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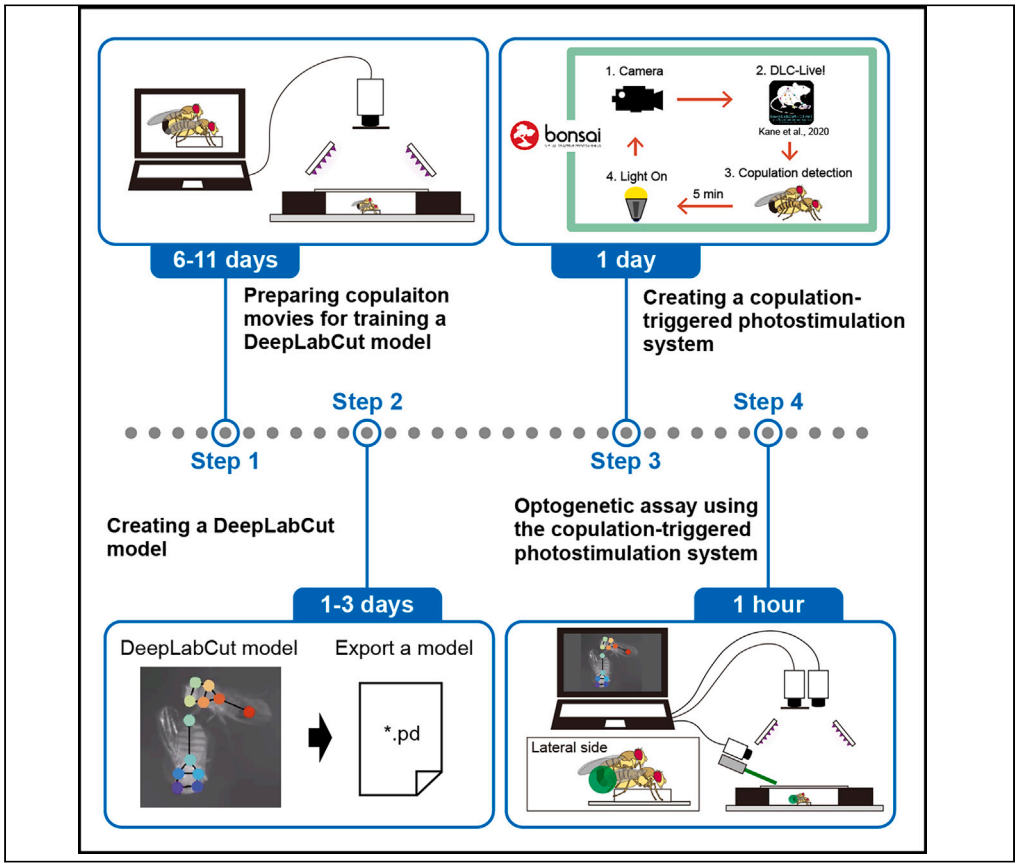
# Protocol to investigate the neural basis for copulation posture of *Drosophila* using a closed-loop real-time optogenetic system




In internal fertilization animals, maintaining a copulation posture facilitates the process of transporting gametes from male to female. Here, we present a protocol to investigate the neural basis for copulation posture of fruit flies using a closed-loop real-time optogenetic system. We describe steps for using deep learning analysis to enable optogenetic manipulation of neural activity only during copulation with high efficiency. This system can be applied to various animal behaviors other than copulation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Automatic detection of male copulation behavior with a fixed female

Details on identifying copulation using a photostimulation system

This system allows neural manipulations during copulation using optogenetics

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# Protocol to investigate the neural basis for copulation posture of *Drosophila* using a closed-loop real-time optogenetic system

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## SUMMARY

**In internal fertilization animals, maintaining a copulation posture facilitates the process of transporting gametes from male to female. Here, we present a protocol to investigate the neural basis for copulation posture of fruit flies using a closed-loop real-time optogenetic system. We describe steps for using deep learning analysis to enable optogenetic manipulation of neural activity only during copulation with high efficiency. This system can be applied to various animal behaviors other than copulation.**

**For complete details on the use and execution of this protocol, please refer to Yamanouchi et al. (2023).[1]**

## BEFORE YOU BEGIN

In this system, DeepLabCut-Live!,[2] a platform that achieves real-time animal pose estimation, is combined with Bonsai[3] to detect copulation initiation in real time. Creation of a training model using DeepLabCut[4] is necessary before starting the protocol. The information on copulation initiation detected by Bonsai needs to be converted into a trigger signal for photostimulation. This section describes three major steps: (1) preparing copulation movies for training the DeepLabCut model, (2) creating the DeepLabCut model, and (3) assembling the software and hardware for the copulation-triggered photostimulation system. Basic coding skills in Terminal, Python, and Arduino are required.

### Prepare copulation movies for training a DeepLabCut model

Timing: 5–8 days (for step 1)

Timing: 10 min (for step 2)

Timing: 1–3 days (for step 3)

All experiments are performed between zeitgeber time (ZT) 1–11 at 25 ± 1°C and 50% ± 10% relative humidity, using wild-type males and females.

