Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62895R1
Full Title:	Electroantennography-based bio-hybrid odor-detecting drone using silkmoth antennae for odor source localization
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Additional Information:	
Question	Response
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TITLE:

Electroantennography-based Bio-hybrid Odor-detecting Drone Using Silkmoth Antennae for Odor Source Localization

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KEYWORDS:

Drone, bio-hybrid robotics, electroantennogram, silkmoth, insect antenna, odorant biosensor, odor source localization, spiral-surge algorithm

SUMMARY:

This study introduces experimental protocols for a bio-hybrid odor-detecting drone based on silkmoth antennae. The operation of an experimental electroantennogram device with silkmoth antennae is presented, in addition to the structure of a bio-hybrid drone designed for odor source localization using the spiral-surge algorithm.

ABSTRACT:

Small drones with chemical or biosensor devices that can detect airborne odorant molecules have attracted considerable attention owing to their applicability in environmental and security monitoring and search-and-rescue operations. Small drones with commercial metal-oxide-semiconductor (MOX) sensors have been developed for odor source localization; however, their real-time–odor-detection performance has proven inadequate. However, biosensing

technologies based on insect olfactory systems exhibit relatively high sensitivity, selectivity, and real-time response with respect to odorant molecules compared to commercial MOX gas sensors. In such devices, excised insect antennae function as portable odorant biosensor elements and have been found to deliver excellent sensing performance. This study presents experimental protocols for odorant-molecule detection in the air using a small autonomous bio-hybrid drone based on a mountable electroantennography (EAG) device incorporating silkmoth antennae.

We developed a mountable EAG device including sensing/processing parts with a Wi-Fi module. The device was equipped with a simple sensor enclosure to enhance the sensor directivity. Thus, odor source localization was conducted using the spiral-surge algorithm, which does not assume an upwind direction. The experimental bio-hybrid odor-detecting drone identified real-time odorant-concentration differences in a pseudo-open environment (outside a wind tunnel) and localized the source. The developed drone and associated system can serve as an efficient odorant molecule-detection tool and a suitable flight platform for developing odor source localization algorithms owing to its high programmability.

INTRODUCTION:

With recent advances, small drones with chemical sensing devices have become highly applicable in environmental and security monitoring and gas-leak detection[1]. Small drones (with a diameter approximately < 20 cm) with commercial MOX gas sensors have been recently applied for performing odor mapping or odor source localization[2–4]. When searching for odor sources, a drone must trace odor plumes; however, odor source localization using small drones presents significant challenges. In an open environment, odor-plume structures are subjected to continual changes due to environmental factors such as the wind or landscape. Hence, drones should be capable of identifying odorant-concentration differences and directions varying over time; however, the odor detection performance of commercial MOX sensors is still inadequate for real-time sensing because of their slow recovery time[5].

Bio-hybrid systems formed by the merging of biological and artificial systems are a recent trend in robotics and sensor technologies[6], showing great potential for surpassing the capabilities of existing approaches. For instance, a bio-robotic sensor network has been developed based on cockroaches for application in disaster situations[7]. Experiments have been conducted in which cyborg rats with computationally enhanced intelligence were tasked with solving mazes[8]. Performance enhancements and the possibility of social integration of biomimetic robots into groups of real zebra fishes have been investigated[9].

Naturally, this trend has been applied to develop odorant sensors[10]. For example, biosensors based on insect olfactory systems have relatively high sensitivity and selectivity with respect to various odorant molecules compared to existing MOX sensors[11]. Along these lines, we had previously developed bio-hybrid odorant biosensor systems based on a combination of insect cells expressing insect odorant receptors and a microscope or electronic devices[12–16]. Moreover, insect antennae can be independently used as portable odorant sensing parts with high sensitivity, selectivity, reproducibility, and quick response/recovery time, using the electroantennography (EAG) technique[17–19]. Several ground-mobile odor-sensing robots with

EAG techniques based on insect antennae[20–23] or small drones with EAG devices[24,25] have been developed for odor detection and odor source localization. These robots displayed sensor sensitivity and real-time sensing ability. However, the mobility of ground mobile robots is significantly influenced by land features or obstacles. In addition, the flight performance and odor source localization algorithms of existing EAG-based bio-hybrid drones remain limited because experimental conditions have been limited to tethered flight[24] or to being conducted in a small wind tunnel[25].

