscript are listed below.

Major Concerns:
Is the analysis software available to other users?

The analysis software is available to other users commercially. The authors have made note of this in the protocol (Protocol 4.2) and have also given suggestions for similar software—also tested in consideration for this set of experiments-- that is freely available.

Minor Concerns:
Protocol:
*Ln 112: It will be helpful for the reader interested in building the device to know how the looming speed (in units of degrees per second) was determined. Perhaps include a diagram

indicating the geometry of the setup and the corresponding calculation relating the linear seed of the looming object and the position of the mouse.

We have added a diagram (Figure 1D) to better explain the calculations to readers. We have also inserted additional stimulus information in section 1.4 of the protocol in order to clarify stimulus parameters.

*Include a diagram illustrating what a looming stimulus looks like and how it moves along the monitor. What is the size of the looming object?

We have included size information (Protocol 1.4) and have also added a diagram (Figure 1C) which illustrates how the stimulus expands on the monitor.

*Indicate the irradiance (illuminance) of the mouse environment and the contrast of the object.

The ambient level of the arena was measured and described in the Representative Results section.

*Ln 114. Says 'we set the looming stimulus 1 second in duration and 10 times in a row'. How was the stimulus introduced into the field? In what region of the field? Does it make a difference? At a speed of 50 degrees per second, the object will only move over a small portion of the overhead field (visual angle) during a one second presentation… See comments below.
*The time between repetitions is not indicated here. How is it controlled? Does it provide enough time for the mice to come out of the refuge? Perhaps refer the reader to the next section (3.3.3 where more details are indicated)
We have added more schemes to explain our stimulus (Figure 1). The looming started expanding from the center to the edge of the monitor (2 to 50 degree). We repeated the stimulus presentation 10 times. This procedure is nowdescribed in Protocol 1.4 and Figure 1. Upon your recommendation, we also refer the reader to section 3.3.

Video analysis
*Ln 4.2: Is this custom software? Is the software available to the readers? Perhaps the info in line 166 could be moved up to this section

The software is not custom, but it is available to readers should they wish to purchase it (Table of Materials). We have clarified this and suggest other possible software that is freely available in Protocol 4.2

.
Representative results:
*Ln 170: unclear how the latency time was measured. Was this from the onset of the looming stimulus? If the duration of the looming stimulus is 1 second (see above) does this mean that the response delay can over-extend the duration of the stimulus (indicated to be between 0.1 to 6 seconds)?

Mice were exposed to the looming stimulus for 10 seconds (250 ms duration, 10 times with an interval of 1 second). The delay time was measured from the onset of the looming stimuli to mouse flight. Therefore, flight occurred during the looming stimuli. We clarified parameters of

the looming stimulus in Protocol 1.4 and modified the latency description in the Representative Results.

Discussion:
*In 220: does this paragraph belong in the introduction?

While we understand the suggestion, we feel the limitations of the study are more appropriately examined in the Discussion section.

*Perhaps include a reference to Huberman's work (Salay et al, 2018) describing the central circuitry involved in looming responses?

We have cited this paper in the last paragraph of Introduction. Thank you for your suggestion.

Figure 2: Legend Fig 2 states that the stimulus duration was 10 seconds not 1 second as indicated in the text.

We clarified the stimulus procedure (Protocol 1.4) in order to avoid confusion about stimulus duration.

How is the 'freezing behavior' defined? By looking at the C and D graphs this mouse seems frozen at 5.5 seconds. Can a more quantitative definition of 'frozen' be introduced. Perhaps by setting noise thresholds for each of the output parameters (velocity and distance)?
We have replaced the figures in question with graphs that have lower noise levels in order to avoid confusion. We have also cited our definitions for both freezing and flight in the manuscript (Protocol section 4.4).

Reviewer #2:
Manuscript Summary:
In this manuscript, the authors used looming visual stimuli to evaluate mouse vision. Take the advantage of a self-built system, they showed that looming visual stimulus induced consistent and robust fear responses in mice, and this behavioral assay could be a useful test of mouse vision which required minimal training of the experimenter.
This method is practical and easy to use. However, I would suggest that the following serious issues should be addressed before the consideration of accepting this manuscript to be published in JOVE.

Major Concerns:
1. The aim of using looming visual stimuli was to evaluate mouse vision. However, the authors did not provide a specific and quantitative definition of the vision. They just showed that naïve healthy-eyed mice exhibited escaping or freezing behaviors and blind mice showed no behavioral response. This was just an all-or-none judgment but not a quantitative evaluation of vision.