1. Prepare virgin flies.
   a. Collect males and females within 8 h after eclosion to ensure their virgin status.

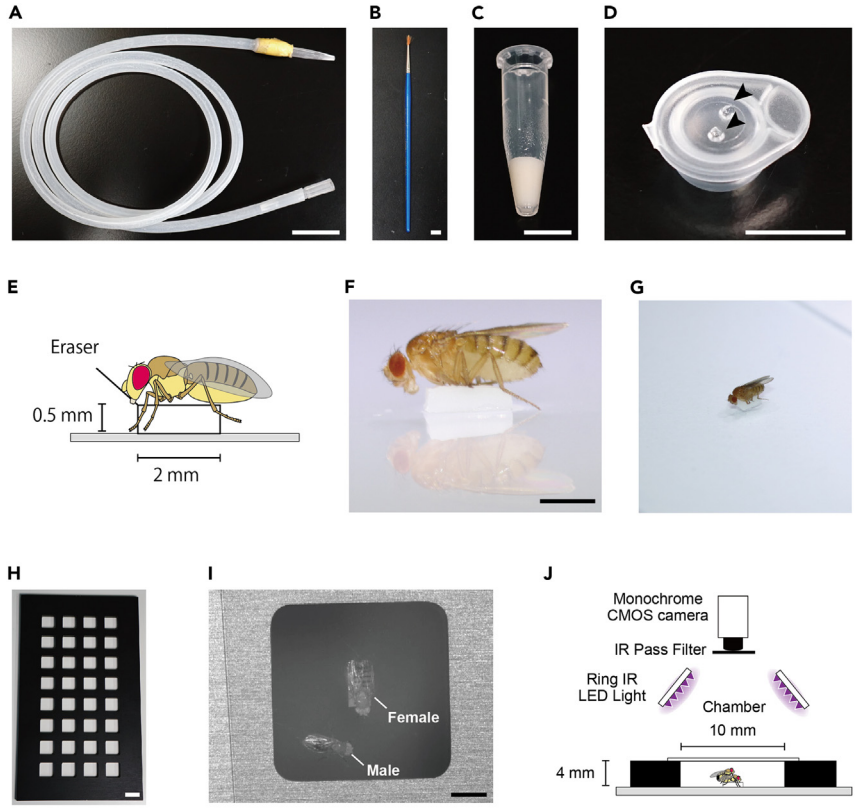


**Figure 1. Preparing copulation movies**

(A) Handmade insect aspirator. Scale bar, 5 cm.

(B) Paintbrush for collecting flies. Scale bar, 1 cm.

(C) Plastic tube containing fly food to keep male flies separately. Scale bar, 1 cm.

(D) The lid of a plastic tube. The black arrowheads indicate small holes to prevent flies from choking. Scale bar, 1 cm.

(E–G) The illustration (E), side view (F), and overview (G) of a fixed female. (F) Scale bar, 1 cm.

(H) Courtship chamber. The chamber is made of an aluminum plate with an anodic oxide coating to prevent light reflection. Scale bar, 1 cm.

(I) Screenshot of copulation assay. Scale bar, 200 μm.

(J) Experimental setup of the copulation assay. An infrared (IR) camera records the flies. The ring IR LED lights that illuminate the chamber enable the IR camera to capture video. The fixed female is placed at the center of the courtship chamber.

i. Anesthetize newly hatched flies on ice for ~1 min.
ii. Separate males and females.

*Note:* We use a handmade insect aspirator and a paintbrush for collecting flies (Figures 1A and 1B).

*Note:* The use of ice anesthesia is recommended in behavioral experiments because previous research suggested that $CO_2$ anesthesia has a more long-term detrimental effect on behavior than ice anesthesia.[5]

b. Females are kept in groups of 10–30 in a fly vial. Males are kept singly in a plastic tube (1.5 mL, Eppendorf; the lid has two small holes to prevent flies from choking) containing ~200 μL fly food (Figures 1C and 1D).

*Note:* Keeping males in isolated conditions increases male courtship activity.[6]

c. Flies are maintained under a 12 h L/D cycle until the copulation assay. Flies are transferred to new tubes or vials every 2–3 days, but not on the day of the experiment. Males and females 5–8 days after eclosion are used in the following experiments.

2. Affix a female fly (Methods video S1).
   a. Prepare the copulation stage. We use a piece of eraser (2 mm in length, 1 mm in width, and 0.5 mm in height).
   b. Glue the copulation stage at the center of a glass slide (Matsunami Glass IND., Ltd, Osaka, Japan) using light-curing adhesives (1771E, ThreeBond Holdings Co., Ltd.).
   c. A female is anesthetized on ice for ~30 s to make it immobile.
   d. The ventral side of its thorax and abdomen is glued to the copulation stage using light-curing adhesives under ice anesthesia for ~30 s (Figures 1E–1G).
   e. After gluing female, the female and copulation stage are irradiated with ultraviolet light for ~5 s by a UV penlight (023BUV, Streamlight, Inc.). The female's genitalia should protrude beyond the copulation stage (Figures 1E and 1F).

⚠ CRITICAL: The glue should attach the female's thorax and part of the abdomen to the copulation stage. The female's forelegs and middle legs should also be fixed to the stage. The adhesive should not stick to other body parts, such as the hindlegs, genitalia, and head (Figure 1F).

*Note:* Gluing the female to the stage, instead of employing a free-moving female, allows us to fix the position at the center of the visual field, facilitating copulation detection in DeepLabCut-Live!. This procedure also enables focusing the laser light for photostimulation on the copulation stage.

3. Perform copulation assay and record copulation movies for creating a DeepLabCut model (Methods video S1, related to steps a-c).
   a. Place the glass slide with a fixed female at the center of an arena of a custom-made courtship chamber (Figures 1H and 1I). See materials and equipment for details on custom-made courtship chamber. The chamber has 32 arenas in total. To eliminate the possible effect of residual pheromones from a previous trial on fly's behavior, each trial is performed in a different arena.
   b. Introduce a male fly into the arena without anesthesia using a handmade insect aspirator.
   c. Enclose the arena with a cover glass (Matsunami Glass IND., Ltd, Osaka, Japan).
   d. Take movies for ~1 h using IC Capture (The Imaging Source Asia Co., Ltd.) by combining the monochrome CMOS Camera (DMK33UX273, The Imaging Source Asia Co., Ltd) with 50 mm focal length camera lens (MVL50M23, Thorlabs, Inc.). The angle of view is adjusted so that a single arena can be enlarged (See Figure 1I). All recordings are performed at a resolution of 640 by 480 pixels and 30 frames/s (Figures 1I and 1J). The arena is illuminated with 850 nm IR light to obtain movies with a monochrome camera equipped with an IR pass filter (IR-82, FUJIFILM Corporation).
   e. Play back the obtained movies and select movies in which copulation occurred. The movies selected here are used in the next step as copulation movies.

*Note:* In our setup, most pairs copulate within 1 h. In our experiment, 10 copulation movies are used to create the DeepLabCut model.

### Create a DeepLabCut model

Timing: 1–3 days

In this step, we create a DeepLabCut model for a real-time optogenetic system. We follow the protocol for training videos in the DeepLabCut workflow.[7] To obtain training data, images are collected from the copulation movies recorded in step 3.