This study presents experimental protocols for odor detection in the air and odor source localization using a recently developed bio-hybrid drone based on silkmoth (*Bombyx mori*) antennae[26]. We developed a mountable-sized and lightweight EAG device with a wireless communication function to detect the odor responses of silkmoth antennae. The EAG device was mounted on a small drone, installed in a simple sensor enclosure to enhance the sensor directivity for odorant molecules and reduce noise. The bio-hybrid drone reproducibly detected airborne odorant molecules and identified the maximum odorant concentration during spiral movements. Moreover, the drone localized the odor source using the spiral-surge algorithm without wind-direction information.

PROTOCOL:

1. Insects

NOTE: Eggs of silkmoths (*Bombyx mori*) were purchased from a domestic company. The silkmoths were used within 10 days after they emerged from cocoons. Prepare three adult silkmoths for the experiments (six antennae); however, this number can be changed depending on the experimental requirements.

1.1. Incubate silkmoth eggs at 15 °C for 24 h and move them to an incubator at 25 °C.

NOTE: The silkworms hatch approximately 10–13 days later.

1.2. Place the silkworms on sliced artificial diets in a plastic dish.

1.3. After 20–25 days of silkworm raising, observe the formation and pupation of the silkworms within cocoons.

NOTE: The cultivation procedure includes feeding, removal, and sanitization in an environment at 25 °C. The silkmoths emerge from the cocoons after 10–15 days.

2. Odorants and odor source preparation

NOTE: The principal component of the female silkmoth sex pheromone, bombykol ((E,Z)-10,12-hexadecadien-1-ol), was used as an odor source to perform stimulation. A male silkmoth (**Figure 1A**) can identify and discriminate bombykol[27], and isolated silkmoth antennae have been used to act as a biosensor on mobile robots[20–22]. Store purified bombykol dissolved in hexane (10 mg/mL) in a high-sealed storage bottle in a refrigerator at −30 °C.

2.1. Insert a syringe into the high-sealed storage bottle and withdraw and inject 2 mL of 2000 ng/µL bombykol into a 10 mL vial. Then, add 8 mL of hexane to the same vial.

2.2. Dilute 400 ng/µL of bombykol to 2 ng/µL of bombykol with hexane in a 1 mL vial.

2.3. Cut filter paper into 10 mm × 10 mm pieces, roll them into a cylindrical shape, and place them in a glass tube (internal diameter [ID]: 5 mm; outer diameter [OD]: 7 mm; length [L]: 100 mm).

2.4. Drop a diluted sample (100 ng bombykol dissolved in 50 µL of hexane) onto a portion of the filter paper in the glass tube.

2.5. Close both ends of the glass tube with the filter paper using poly-droppers cut in the middle.

3. EAG experiments on a fixed desk surface

NOTE: The mountable EAG device, which functions as a portable biosensor on a small drone, is shown in **Figure 1B**. The device included high-pass (0.1 Hz) and low-pass (300 Hz) filters. The detailed information of the electrical circuit is described in Terutsuki et al.[26]

3.1. Perform data acquisition and analysis on a personal computer (PC) after the EAG device has sent the measurement data.

3.2. To generate purified air, pass the airflow generated by a compact air pump with a cooling fan through cotton, activated carbon granules, and distilled water. Then, pass the purified air through a glass tube for stimulation.

NOTE: A photograph of the odor stimulation system is shown in **Figure 1C**. The airflow path is indicated by black arrows. The airflow path of the exhaust port of the solenoid valve is indicated by the dashed black arrow.

3.3. Set the flow rate as 5 L min^{-1} using a flowmeter for odor stimulation in the fixed experimental setup. Set a higher flow rate for generation, assuming odor stimulations of several meters for the drone experiments.

NOTE: That the flow rate (5 L min^{-1}) did not affect the signal detection of the EAG device had been previously confirmed[26]. The maximum airflow velocity at the EAG device position during stimulation was measured as 3.9 m s^{-1} using an anemometer.

3.4. Use a solenoid valve with a microcontroller to stimulate the EAG device and conduct the stimulations automatically.

3.5. Set the stimulation time to 0.5 s using the solenoid valve.

3.6. Use electrically conductive gel to attach a silkmoth antenna to the electrode.

NOTE: This procedure does not require the insertion of micrometer-scale wires to both ends of a silkmoth antenna to attach it to the EAG device.

3.6.1. Isolate silkmoth antennae using postmortem scissors (**Figure 2A,B**) without anesthesia. See **Figure 2C** for an enlarged view of the antenna.

