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Title: Non-Aversive Animal Restraint Enabling Recording of Optomotor Reflex in Ground Squirrels

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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 something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**
3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 22
Number of Shots: 40 (28 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Videographer's NOTE:
- Recorded at 23.98
- Recorded in Rec709
Sound Channels
- 1:internal mic, 2: internal mic, 3: Boom mic
-Authors will send headshot photos

1.1. **Kiyoharu Miyagishima:** The OMR is widely used to screen visual impairments but has been limited to traditional models like mice and rats. Our redesigned platform enables recordings from 13-lined ground squirrels which have a cone-rich visual streak resembling the human macula.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.2*

What are the current experimental challenges?

1.2. **Francisco Nadal-Nicolás:** Repeated escape attempts or inattentive exploratory behaviors can disrupt recordings compromising accuracy and limiting sample sizes.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

What research gap are you addressing with your protocol?

1.3. **John Ball:** Combining the platform with a familiar transfer-like tube, promotes quick habituation, reduces anxiety-like behaviors, minimizes stress, and lowers the risk of falls.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.4.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Francisco Nadal-Nicolás:** Ground squirrels often struggle with stability on standard round flat platforms, but the new semi-enclosed design improves engagement and enables reliable data collection without extensive acclimation or positive reinforcement.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

What research questions will your laboratory focus on in the future?

1.5. **Kiyoharu Miyagishima:** The ability to record from cone-dominant mammals opens new avenues to investigate drug-induced ocular side effects, including changes in color vision.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.4.1*

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL): **Videographer's NOTE:** Not filmed

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Francisco Nadal-Nicolás:** Publishing with JoVE will enhance the visibility and impact of our research by providing a clear, visual demonstration and personalizes the interaction beyond what a traditional manuscript allows.

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.4.1*

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE?

1.7. **Kiyoharu Miyagishima:** JoVE previously helped document our lab's DC-ERG method, making the technique more accessible to other researchers which led to more collaborations and inquiries about its implementation.

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.2*

Ethics Title Card

This research has been approved by the Animal and Care and Use Committee at the National Eye Institute at the National Institutes of Health

Protocol

2. Setting up the Optomotor Reflex (OMR) Arena

Demonstrator: Francisco Nadal-Nicolás

2.1. To begin, wipe the platform and the square tube enclosure assembly using a cleaner and disinfectant to eliminate unwanted olfactory cues **[1-TXT]**.

2.1.1. WIDE: Talent spraying and wiping down the platform and square tube enclosure with disinfectant. **TXT: Disinfect before and between each animal**

2.2. Tear a piece of paper towel approximately 50 by 50 millimeters **[1]** and fold it to cover the top surface of the pedestal **[2]**. Place the 3D-printed platform onto the 14-inch high pedestal with the folded paper towel in place **[2]**.

2.2.1. Talent tearing the paper towel.

2.2.2. Talent folding the paper towel to cover the pedestal top. **Videographer's NOTE**: Shot 2.2.2 & 2.2.3 were filmed together

2.2.3. Talent placing the 3D-printed platform onto the pedestal with the paper towel underneath.

2.3. Wearing leather work gloves **[1]**, gently guide the animal into the square tube enclosure assembly **[1]**.

2.3.1. Talent wearing leather gloves.

2.3.2. Talent gently directing the animal into the enclosure.

2.4. Transfer the animal to the arena by positioning the enclosure assembly onto the platform **[1]**.

2.4.1. Talent lowering the enclosure assembly with the animal onto the platform.

2.5. Now, carefully detach the removable top enclosure, leaving the animal inside the acrylic extruded clear square tube on the platform **[1]**. Close the door to the optomotor reflex system **[2]**.

2.5.1. Talent lifting off the top enclosure from the tube assembly, animal remaining inside.

2.5.2. Talent closing the door to the OMR system.

3. Setting up the OMR Software Parameters and Data Acquisition

Demonstrator: Kiyoharu Miyagishima

3.1. Typically visual acuity tests on rodents are performed using the standard 100 percent contrast setting **[1]**. However, squirrels can see well beyond the spatial frequency of mice and rats - even beyond the limits of the software which is a maximum of 2 cycles per degree **[2]**.