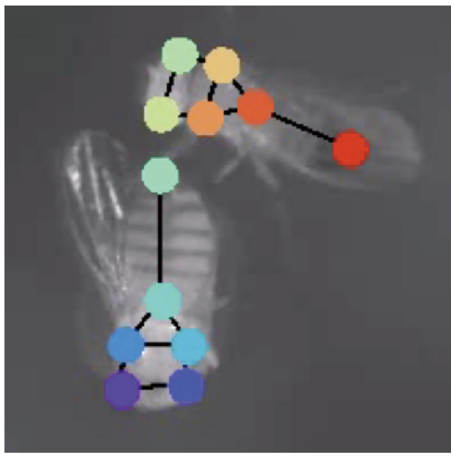

**Figure 2. Key points detected by DeepLabCut model**
The color points indicate 12 key points on eyes, bases of the wings, scutellums, and genitalia of both male and female flies being detected by the DeepLabCut model.

4. Collect movie clips for ~5 min before, during, and ~15 min after copulation from the copulation movie using a video editing software such as VideoProc Converter (Chengdu Digiarty Software, Inc.).

   *Note:* If clips cannot be collected using the above rule due to the timing of the copulation initiation, we adjusted the total time before and after copulation to be 20 min (e.g., When movies end less than 15 min after copulation, we increase the time of pre-copulation clipping.

5. Extract 20 training frames per movie clip using the k-means algorithm in DeepLabCut for the training dataset. The images in the training dataset should include both with and without copulation.
6. Manually label the following 12 key points in each image of the training dataset: eyes, bases of the wings, scutellums, and genitalia of both male and female flies (Figure 2). These images with key points serve as the training dataset.

   *Note:* Label only the key points that can be identified in each movie image; do not label invisible body parts due to body orientation or overlapping male and female flies.

   *Note:* To increase the accuracy of the training dataset, use images of flies in various orientations and locations to improve the DeepLabCut model.

7. Train the model using the training dataset pre-trained with the ResNet50 (epoc = 500000). For the copulation detection, approximately 200 images should be used for creating the DeepLabCut model.
8. Compare the RMSE value (Table S1), the evaluation index implemented in DeepLabCut, and confirm that this value is sufficiently small. Furthermore, analyze five copulation movies that were not used for training dataset to confirm whether the prepared model can detect copulation well.
9. Export the DeepLabCut model in the protocol buffer format (.pb file) to adopt DeepLabCut-Live!.[2] This can be performed using the following command line:

```
dlc model-export /path/to/config.yaml
```

*Note:* To export models as .pb files, DeepLabCut package version 2.1.8 or later is required.[2]

### Creating a copulation-triggered photostimulation system

Timing: 1 day

Optogenetic assays are performed using a copulation-triggered photostimulation system. This step describes (1) creating a custom Bonsai[3] program for real-time analysis, (2) creating an Arduino program for laser regulation, and (3) assembling the devices for the copulation-triggered photostimulation system. In this system, Bonsai refers to DeepLabCut-Live![2] to incorporate coordinates information on male and female flies. Bonsai then calculates the distance between the male and female to generate a binary signal (copulate or not-copulate) for each video frame, which is then sent to the Arduino. The Arduino processes the binary signals and operates a photostimulation system. All original codes are publicly available at Zenodo (https://doi.org/10.5281/zenodo.7780536). The experimenter can use and modify these program codes freely. This real-time optogenetic system can also be utilized for other types of behaviors.

10. Create a custom Bonsai program by connecting nodes. The program scheme is shown in Figures 3A and 3B. This custom Bonsai program imports camera frames, obtains coordinates information on male and female flies by DeepLabCut-Live!, and calculates these coordinates.

*Note:* If (1) the distance between the middle points of female wings and male eyes and (2) the distance between the middle point of male wings and the female scutellums (Figure 4A) are both shorter than the threshold (~1.6 mm, about the half of a fly body length), the Bonsai program classifies this pair into the "copulate" state and generates a "copulate" signal to Arduino. If not, the Bonsai program generates a "not-copulate" signal. The threshold is determined by measuring the distances in copulation movie clips in the DeepLabCut training dataset.

a. Add three "Camera Capture" nodes to import frames from CMOS cameras. One node is for a monochrome CMOS camera to record the fly behavior and the other two are for color CMOS cameras to confirm the time stamp of photostimulations and the photostimulation area, respectively.
b. Add "VideoWriter" nodes to save frames as movie flies from three CMOS cameras.
c. Create a downstream flow for the monochrome CMOS camera (Figure 3A, top). Add a "PredictPose" node to adopt the training model for DeepLabCut-Live! to Bonsai program. By operating this node, DeepLabCut-Live! detects the coordinates information on 12 key points in Bonsai.
d. Add a "Defer" node so that the Bonsai program calculates the coordinates of body parts in real time and recognizes the movie frames that potentially show copulation. The "Defer" node contains the program from a "Source1" node to a "Workflow Output" node shown in Figure 3B. This node is set to classify the fly pair into the binary state, copulate or not-copulate.
e. Add a "DigitalOutput" node to send signals to Arduino.

*Optional:* To control signal timing, add "Delay" nodes between "Defer" and "DigitalOutput". In *Drosophila* copulation, coupling between males and females is unstable for the first 5 min after initiation and stabilizes afterward.[8,9] Therefore, we introduced a 5 min delay to trigger the Arduino-based photostimulation system after copulation detection by Bonsai calculation.

11. Prepare two Arduinos: "Arduino-a" converts the signal that is generated by the Bonsai program into pulse signals ("copulate" or "not-copulate" signal), and "Arduino-b" receives the "copulate" or "not-copulate" pulse signals and begins counting checkpoints to judge the copulation event. When "Arduino-b" judges the copulation event occurred, it generates a trigger signal for turning on the green laser.