3.6.2. Cut both sides of the isolated silkmoth antenna and attach it to the Ag/AgCl-coated electrodes of the sensing part of the EAG device (**Figure 3A**) using electrically conductive gel.

3.7. Connect the glass tube containing bombykol to the odor stimulation system (ensure that the pump is already switched on).

3.8. Fix the glass tube such that its tip is 10 mm from the silkmoth antenna on the EAG device (**Figure 3B**).

3.9. Set the exhaust port (diameter of 60 mm) at 30 mm behind the EAG device to stabilize the airflow and prevent pheromone stagnation (**Figure 3B**).

3.10. Switch on the EAG device. Connect the PC to the Wi-Fi access point.

3.11. Run the data acquisition program on the PC. See **Figure 3C** for the graphical user interface (GUI) on the PC for the experiments.

3.12. After pressing the **Ground** button in the **Log menu** to decide the experimental state, press the **Log start** button for data acquisition. Five seconds after pressing the **Log start** button, initiate odor stimulations.

3.13. Press the **Log stop** button on the GUI to stop recording.

4. Drone

NOTE: A commercial drone flight platform (98 mm x 93 mm x 41 mm; weight 87 g; maximum flight time 13 min) was used in this study. The payload of the drone was approximately 30 g based on the experiments. The drone was equipped with a vision positioning system (VPS) consisting of a camera and an infrared sensor under its body, which allowed for stable hovering without an external positioning system.

4.1. Remove the top cover of the drone and add a custom carbon fiber-reinforced plastic (CFRP) board using a three-dimensional (3D)-printed mount to attach the EAG device. See **Figure 4A** for an image of the bio-hybrid drone.

NOTE: The drone developer offers a software development kit (SDK) and sample Python programs (see the **Table of Materials**); therefore, the drone control program for flight experiments was based on these.

4.2. Send flight commands through the PC to control the drone.

NOTE: For safety, cut-resistant gloves are required for stopping (catching) the drone in an emergency abort. The GUI is equipped with an emergency stop button to immediately stop the rotation of the propellers of the drone (**Figure 3C**).

5. Flight experimental area preparation

5.1. Prepare an experimental flight area (5.0 m x 3.2 m x 3.0 m) and equip it with a commercial surveillance camera on the ceiling.

5.2. Set the flow rate of the odor stimulation system as 5 L min^{-1} and the stimulation time to 0.5 s using the solenoid valve.

6. EAG experiments on the drone

6.1 Isolate silkmoth antennae using postmortem scissors and cut both sides of the antenna.

6.2 Attach the isolated antennae to the Ag/AgCl-coated electrodes of the sensing part of the EAG device using electrically conductive gel.

6.3 Connect the glass tube containing bombykol (50,000 ng in 250 µL of hexane/filter paper) to the odor stimulation system (with the pump already switched on).

6.4 Set the glass tube so that the tube and its tip are parallel to and directly above the edge of the desk, respectively.

6.5 Set the circulator so that the most protruding part (the center of the fan) is 15 cm from the edge of the desk.

6.6 Set the wind speed of the circulator to **1 (minimum power)** by pushing the button on the console.

6.7 Mount the EAG device on the drone. Connect the PC to the Wi-Fi access point. Switch on the EAG device and the drone.

NOTE: The switch of the EAG device is in the processing part.

6.8 Run the drone control program on the PC.

6.8.1. After the light on the drone blinks yellow, press the appropriate button in the **Command menu** on the GUI (**Figure 3C**) of the PC to execute the command.

NOTE: After the drone is connected to the PC, the GUI displays the drone status values (including the position, altitude, and temperature information obtained by the drone), and the light on the drone will turn purple.

6.8.2. Press the **Take off** button on the GUI to hover the drone above the ground.

6.8.3. After pressing the **Flight** button in the **Log menu** to decide the experimental state, press the **Log start** button for data acquisition.

NOTE: Odor stimulation will be initiated 5 s after pressing the **Log start** button.

6.8.4. Press the **Log stop** button on the GUI to stop recording.

6.8.5. Send the **Stop** command in intervals of 5 s after the lift-off of the drone to maintain the hovering state, as the drone automatically lands if not operated for approximately 15 s.

7. Sensor enclosure

7.1. Develop a sensor enclosure (L: 40 mm; ID: 20 mm; OD: 22 mm) based on a carbon fiber tube to enhance the sensor directivity. See **Figure 4B,C** for an image of the bio-hybrid drone with its sensor enclosure and configuration.