A few research articles have investigated the mechanisms of looming experiment, which revealed that the looming stimulus evokes the fear response via the image-forming pathway. Although multiple methods to examine mouse vision are available, many of them, such as pupil light reflex and optomotor reflex, are induced by sub-cortical pathways. The looming

experiments is one of few methods to test the image-forming pathway that arises through the visual cortex and the limbic system. Because JoVE is a methodology journal, we do not show any results regarding the mechanism of the looming responses. However, the looming experiments are unique and important vision tests, and thus this looming test merits introduction as a means of assessing vision. We expanded the last paragraph of Introduction to address to your concern.

2. Some logical mistakes appeared in the manuscript. For example, lines 71 "…measuring mouse behavior, such as electroretinogram (ERG)...", ERG is not a behavior.

The authors have deleted ERG in line 71 and have also read through the manuscript and edited other similar logical errors.

3. In the "protocol" session, the authors did not describe how blind mice were prepared.

This was an oversight on our part, thank you for finding this error. These mice were blind due to severe cataract for both eyes, which was induced spontaneously or by ketamine anesthesia. These mice did not show pupil light reflex and OMR. We have included the preparation of blind mice (Protocol 1.5).

4. The authors did not show whether this study has been approved by an ethics committee.

We have now included the Statement at the beginning of the Protocol section.

5. Many descriptions were not accurate enough in this manuscript. The parameters for displaying looming stimuli were not accurate enough. Was there any time interval between the adjacent two looming stimuli? The visual angles for the same looming stimulus at different places of the paper floor should be different. What were the exact size and shape of a looming stimulus?

We apologize for the lack of appropriate description contained within the manuscript and thank you for your insight. We have added further detail on the stimulus presentation in section 1.4 and 1.4.1. Additionally, we have added information concerning the location of the mice in relation to the stimulus (presented in section 3.3.1). Additional panels (Figure 1C-D) were also included in figure 1 to show the shape of the stimulus. The stimulus size parameters are now included in Protocol 1.4.

6. In Chapter 4.2, the authors did not introduce what and how analysis software was used.

We expanded the Protocol 4.2 to fully describe the mouse track analysis.

7. In line 171, did "The latency from the looming stimuli to flight" mean the latency from the onset of the first looming stimulus to mouse fight?

Yes, this was what we meant. We have modified the description in the Representative Results section to clarify the meaning of latency.

8. The camera was placed near one side of the arena. Therefore, the shape of the paper floor in captured images should be a trapezoid rather than a rectangle. Was there any follow-up correction of the shape of the paper floor?

We have corrected the skewed image by simple calculation. This calculation is now shown in Figure 2, and the description is given in Protocol 4.3.

9. Some figures are presented in an appropriate or inaccurate way. In figure 2A, the readers cannot distinguish the boundaries between the adjacent two stages. Some markers should be added, or different colors should be used.

The panel has been edited to more clearly demarcate the pre-stimulus (black solid line) and during/post-stimulus phase (red dashed line). We also revised the figure legend to further clarify this panel.

10. From Figure 2B to 2E, only the first 3 seconds of looming stimuli were shown. The authors should show not only the entire stage of looming stimuli but also a few seconds after the end of looming stimuli.

While we understand why this may be helpful in denoting periods before, during, and after the stimulus, all animals remained in the refuge once they fled until well past the end of the stimulus duration. These figures are simply meant to depict representatively the immediate behavioral response to the stimulus. Since the animals remain stationary in the hut following stimulus termination, both figures would show a line hovering on the x-axis if extended any further. We have added the description of stationary mouse behavior after the flight to Figure 3 legend.

Minor Concerns:
1. Figure 2D and 2E had no Y-axis label.

The authors have made sure to correctly format the PDF so that all axes are showing.

2. In figure 2D, a blue arrow could not quantitatively describe the time period of freezing, especially the start and end of freezing. Therefore, the authors should use two arrows or a background color to illustrate the exact time period of freezing.

In response to this commentary, the authors have added a pale yellow background during the stimulus duration of Figure 3B-E and have inserted a double sided red arrow to denote the freezing period in Figure 3D-E.

3. Many grammatical mistakes exist in the manuscript. For example, lines70 and 71 "They conducted not only cellular experiments, but also measuring mouse behavior, such as…" should be "They not only conducted cellular experiments but also measured mouse behaviors, such as…". Line 93 "mouse visual and emotional system" should be "mouse visual and emotional systems". Besides, confounding use of present tense and past tense was frequent in this manuscript. For example, line 108 and 109 "Because the monitor cannot be seen by the experimenter from the outside, a second monitor was prepared, which duplicates..."

The authors thank the reviewer for finding these grammatical errors and have taken care to comb the manuscript to eliminate further errors. All grammatical errors from the above comment have been fixed accordingly.