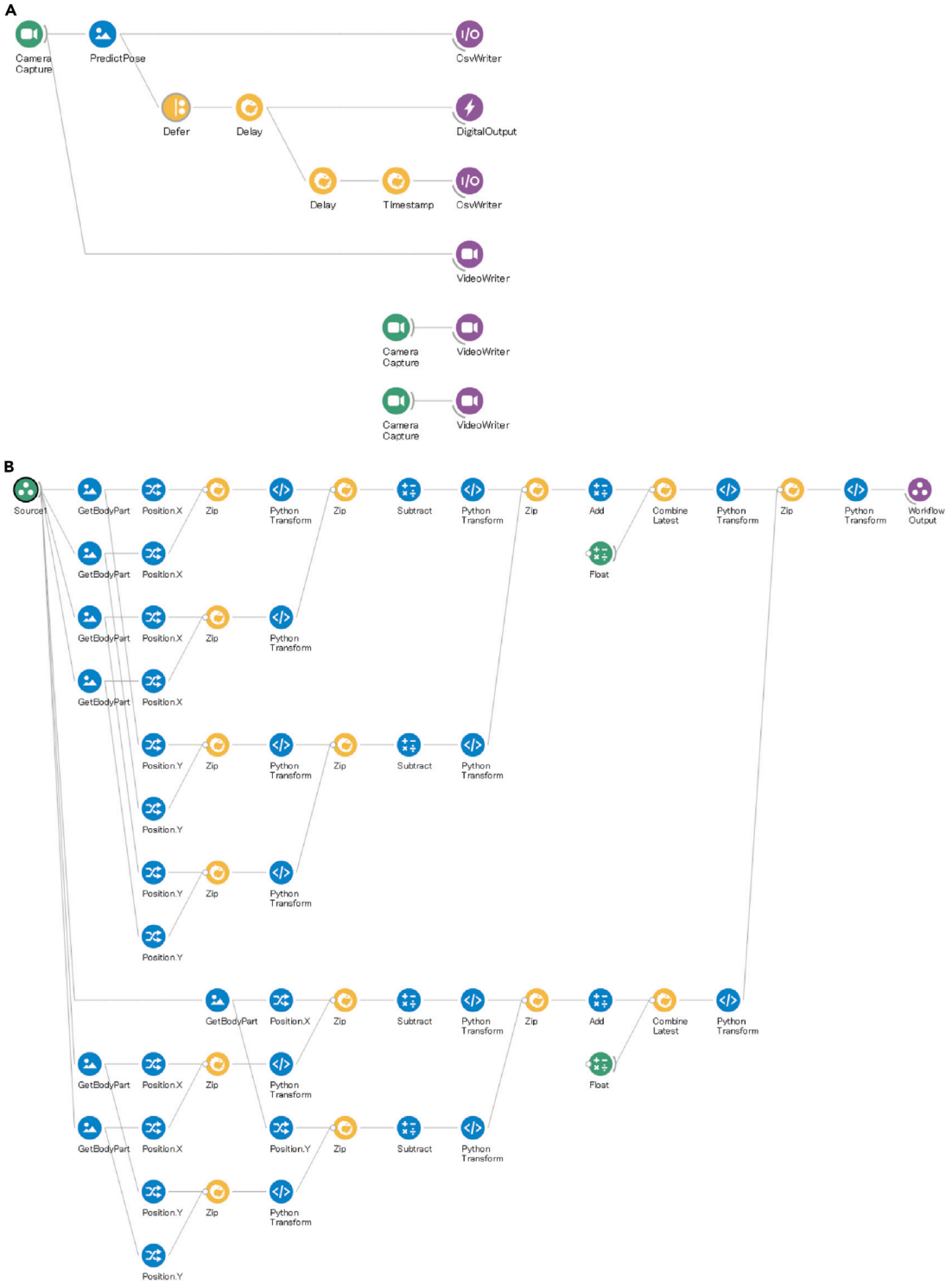


**Figure 3. Bonsai program workflow of the copulation-triggered photostimulation system**

(A) Workflow of the main Bonsai program.

(B) Workflow of the "Defer" node in the main Bonsai program.