7.2. Cover the sensing part with a heat-shrink insulation tube and fix it to the inner wall of the enclosure using double-sided tape.

7.3. Insert the sensing part of the EAG device into the sensor enclosure.

7.4. Set the distance between the tip of the electrodes and the tip of the enclosure as 10 mm.

8. Odor tracing demonstration using the bio-hybrid drone

8.1. Isolate silkmoth antennae using postmortem scissors and cut both sides of the antenna.

8.2. Attach the isolated antenna to the Ag/AgCl-coated electrodes of the sensing part of the EAG device using electrically conductive gel.

8.3. Mount the EAG device with the sensor enclosure on the drone.

8.4. Hover the drone so that it begins an approximately 90° pivoting motion to the left and right.

8.5. Stimulate the EAG device on the drone using poly-droppers containing bombykol during these movements.

8.6. Conduct four cycles of step 8.5.

NOTE: After step 8.6, the drone will rotate clockwise. When conducting the stimulation during this movement, the drone will perform one counterclockwise rotation and land.

9. Odor source localization using the bio-hybrid drone

9.1. Connect the glass tube containing bombykol (50,000 ng in 250 µL of hexane/filter paper) to the pump that is already switched on.

9.2. Fix the glass tube such that its tip is 150 mm from the circulator.

9.3. Define the direction toward the odor source as 0°, and set the drone at an angle of 270° from the odor source at the starting point.

9.4. Connect the PC to the Wi-Fi access point, and switch on the EAG device and the drone.

9.5. Run the drone control program on the PC.

9.5.1. After the light on the drone blinks yellow, press the appropriate button in the **Command menu** on the GUI of the PC (**Figure 3C**) to execute the command.

NOTE: After the drone is connected to the PC, the GUI will display the drone status values (including the position, altitude, and temperature information obtained by the drone), and the light on the drone will turn purple.

9.5.2. Press the **Take off** button on the GUI to hover the drone above the ground.

9.5.3. After pressing the **Search** button in the **Log menu** to decide the experimental state, press the **Log start** button for data acquisition. Then, press the **Search start** button in the **Command menu** to initiate odor source localization using the spiral-surge algorithm and cyclic odor stimulations (opened: 0.5 s; closed: 2.0 s) of the odor source.

9.5.4. After landing the drone, press the **Log stop** button on the GUI to stop recording.

REPRESENTATIVE RESULTS:

This paper describes the protocols for signal measurements using the proposed EAG device mounted on a desk and drone. First, we evaluated the performance of the EAG device on a desk. A silkmoth antenna on the EAG device was stimulated by bombykol. Twenty continuous stimulations were conducted using 100 ng of bombykol dissolved in 50 µL of hexane with intervals of 5 s, as controlled by a microcontroller. The results indicated that the proposed EAG device reproducibly responded to the stimulations (**Figure 5**).

The odor detection performance of the EAG device was subsequently evaluated on the drone. The drone equipped with the EAG device hovered at the height of 95 cm from the floor and at a distance of 90 cm from the odor source (**Figure 6A**). By following the procedure described in section 6, the signals of the EAG device on the drone were measured relative to bombykol (50,000 ng in 250 µL of hexane/filter paper). The sensor performance of a commercial gas sensor on a drone was evaluated for comparison. A digital multi-pixel gas sensor[28] was used to detect ethanol vapors. This sensor can be used for the detection of total volatile organic compounds (TVOCs).

According to the datasheet, the TVOC signal range of the sensor was 0–60,000 ppb. The drone with the gas sensor breakout board hovered under the same conditions as the EAG device. Moreover, 500 µL of ethanol (99.5% purity) was used as the odor source instead of bombykol. The typical signals of the EAG device and gas sensor on the drone are shown in **Figure 6B**. As the odorant molecules and sensor devices differed in this comparison, quantitative comparisons could not be performed. However, the experimental results suggest that it may be difficult for a drone with a commercial gas sensor to detect odorant molecules with a rapid response/recovery speed. In particular, the recovery time of the gas sensor in this study was significantly higher than that of the EAG device with silkmoth antennae.