3.1.1. SCREEN: Screen-3.1.1-(Set-100-percent-contrast).mov.

3.1.2. SCREEN: Screen-3.1.2-(Set-spatial-freq-to-2-cyc-per-deg).mov.

3.2. At 2 cycles per degree, each stripe averages 5.3 pixels wide. Now, set the rotation speed to 12 degrees per second **[1-TXT]**.

3.2.1. SCREEN: Screen-3.2-DEMO-Squirrel-tracking-2cycdeg_BEST.mov. **TXT: Stimulus presented on all four displays (shown side by side)**

~~3.3. Download which contains the suggested default settings and open the OptoDrum software **[1]**.~~

~~3.3.1. SCREEN: Talent navigating to download Supplementary Coding File 3 and launching the OptoDrum software.~~

~~3.4. Under the **Settings** tab in the Squirrel.track file, click on **1]**.~~

~~3.4.1. SCREEN: Click on **Settings**, then on **Import Settings**, and select Squirrel.track from file explorer.~~

3.5. After importing the Squirrel.track *(squirrel dot track)* **file,** go the **Settings** tab, verify the **Background threshold offset** to 14 to 49, **minimum animal size** to 30 to 100 pixels, and **maximum tail width** to 1 percent of size **[1]**.

3.5.1. SCREEN: Screen-3.5.1-3-and-3.6.1-and-3.7.1-(Verify-ROI-Positions).png. *Video editor: please highlight the box below "tracking" containing Background threshold offset, minimum animal size and maximum tail width*.

3.6. Under **Camera Settings**, ensure that **Invert Video** and **Manual Camera Control** are unchecked and **IR Light** is off **[1]**.

3.6.1. SCREEN: Screen-3.5.1-3-and-3.6.1-and-3.7.1-(Verify-ROI-Positions).png. *Video editor: please highlight the box below "camera settings" focusing on invert video and manual camera control*.

3.7. Widen the region of interest to accommodate the squirrel if it leans outside the tube opening. Set **Position** to X 359, Y 237 and **Size** to X 570, Y 455 **[1]**.

3.7.1. SCREEN: Screen-3.5.1-3-and-3.6.1-and-3.7.1-(Verify-ROI-Positions).png. *Video editor: please highlight the box below "POSITION and SIZE" focusing on 359 and 237 AND 570 and 455*. **TXT: Ensure the region of interest does not cover the stimulus screen**

3.8. Next, select **Session Configuration** from the top panel **[1]**. Under the **Staircase** section, set the estimated spatial acuity values. Enter 1.8 cycles per degree for expected acuity, 0.5 cycles per degree for optimal stimulus resolution, and 0.1 cycles per degree for measurement resolution **[2]**.

3.8.1. SCREEN: Session-3.8.1-2-and-3.9.1-and-3.10.1.mov. 00:00-00:06

3.8.2. SCREEN: Session-3.8.1-2-and-3.9.1-and-3.10.1.mov 00:07-00:19.

3.9. Then, set the number of required confirmations in the staircase settings. Enter 3 confirmations for failed trials and 2 for successful trials **[1]**.

3.9.1. SCREEN:: Session-3.8.1-2-and-3.9.1-and-3.10.1.mov 00:20-00:25.

~~3.10. In the **Staircase** tab, set antialiasing width to 3 pixels and leave the **Sinusoidal** option unchecked **[1]**.~~

~~3.10.1. SCREEN:: Cursor entering "3 px" in antialiasing width and showing **Sinusoidal** unchecked.~~

3.11. For testing criteria, lock the contrast at 12.78 percent and the rotation speed at 12 degrees per second **[1]**. Once two of the three parameters are locked, check the box next to **Auto Set the Parameters** once it becomes active **[2]**.