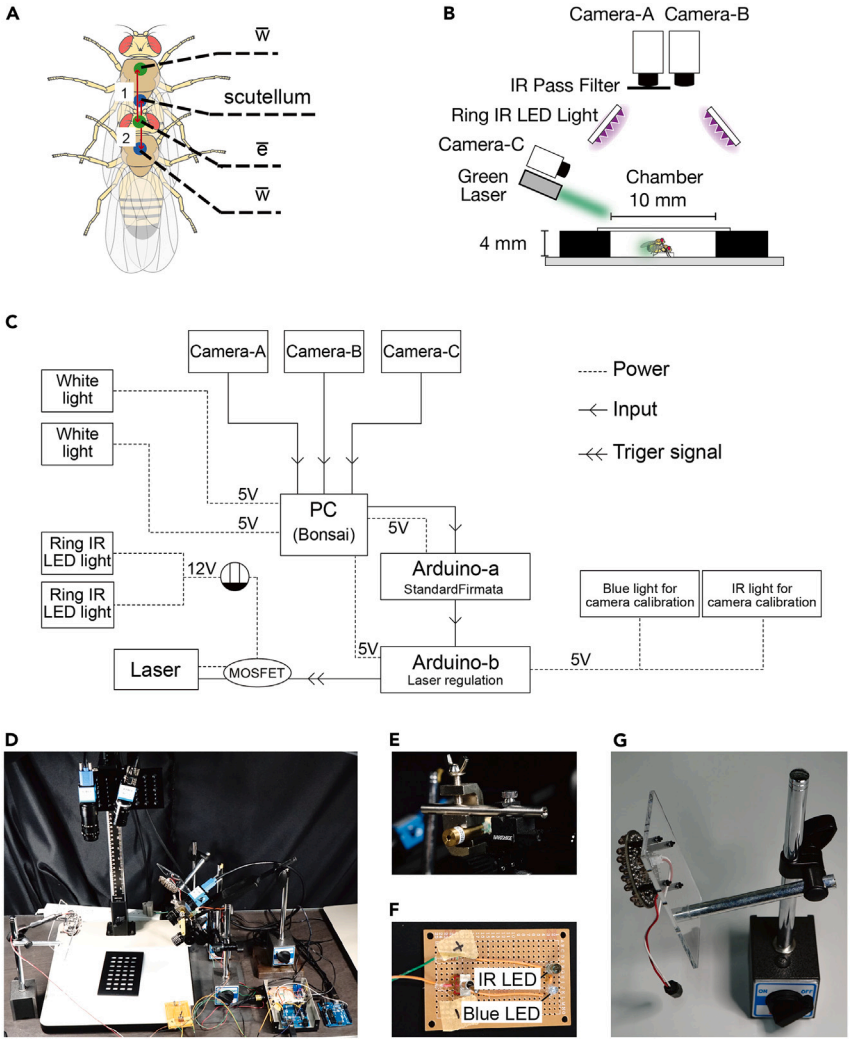


**Figure 4. Experimental setup of the copulation-triggered photostimulation system**

(A) Parameters used in the automatic copulation detection. We obtained the coordinate of the middle point of wing bases, $\bar{w}$, as well as that of eyes, $\bar{e}$. To generate "copulate" or "not-copulate" signals, two distance values were used (1, the distance between the female $\bar{w}$ and male $\bar{e}$ ; 2, the distance between the male $\bar{e}$ and the female scutellum). The "copulate" signal was generated when both values were less than the threshold.

(B) Experimental setup of the copulation-triggered photostimulation system. A monochrome CMOS camera (Camera-A) with a light-absorbing and infrared (IR) pass filter is set above the chamber to record the fly behavior (see Figure 1I for the camera angle). One color CMOS camera (Camera-B) is used to obtain time stamps of photostimulations. Another color CMOS camera (Camera-C) is placed on the same side as the laser and used for confirming the photostimulation area. The ring IR LED light for illuminating the chamber enables the IR camera to capture video. The green laser is used for photostimulation. The fixed female is placed in the center of the courtship chamber.

(C) Schematic of the copulation-triggered photostimulation system.

(D) Overview of the copulation-triggered photostimulation system.

(E) The laser and the manipulator.

(F) Calibration LED light.

(G) Ring IR LED light.

*Note:* Alternatively, you can use the raw Bonsai data to pass all three 'Checkpoints' in the Bonsai script instead of the custom Arduino program.

a. Write the "StandardFirmata" program of Arduino sketch examples in "Arduino-a". "Arduino-a" with "StandardFirmata" program converts the signal from Bonsai into pulse signals. This conversion allows "Arduino-b" to start checkpoint evaluation.
b. Prepare a custom Arduino program for laser regulation. This program has three checkpoints (2, 5, and 15 s after the first detection of "copulate" signal) to reduce the false positives. If the "copulate" signal from Bonsai program is detected at all three checkpoints, the system judges that a copulation event has occurred. The custom Arduino program for laser regulation then creates a trigger signal that turns on the green laser for photostimulation. Although we used three checkpoints of 2, 5, 15 s, each experimenter should define the appropriate checkpoints suitable for the purpose and experimental setup.
c. Write the custom Arduino program in "Arduino-b" to regulate photostimulation after checkpoint evaluation.

12. Assemble the devices for the copulation-triggered photostimulation system (Figures 4B–4D).
    a. Connect "Arduino-a" to a desktop PC.
    b. Connect "Arduino-b" to "Arduino-a".
    c. Set the green laser (wavelength, 532 nm; light intensity, 5.6 $mW/cm^2$) from the side of the female's body. The green laser is regulated using MOSFET which receives signals from "Arduino-b". The precise position of the green laser for irradiating on the male genitalia need to be adjusted just before the experiments.

*Note:* Laser is used to photostimulate a limited area. Alternatively, other light sources can be used. When suppressing *piezo*-expressing neurons throughout the male body, we used a green LED for illuminating the entire chamber by setting the LED at the bottom of the chamber.[1]

d. Set one monochrome CMOS camera and two color CMOS cameras. All CMOS cameras are equipped with a 50 mm focal length lens and operated at a resolution of 640 × 480 pixels at 30 frames per second.

*Note:* A monochrome CMOS camera (Camera-A) with a light-absorbing and IR pass filter is set above the chamber to record the fly behavior (see Figure 1I for the camera angle). One color CMOS camera (Camera-B) is used to time stamp photostimulations. Another color CMOS camera (Camera-C) is placed on the same side as the laser and used for confirming the photostimulation area.

e. Connect two calibration LED lights to "Arduino-b" (Figures 4C and 4F). One of these lights is IR and the other is blue (Figure 4F).

*Note:* Turning these lights on simultaneously enables synchronization of the frames in the movie files recorded by three cameras. Camera-A detects IR light, whereas Camera-B and Camera-C detect blue light.

f. Prepare the Bonsai program. In the "DigitalOutput" node, select the COM connected to "Arduino-a".

*Note:* For the system using DeepLabCut-Live!, a computer equipped with an NVIDIA GeForce GPU or equivalent is preferred.[2]

g. Set two sets of the ring IR LED light above the chamber to enable recordings in "dark" conditions (Figures 4C, 4D, and 4G).

h. Set two white LED lights above the chamber.