We also evaluated the sensor directivity of the EAG device on the drone. In this study, the direction toward the odor source was defined as 0°, and the drone was rotated clockwise by 60° intervals to evaluate signal intensities at each angle. For the drone without a sensor enclosure, the signal intensity at 180°, while the drone faced in the opposite direction from the odor source, was occasionally higher than that at 0° (**Figure 6C**). However, for the drone equipped with the enclosure, the signal intensity of the EAG at 0° became higher than that at 180° (**Figure 6D**). Consequently, the sensor enclosure enhanced the sensor directivity of the EAG device on the drone.

An odor-tracing demonstration was conducted using the bio-hybrid drone with the sensor enclosure. The results indicated that the drone detected bombykol in the air outside a wind tunnel and identified the direction of the odor plume by pivoting movements (**Figure 7**). Finally, odor source localization was conducted based on the spiral-surge algorithm using the bio-hybrid drone (**Figure 8A**). The drone was set at 270° from the odor source at the starting point. After hovering, the drone started searching for the maximum value of the signal intensity during clockwise or counterclockwise spiral movements. Then, the drone moved forward in the direction of the maximum value of the signal intensity. After repeating the odor-searching spiral and surge movements six times, the drone landed on the ground. The flowchart of the spiral-surge algorithm is described in Terutsuki et al.[26]

The trajectory, yaw angles, and EAG signals during the odor source localization are presented in **Figure 8B–D**. **Figure 8D** shows that the detection time, including response and recover times of the EAG device on the drone, was approximately 1 s. The drone autonomously modified its movement by searching for the maximum odor concentration during the spiral movements. Readers can view videos of the odor source localization by the bio-hybrid drone described by Terutsuki et al.[26].

FIGURE AND TABLE LEGENDS:

Figure 1: The silkmoth, EAG device, and odor stimulation system. (**A**) Image of a male silkmoth. (**B**) Image of the mountable EAG device for a small drone. (**C**) Image of the odor stimulation system with airflow directions. Abbreviation: EAG = electroantennography.

Figure 2: Isolation of silkmoth antenna. (**A**) Isolation of a silkmoth antenna using postmortem scissors. (**B**) Typical isolated silkmoth antenna. (**C**) Enlarged view of an isolated silkmoth antenna; scale bar = 0.5 mm.

Figure 3: EAG device set up and GUI. (**A**) Installation of an isolated silkmoth antenna on the electrodes of the EAG device using gel. (**B**) Setup for odor stimulation using the EAG device on the desk. (**C**) The GUI for the experiments. Abbreviations: EAG = electroantennography; GUI = graphical user interface.

Figure 4: Bio-hybrid drone. (**A**) Bio-hybrid drone based on a silkmoth antenna. (**B**) Bio-hybrid drone with the sensor enclosure. (**C**) Configuration of the bio-hybrid drone. Scale bars (**A, B**) = 50 mm. Abbreviation: CFRP = carbon fiber-reinforced plastic.

Figure 5: Typical continuous response profile of the EAG device on the desk stimulated by bombykol. Abbreviation: EAG = electroantennography.

Figure 6: Experimental environment of the bio-hybrid drone and signal intensity of the EAG device. (**A**) Image of the experimental environment with the bio-hybrid drone, which autonomously hovered 95 cm above the ground at a distance of 90 cm from the odor source. (**B**) Comparison between the typical signals of the EAG device and commercial gas sensor on the drone. (**C**) Typical signal intensity of the EAG device without equipping the sensor enclosure on the drone at each angle (N = 1). (**D**) Average signal intensity of the EAG device with the enclosure on the drone at each angle (N = 3; individual tests). The unit of the signal intensities is V. **C** and **D** have been modified from Terutsuki et al.[26]. Abbreviations: EAG = electroantennography; TVOC = total volatile organic compounds.

Figure 7: Manual odor stimulation to demonstrate detection and tracing of odor in a room by the bio-hybrid drone.

Figure 8: Odor source localization by the bio-hybrid drone. (**A**) Viewpoint from the ceiling camera of the flight area of the bio-hybrid drone. (**B**) Typical flight trajectory, (**C**) yaw angles, and (**D**) EAG signal intensities during odor source localization using the spiral-surge algorithm. These figures are representative results (N=1). **A–D** have been modified from Terutsuki et al.[26].

Supplemental Video S1: Demonstration of manual odor stimulation using the bio-hybrid drone.

DISCUSSION:

Mobile robots with EAG devices were first developed 25 years ago[20]. Since then, there have been significant advancements in robotic technologies, including drones. Considering these technological advancements, we developed an autonomous bio-hybrid drone with an EAG device based on a silkmoth antenna for odor detection and localization in air[26]. This study demonstrates the operation of the developed bio-hybrid drone and the tracing of manual stimulation of odors in a room using the drone.