3.11.1. SCREEN: Screen-3.11.1.mov.

3.11.2. SCREEN: Screen-3.11.2.mov 00:05-00:11.

3.12. Then, set the rotation direction based on the expected location of visual impairment. Use clockwise stimulation to assess the left eye, **[1]** or use both clockwise and counterclockwise stimulation for bilateral assessment **[2]**.

3.12.1. SCREEN: Screen-3.12.1-(Select-CW).mov.

3.12.2. SCREEN: Screen-3.12.2-(Select-Both-directions).mov.

3.13. Now, click on **Start Trial** to begin stimulus presentation **[1]**. As the stimulus plays, click on the **Analysis Tab** to monitor angular velocities, track quality, and score in real time **[2]**. At the end of each trial, the software will automatically indicate whether to advance, repeat, or reduce the cycle per degree based on performance **[3]**.

3.13.1. SCREEN: Screen-3.13.1-3-and-3.14.1.mov 00:00-00:05.

3.13.2. SCREEN: Screen-3.13.1-3-and-3.14.1.mov 00:06-00:10.

3.13.3. SCREEN: Screen-3.13.1-3-and-3.14.1.mov 00:25-00:30.

3.14. If the software incorrectly switches the red nose and green tail markers during tracking, press **Ctrl** *(control)* to activate manual override and correct the head-tail orientation **[1]**.

3.14.1. SCREEN: Screen-3.13.1-3-and-3.14.1.mov 00:31-00:37.

3.15. If the animal falls from the platform, pause the trial with the **Pause Evaluation** option or the **Space Bar [1]**. Use the handling tunnel with the lid to safely return the animal to the platform **[2]** and click on **Resume Evaluation** to continue the trial **[3]**.

3.15.1. SCREEN: Screen-3.15.1-(Pause_Return-to-platform_Resume).mov 00:02-00:07.

3.15.2. SCREEN: Screen-3.15.1-(Pause_Return-to-platform_Resume).mov 00:35-00:41.

3.15.3. SCREEN: Screen-3.15.1-(Pause_Return-to-platform_Resume).mov 01:03-01:10.

3.16. After all trials are complete, click on the **Summary Tab** to view results **[1]**. Successful trials will appear in green and unsuccessful ones in red **[2]**. The green-circled trial indicates the visual acuity threshold **[3]**.

3.16.1. SCREEN: Screen-3.16.1-3-Summary-Tab.mov 00:00-00:05.

3.16.2. SCREEN: Screen-3.16.1-3-Summary-Tab.mov 00:05-00:08.

3.16.3. SCREEN: Screen-3.16.1-3-Summary-Tab.mov 00:08-00:12.

3.17. Finally, return the animal to its home cage carefully **[1]**.

3.17.1. Talent placing the animal in its home cage. **Videographer's NOTE**: 3.17.1 was miss slated as 2.5.2; Added another detail shot after Step 3.17.1 and slated it as 3.17.2.

Results

4. Results

4.1. Animals placed on the standard round flat platform for the OMR test invariably fell off and were unable to complete the automated visual threshold assessment within the allotted 10 minutes **[1]**.
4.1.1. LAB MEDIA: Figure 3A.

4.2. In contrast, animals placed on the non-aversive platform with the handling tunnel completed OMR measurements successfully in all cases without prior acclimation or reward conditioning **[1]**.
4.2.1. LAB MEDIA: Figure 3B.

4.3. The modified tunnel allowed the animal to reorient and view stimuli from any direction, enhancing natural behavior during testing **[1]**. The tracking software successfully monitored head movement as the animal responded to directional stimuli **[2]**.
4.3.1. LAB MEDIA: Figure 4A. Video editor: Show the squirrel inside the transparent tunnel with freedom of movement.
4.3.2. LAB MEDIA: Figure 4B, C.

4.4. Overlaying frames taken two seconds apart showed reflexive head tracking in response to visual stimuli **[1]**.
4.4.1. LAB MEDIA: Figure 4D. *Video editor: Focus on the overlay showing the head's position change*.