*Note:* Instead of two color cameras, a data acquisition device (DAQ) can be used to monitor camera time stamps and record the trigger to regulate the laser photostimulation.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| All trans-retinal | Sigma-Aldrich | Cat# R2500 |
| 99.5% Ethanol | Kanto Kagaku | Cat# 14033-80 |
| Experimental models: Organisms/strains | | |
| *D. melanogaster*: Canton-S | Hotta Lab strain, a gift from Dr. Kei Ito | N/A |
| *D. melanogaster*: *piezo-GAL4* (*Chr 3*) | Bloomington Drosophila Stock Center | BDSC: 59266; RRID: BDSC_59266 |
| *D. melanogaster*: *UAS-GtACR1. d.EYFP* (attP2) | Bloomington Drosophila Stock Center | BDSC: 92983; RRID: BDSC_92983 |
| Software and algorithms | | |
| VideoProc Converter | Chengdu Digiarty Software, Inc. | N/A |
| DeepLabCut | Mathis et al.[4] | https://github.com/DeepLabCut/DeepLabCut |
| IC Capture | The Imaging Source Asia Co., Ltd. | N/A |
| Python v3.8.13 | Python | N/A |
| Bonsai | Bonsai Foundation CIC | https://github.com/bonsai-rx/bonsai |
| Bonsai-DeepLabCut Library | Bonsai Foundation CIC[2] | https://github.com/bonsai-rx/deeplabcut |
| Bonsai-DeepLabCut Design Library | Bonsai Foundation CIC[2] | https://github.com/bonsai-rx/deeplabcut |
| R v4.2.1 | R Project | N/A |
| Arduino IDE | Arduino | N/A |
| ARTool package v0.11.1 | Kay et al.[10] | https://github.com/mjskay/ARTool/ |
| brunnermunzel package v1.4.1 | N/A | https://github.com/toshi-ara/brunnermunzel |
| ggplot2 | N/A | https://ggplot2.tidyverse.org/ |
| Source codes | Yamanouchi et al.[1] | https://github.com/HMYamano/piezo_copulation_source_code |
| Others | | |
| Light-curing adhesives | ThreeBond Holdings Co., Ltd. | Cat# 1771E |
| UV penlight | Streamlight, Inc. | Cat# 023BUV |
| Copulation assay chamber for optogenetic assay | Custom made | N/A |
| Monochrome CMOS camera (DMK33UX273) | The Imaging Source Asia Co., Ltd. | Cat# DMK33UX273 |
| Color CMOS camera (DFK 33UP1300) | The Imaging Source Asia Co., Ltd. | Cat# DFK 33UP1300 |
| 50 mm focal length camera lens (MVL50M23) | Thorlabs, Inc. | Cat# MVL50M23 |
| 50 mm focal length camera lens (VS-5026VM) | VS Technology Corporation | Cat# VS-5026VM |
| IR pass filter (IR-82) | Fujifilm Corporation | Cat# IR-82 |
| 850 nm infrared LED ring light (FRS5CS) | OptoSupply | Cat# FRS5CS |
| 525 nm green LED (OSG5XNE3E1E) | OptoSupply | Cat# OSG5XNE3E1E |
| 532 nm green laser light (JPM-1-3) | LightVision Technologies, Corp. | Cat# JPM-1-3 |
| Magnet stand (MB-BV) | MonotaRO Co., Ltd. | Cat# MB-BV |
| Manipulator (UM-3C) | Narishige Group | Cat# UM-3C |
| Photometer (PM100D) | Thorlabs, Inc. | Cat# PM100D |
| AE-ATMEGA-UNO-R3 (based on Arduino UNO) | Akizuki Denshi Tsusho Co., Ltd. | Cat# AE-ATMEGA-UNO-R3 |
| Cover glass | Matsunami Glass Ind. | Cat# C018181 |
| Slide glass | Matsunami Glass Ind. | Cat# S024410 |

## MATERIALS AND EQUIPMENT

| Standard fly food | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Agar | 0.8% | 8 g |
| Cornmeal | 4.0% | 40 g |
| Yeast | 4.5% | 45 g |
| Glucose | 10% | 100 g |
| Propionic acid | 0.4% | 4 mL |
| 10% Methyl p-hydroxybenzoate in 70% EtOH | 0.3% | 3 mL |
| $dH_2O$ | N/A | Up to 1 L |
| **Total** | **N/A** | **1000 mL** |

*Note:* Maintain the fly food at 4°C for up to 1 month or until it becomes dry.

**Computer Software:** DeepLabCut requires Python/Anaconda.[4] We used a custom-script for Bonsai to perform real-time analysis using DeepLabCut-Live!, and a custom Arduino sketch for controlling LED lights.

**Computer Hardware:** As suggested in the DeepLabCut manual,[4] a PC equipped with NVIDIA GPU (at least 8 GB memory) should be used to train the GPU-based network. You can use Google Colaboratory (https://colab.research.google.com/) instead of a PC equipped with GPU. The real-time analysis using DeepLabCut-Live! also requires a PC equipped with GPU.

**Courtship chamber:** A courtship chamber is made of an aluminum plate with an anodic oxide coating to prevent light reflection. The chamber size is 10 mm × 10 mm × 4 mm with a 1 mm fillet radius for laser photostimulation (Figure 1H).

## STEP-BY-STEP METHOD DETAILS

### Optogenetic assay using the copulation-triggered photostimulation system

**Timing: 15–20 days (for step 1)**

**Timing: 1–2 days (for step 2)**

**Timing: 1 h per experiment (for step 3)**

The copulation-triggered photostimulation system detects copulation events in real time and turns on the green laser required for GtACR1-mediated inhibition of neural activity. This system allows for the inhibition of specific neural circuits in males only during copulation. In this assay, we use males that express GtACR1[11] in *piezo*-expressing neurons (*$w^{1118}$; +/+; piezo-GAL4/UAS-GtACR1.d.EYFP*). As a mating partner, we use wild-type females. See key resources table for details on fly strains.

1. Prepare *GtACR1*-expressing male flies.
   a. Set up the fly crosses. Introduce parental flies (4–5 *piezo-GAL4* males and *UAS-GtACR1* virgin females) into vials containing fly media. The detailed genotypes are listed in the key resources table.
   b. Transfer parental flies (F0 fly) to fresh vials every 5 days.
   c. Collect F1 males of the desired genotypes according to step 1 in before you begin.

*Note:* F0 flies are transferred to fresh vials up to 2 times.

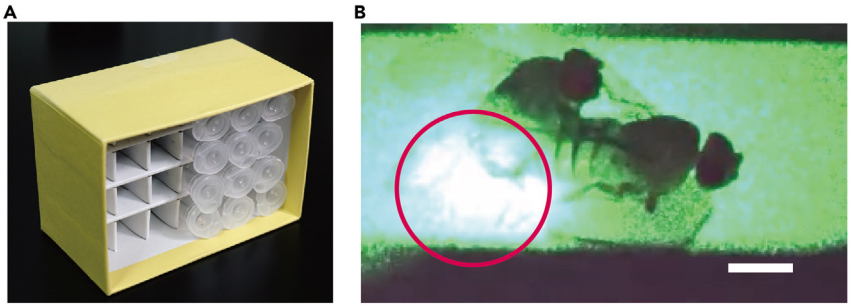


**Figure 5. Maintenance conditions for *GtACR1*-expressing male flies while feeding ATR or ethanol**

(A) Put the plastic tubes horizontally in the box to feed ATR or ethanol to the flies and keep them in the dark.