In this study, as silkmoth antennae were attached to electrodes using electrically conductive gel, we verified that both ends of each antenna made contact with the electrodes securely before beginning EAG experiments on the desk or the drone. If signals from the EAG device were suddenly lost during the experiment, a researcher would first check the connection of the antenna with the electrodes. It is possible that this problem occurred with a higher probability in the EAG experiments on the drone. While the lifespan of isolated silkmoth antennae is more than an hour, because the gel dried out in a dozen to dozens of minutes in this study, the addition of gel to the connecting points of the antennae and the electrodes may help recover signal intensities.

The drone in this study was equipped with the VPS comprising a camera and an infrared sensor for flight stabilization. We found that the drone drifted during hovering on a smooth floor, which may have caused the instability of an infrared sensor under the body of the drone. The same problem sometimes arose when experiments were conducted using this drone in a room with a smooth floor such as tile. Therefore, we covered the floor with raised carpets (we used four-color carpets of 45 cm × 45 cm area) and reduced the drift of the drone. This process was found to be useful for flight stabilization of the EAG experiments on the drone.

The significance of the bio-hybrid drone in this study lies in its ability to recognize odor concentration and its sensor directivity toward odor sources. The drone identified real-time odorant-concentration differences outside a wind tunnel and localized the source using the spiral-surge algorithm (**Figure 8**). The spiral-surge algorithm[29,30] does not require plume-location information during plume reacquisition and exhibits its relatively high reliability, compared to that of the casting algorithm, in a low-speed laminar flow[30]. This algorithm was previously installed on a ground mobile robot[30]; however, a wind direction sensor was required to recognize the upwind direction. Odor information was binarized, and concentration was ignored.

For the insect antenna-based drone, mounting additional sensors, such as wind sensors, is a trade-off between payload and battery consumption. In addition, odor information detected by the EAG on the drone was still assessed to determine whether it exceeded a threshold[25]. The bio-hybrid drone design used in this study enhanced the directivity of the EAG device itself and did not require a wind direction sensor. The sensor directivity enabled the drone to utilize odor concentration information during spiral movements in a room environment that was more complex than a wind tunnel. A cylindrical enclosure was used in this study; however, a more elaborate and lightweight enclosure should be developed in the future.

However, the bio-hybrid drone examined in this study has some limitations. For example, the distance of odor source localization was still limited. Owing to their high mobility, drones should be capable of searching for odors over long distances in the order of several tens of meters. However, the distance achieved by the insect antenna-based bio-hybrid drone was limited to 2 m[26], and odor source localization tests were conducted in a wind tunnel with limited space[25]. Extending the searching distance is essential for the development of a practical odor-detecting flight platform.

For long-distance searches (over 10 m), a high sensor directivity and an efficient odor source localization algorithm are required, given that dilution of the odor concentration and complex distribution of the odor plume are expected. Stereo sensing using two antennae of the same insect can increase directionality[23]. Most odor source localization experiments using small drones with commercial gas sensors were conducted using a single sensor, and an EAG device array on drones was not conducted. Therefore, an EAG device array must be developed for small drones to increase their odor-sensing-application potential. The EAG device array would also facilitate the development of an efficient odor source localization algorithm as it allows for more precise localization of an odor plume.

Insect antenna-based bio-hybrid odor-detecting drones contribute to both fundamental and applied research. From the perspective of fundamental research, such drones can be used as test platforms to develop odor source localization algorithms. Various algorithms have been previously proposed[31]; however, test platforms using a mobile robot that conducted two-dimensional odor searches or commercial gas sensors have exhibited limited performance. In these setups, it is difficult for proposed algorithms to demonstrate their performance. The bio-hybrid drone in this study demonstrated odor concentration recognition ability as well as sensor directivity, sensitivity, and selectivity. Therefore, it shows great promise for installation in more advanced or three-dimensional odor source localization algorithms.

In terms of applications, bio-hybrid drones can be deployed on missions that living animals may have difficulty approaching, such as detecting toxic chemical/biological leaks, explosive materials, and search-and-rescue operations. To apply such drones to these missions, the insect antennae need to detect odorant molecules included in target odor sources. Silkmoth antennae can be genetically modified[32] to have the potential to detect odorant molecules other than the female silkmoth sex pheromone; thus, these applications are now becoming reality.