4.5. The software's real-time analysis differentiated visually-evoked from random head movements, correlating with the stimulus presentation windows **[1]**.
4.5.1. LAB MEDIA: Figure 4E. *Video editor: Focus on the yellow circles in the second row*.

4.6. Using the staircase method, the software quickly determined visual thresholds, with most stimuli requiring less than 30 seconds of presentation **[1]**.
4.6.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the duration column* .

Pronunciation guide :

1. Olfactory

Pronunciation link:
https://www.merriam-webster.com/dictionary/olfactory
IPA: /ˈɑːlˌfæk.tɚ.i/ or /ˈoʊlˌfæk.tɚ.i/
Phonetic Spelling: awl-fak-tuh-ree or ohl-fak-tuh-ree

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2. Pedestal

Pronunciation link:
https://www.merriam-webster.com/dictionary/pedestal
IPA: /ˈpɛdɪstəl/
Phonetic Spelling: peh-duh-stuhl

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3. Acrylic

Pronunciation link:
https://www.merriam-webster.com/dictionary/acrylic
IPA: /əˈkrɪlɪk/
Phonetic Spelling: uh-kril-ik

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4. Optomotor

Pronunciation link:
https://www.howtopronounce.com/optomotor
IPA: /ˌɑːp.təˈmoʊ.tɚ/
Phonetic Spelling: op-tuh-moh-ter

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5. Reflex

Pronunciation link:
https://www.merriam-webster.com/dictionary/reflex
IPA: /ˈriːˌflɛks/
Phonetic Spelling: ree-fleks

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6. Spatial

Pronunciation link:
https://www.merriam-webster.com/dictionary/spatial

IPA: /ˈspeɪʃəl/
Phonetic Spelling: spay-shuhl

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7. Frequency

Pronunciation link:
https://www.merriam-webster.com/dictionary/frequency
IPA: /ˈfriː.kwən.si/
Phonetic Spelling: free-kwuhn-see

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8. Pixels

Pronunciation link:
https://www.merriam-webster.com/dictionary/pixel
IPA: /ˈpɪk.səlz/
Phonetic Spelling: pik-suhlz

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9. Stimulus

Pronunciation link:
https://www.merriam-webster.com/dictionary/stimulus
IPA: /ˈstɪmjələs/
Phonetic Spelling: stim-yuh-luhs

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10. OptoDrum

Pronunciation link:
No confirmed link found
IPA: /ˈɑːp.toʊ.drʌm/ (constructed)
Phonetic Spelling: op-toh-drum

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11. Threshold

Pronunciation link:
https://www.merriam-webster.com/dictionary/threshold
IPA: /ˈθrɛʃˌhoʊld/
Phonetic Spelling: thresh-hohld

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12. Antialiasing

Pronunciation link:
https://www.howtopronounce.com/antialiasing
IPA: /ˌæn.tiˈeɪ.li.ə.sɪŋ/
Phonetic Spelling: an-tee-ay-lee-uh-sing

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13. Sinusoidal

Pronunciation link:
https://www.merriam-webster.com/dictionary/sinusoidal
IPA: /ˌsaɪ.nəˈsɔɪ.dəl/
Phonetic Spelling: sigh-nuh-soy-duhl

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14. Bilateral

Pronunciation link:
https://www.merriam-webster.com/dictionary/bilateral
IPA: /ˌbaɪˈlætərəl/
Phonetic Spelling: bye-lat-er-uhl

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15. Evaluation

Pronunciation link:
https://www.merriam-webster.com/dictionary/evaluation
IPA: /ɪˌvæljuˈeɪʃən/
Phonetic Spelling: ee-val-yoo-ay-shun

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16. Acuity

Pronunciation link:
https://www.merriam-webster.com/dictionary/acuity
IPA: /əˈkjuːəti/
Phonetic Spelling: uh-kyoo-uh-tee