(B) Lateral view of the optogenetic assay. Red circle indicates the laser focusing area. Scale bar, 1 mm.

*Note:* Set-up several vials at a time to obtain enough F1 male flies.

*Note:* Wild-type females are prepared as mating partners according to step 1 in *Before you begin*.

**Pause point:** The experimenter can decide the timing of subsequent feeding of all-trans-retinal (ATR) as long as the tested flies are within 5–8 days after eclosion at the time of the optogenetic assay.

2. Feeding of ATR. Male flies of the experimental group are fed food containing ATR. Control males are fed food containing ethanol (EtOH) solvent.
   a. Prepare plastic tubes containing ~150 μL of fly food.
   b. For the experimental group, add 2 μL of ATR, 25 mg/mL dissolved in 99.5% EtOH (need to be prepared just before use) on the food surface. For the control group, add 2 μL of 99.5% EtOH on the food surface instead of ATR.
   c. Transfer males to a plastic tube containing fly food with the surface covered with ATR (or EtOH) 24–36 h before the experiment.
   d. Keep the male flies on prepared food for 24–36 h in the dark before being used for the assays.

*Note:* ATR is a light-sensitive pigment that enables ion flux through green-light absorption of GtACR1.[11]

⚠ CRITICAL: After placing a male fly in a plastic tube containing ATR or EtOH, the tubes should be stored horizontally to prevent the male fly from sticking to the food surface (Figure 5).

3. Perform optogenetic assay.
   a. Put a fixed female at the center of a courtship chamber (Figure 1H, see step 2 in before you begin).
   b. The green laser is positioned using a manipulator to adjust the focusing area to the female genitalia. This adjustment allows irradiation on the male genitalia during copulation (Figure 4B).
   c. Introduce a male fly into the chamber without anesthesia.
   d. Enclose them with a cover glass.
   e. Run a custom Bonsai script and take movies. The Bonsai script operates a monochrome CMOS camera and two color CMOS cameras.
   f. The two calibration LEDs are turned on at the beginning of each recording to calibrate the start timing of movies recorded by each camera (Figure 4F).

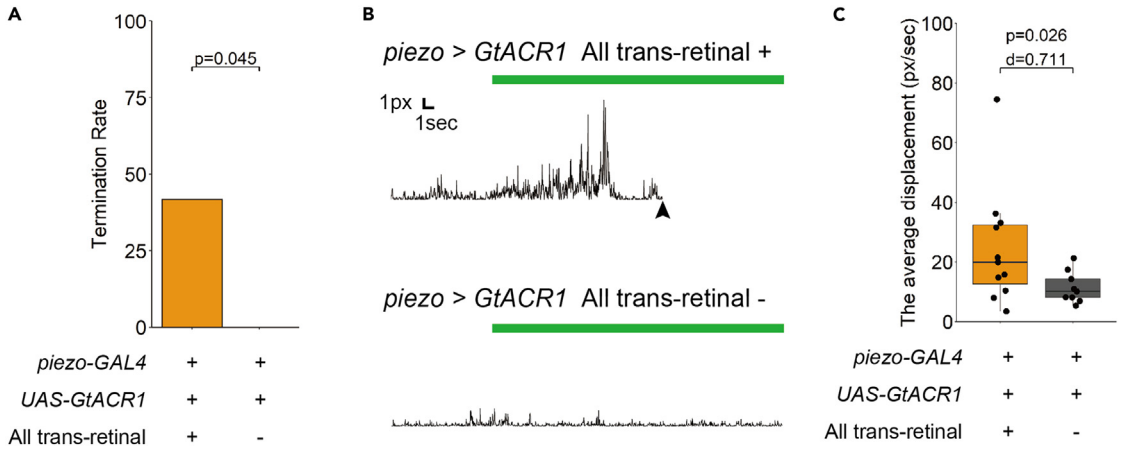


**Figure 6. Male copulation behavior analysis during optogenetic inhibition**

(A) Rate of copulation termination during optogenetic inhibition. *piezo-GAL4* expressing neurons of the posterior side of male bodies are inhibited. Fisher's exact test was used for statistical analysis. p indicates p value. Sample sizes: Experimental, n = 12; Control, n = 9. Reprinted with permission from Yamanouchi et al., 2023.[1]

(B) Example time traces of male copulation positions during laser photostimulation. Data for *piezo-GAL4>UAS-GtACR1* males in experimental (Top; All trans-retinal+) and control (Bottom; All trans-retinal−) conditions are shown. Green horizontal bars indicate the period of the laser photostimulation, which starts 5 min after the copulation initiation. Black arrowhead shows the termination of copulation. Reprinted with permission from Yamanouchi et al., 2023.[1]

(C) Average displacement of the male copulation position for *piezo-GAL4>UAS-GtACR1* males during laser photostimulation. ART one-way ANOVA test was used for statistical analysis. Boxplots display medians (horizontal white line in each box) with 25th and 75th percentiles and whiskers denote 1.5× the interquartile range. Each point indicates individual data. d and p indicate Cohen's d effect size and p value, respectively. Sample sizes: Experimental, n = 12; and Control, n = 9. Reprinted with permission from Yamanouchi et al., 2023.[1]

i. For calibration of time-stamps in each movie, blink the two calibration LEDs three times so that LED lights appear on two cameras (Camera-A and Camera-B) at the beginning of each movie file.
ii. In the analysis step, the onsets of movies are aligned based on the calibration LED of each movie.

***Note:*** When a copulation event is detected by "Arduino-b", laser photostimulation is turned on after a predetermined delay period (5 min in our case). During photostimulation, male *piezo*-expressing neurons are suppressed.

g. Stop the Bonsai program after ~1 h or copulation has finished.