ACKNOWLEDGMENTS:

This work was supported in part by a research grant from The Murata Science Foundation. The authors would like to acknowledge Smart Robotics Co. Ltd., Tokyo, Japan, for assisting in the development of the drone platforms and programming and Assist Technology Co., Ltd., Osaka, Japan, for assisting with the design of the electronic circuits. The authors would also like to thank Dr. Shigeru Matsuyama (Graduate School of Life and Environmental Sciences, University of Tsukuba) for providing purified bombykol; Mr. Takuya Nakajo (RCAST, The University of Tokyo) for support for silkmoth breeding; and Mr. Yusuke Notomi (Graduate School of Science and Technology, Tokyo University of Science) for supporting the acquisition of silkmoth images.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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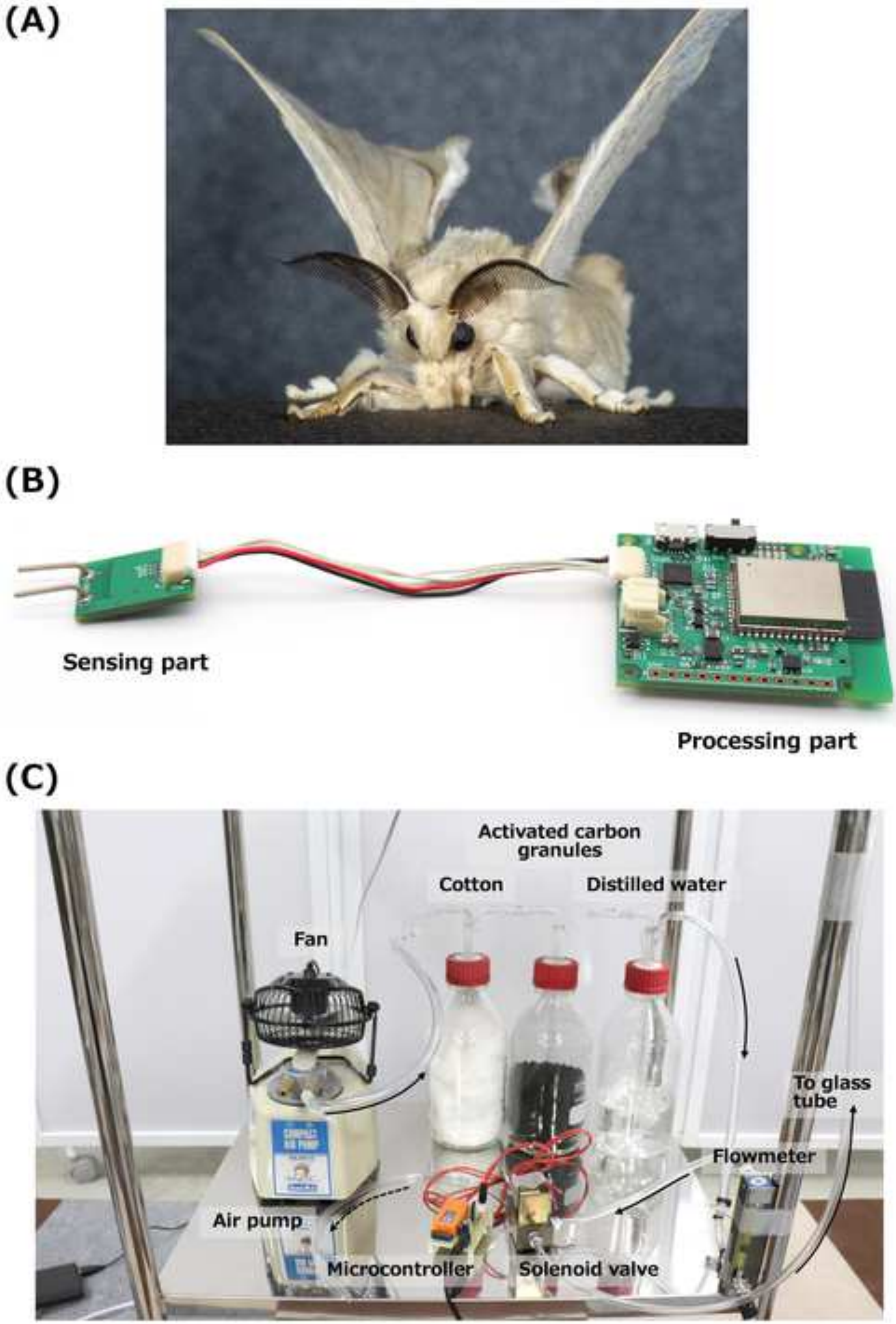