## EXPECTED OUTCOMES

In adult *D. melanogaster*, Piezo channels are responsible for internal and external mechanosensation.[12–15] $Piezo^{KO}$ males tend to tilt during the entire copulation period, indicating the contribution of the Piezo channel to stabilizing copulation posture. In our optogenetic assay shown here, we used a male fly expressing the green-light-gated anion-channel GtACR1 in *piezo*-expressing cells. Five min after this system had detected copulation initiation in each pair, the activity of *piezo*-expressing neurons at the posterior side (focusing on male genitalia) of males was suppressed using a green laser. In the experimental group, this targeted photostimulation destabilized the male's copulation posture and significantly terminated copulation (Figure 6A). In contrast, no such tendency was observed in control males (Figure 6A).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Quantification

Here we describe a method to evaluate whether copulation is terminated by optogenetic inhibition of the male *piezo*-expressing neurons. First, the time stamps of videos acquired by three cameras are

synchronized by the frames capturing blinks of two calibration LED lights. Next, the video acquired by Camera-A is replayed, and data in which copulation occurred and continued for more than 5 min are selected. For the selected data, videos recorded by Camera-C allow for confirmation of whether the laser is irradiated to the male genitalia for 30 s from 5 min after copulation initiation. Data with laser irradiation problems are excluded. The timing of laser irradiation cannot be determined from the video acquired by Camera-A since this is an IR camera. Therefore, we determine when the laser photostimulation occurs in the video acquired by Camera-B as a reference. If the male dismounted from the female during the laser photostimulation, the pair was judged to have terminated copulation (Figure 6A).

*Optional:* In addition, we analyzed the body movements of males during photostimulation using DeepLabCut. We also found that the average movement distance during photostimulation was greater when compared to the average movement distance during the no-photostimulation period (Figure 6B). In the experimental group, this targeted photostimulation destabilized the male's copulation posture significantly after the initiation of photostimulation (Figure 6C).

### Statistical analysis

To conduct statistical analyses, we used R (version 4.2.1) and Python (version 3.8.13). After verifying the equality of variance (Bartlett's test for three-groups comparisons; F-tests for two-groups comparisons) and normality of the values (Shapiro-Wilk test), we compared between conditions by using Aligned rank transform one-way analysis of variance (ART one-way ANOVA) tests. The p values were adjusted using the Benjamini-Hochberg method in the post hoc test. We used the ARTool package (version 0.11.1)[15] for the ART one-way ANOVA. The graphs shown in Figure 6 were drawn using the R package ggplot2.

## LIMITATIONS

The Bonsai program generates "copulate" signals based on distances between the male's and female's body parts. Therefore, false positives of "copulate" signal may occur, especially if the male stays beside the fixed female and the male attempts to mate but fails to copulate. Such false-positive signals would affect the accuracy of detecting copulation events to generate trigger signals for the laser control in the Arduino programs. We set several checkpoints in the Arduino program to reduce false copulation detection; only if copulation signals are detected at all checkpoints does our system determine that a copulation event has occurred. Although checkpointing can improve the accuracy of copulation event detections, this system still generates a trigger signal without an actual copulation event. See Table S2 for a list of false negatives and false positives that indicate the evaluation of the copulation-triggered photostimulation system.

Another problem with the copulation-triggered photostimulation system is that copulation detection requires fixing the female, which may reduce the copulation rate. Fixation of the female is desirable as DeepLabCut-Live! is difficult to use for tracking freely moving multi-animals. Although DeepLabCut offers an algorithm suitable for multi-animal tracking,[16] this algorithm is currently not applicable to DeepLabCut-Live!. Thus if a freely-moving female is used as the male's mating partner, DeepLabCut-Live! fails to acquire coordinate information on two individual flies. Fixing a female restricts its locomotion, allowing DeepLabCut-Live! to recognize the female as a different object from a free-walking male.

Another drawback of fixing a female is that it precludes observation of the post-mating behaviors of females, such as egg laying, because a fixed female cannot be removed from the stage.[17] If post-mating behavior is required to be observed, it would be helpful to use a real-time copulation detection system which allows for multiple freely moving flies, such as a system based on YOLO,[18] an

object detection algorithm,[19] and a system defined by the individual distance between two individuals.[20]

## TROUBLESHOOTING

### Problem 1

Low copulation rates during the experiment (related to Step 3 in before you begin and Step 3 in step-by-step method details).

### Potential solution

Keep the system illuminated with a faint white light, as one factor contributing to low copulation rate may be dark experimental conditions. In addition, transgenic male flies that have *white* gene mutations on the X chromosome have a low copulation success rate.[21] In this case, the male genotype should be re-examined.

### Problem 2

Different playback speeds between movies recorded in three cameras for the optogenetic assay (related to Step 3 in step-by-step method details).

### Potential solution

This is a possible problem due to the workflow in Bonsai. The capture workflow of Camera-A contains DeepLabCut-Live! image processing and copulation detection, which could delay video recording. To correct the playback speeds of each movie, calculate the relationship between the playback speed of each movie using the calibration light at the start of the experiment and at the start of copulation. Camera-B and Camera-C can be used as a trustworthy time reference, so we match the speed of Camera-A's video to these cameras.

Another possible solution is to use a DAQ device. The DAQ is able to monitor camera timestamps and measure the playback speed relationship between each camera. In addition, the DAQ can monitor the triggers to turn on the laser photostimulation. Employing DAQ allows simplification of the hardware for this system, such as reducing the number of cameras in use, but requires more advanced programming skills.

### Problem 3

Too many false positives in copulation detection by Bonsai (related to Step 3 in step-by-step method details).

### Potential solution

Modify the definition of the copulation state decision making in the Bonsai program. In addition, revise the number and duration of checkpoints in the Arduino sketch.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Ryoya Tanaka (tanaka.ryoya.z3@f.mail.nagoya-u.ac.jp).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- DeepLabCut models, training data, and all the other codes have been deposited at Zenodo and are publicly available (https://doi.org/10.5281/zenodo.7780536). The DOI is listed in the key resources table. Any additional original/source data are available from the lead contact on request.

- Any additional information required to reanalyze the data reported in this study is available from the lead contact upon request.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102623.

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## AUTHOR CONTRIBUTIONS

H.M.Y.: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, visualization, project administration, and writing – original draft; A.K.: supervision, funding acquisition, and writing – original draft; R.T.: supervision, project administration, funding acquisition, and writing – original draft.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used DeepL in order to assist in translating texts from Japanese to English. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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