(A)
(B)
Sensing part
Processing part
(C)
Activated carbon granules
Cotton
Distilled water
Fan
To glass tube
Flowmeter
Air pump
Microcontroller
Solenoid valve

Fig.2

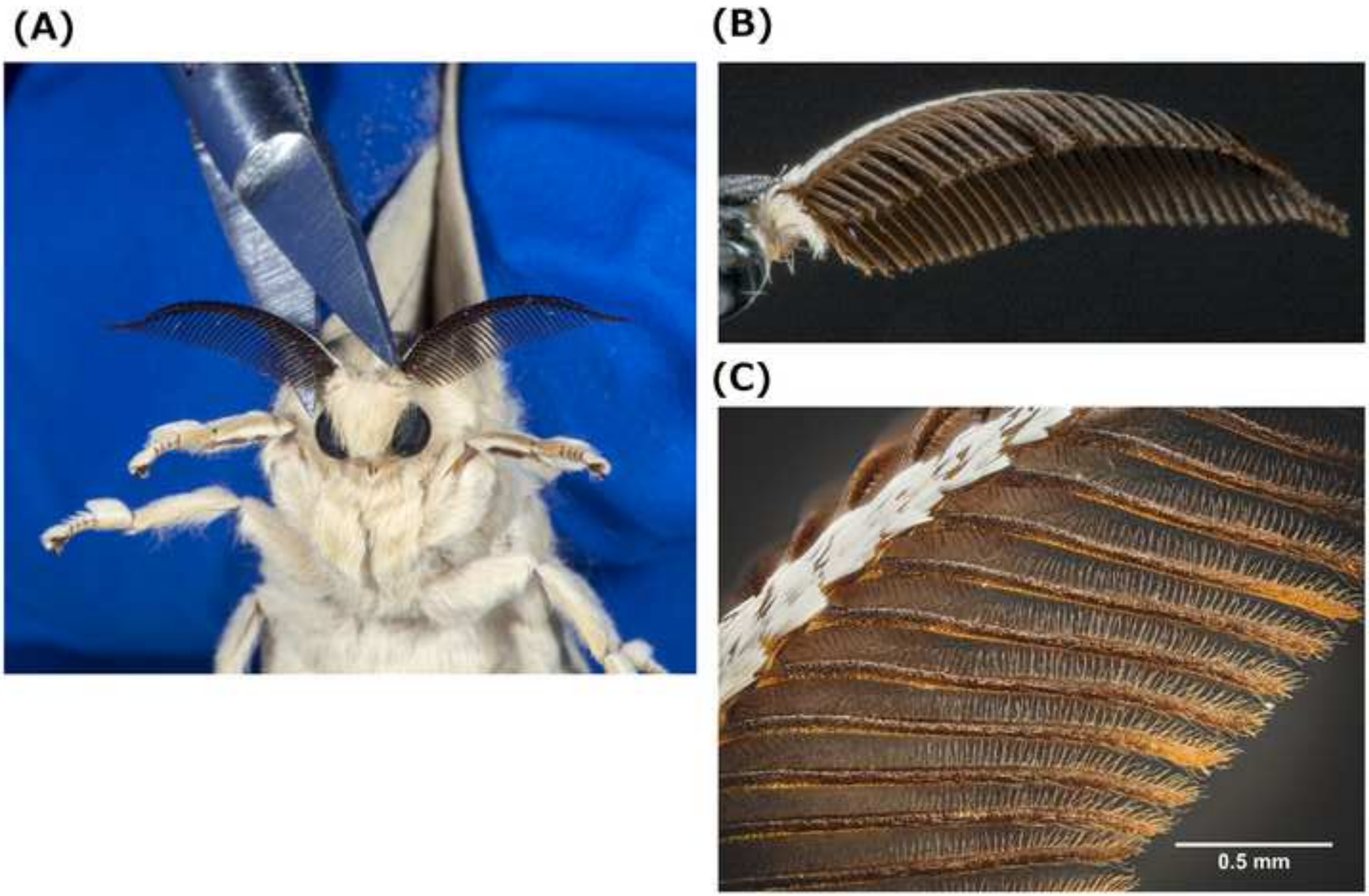


Fig.3

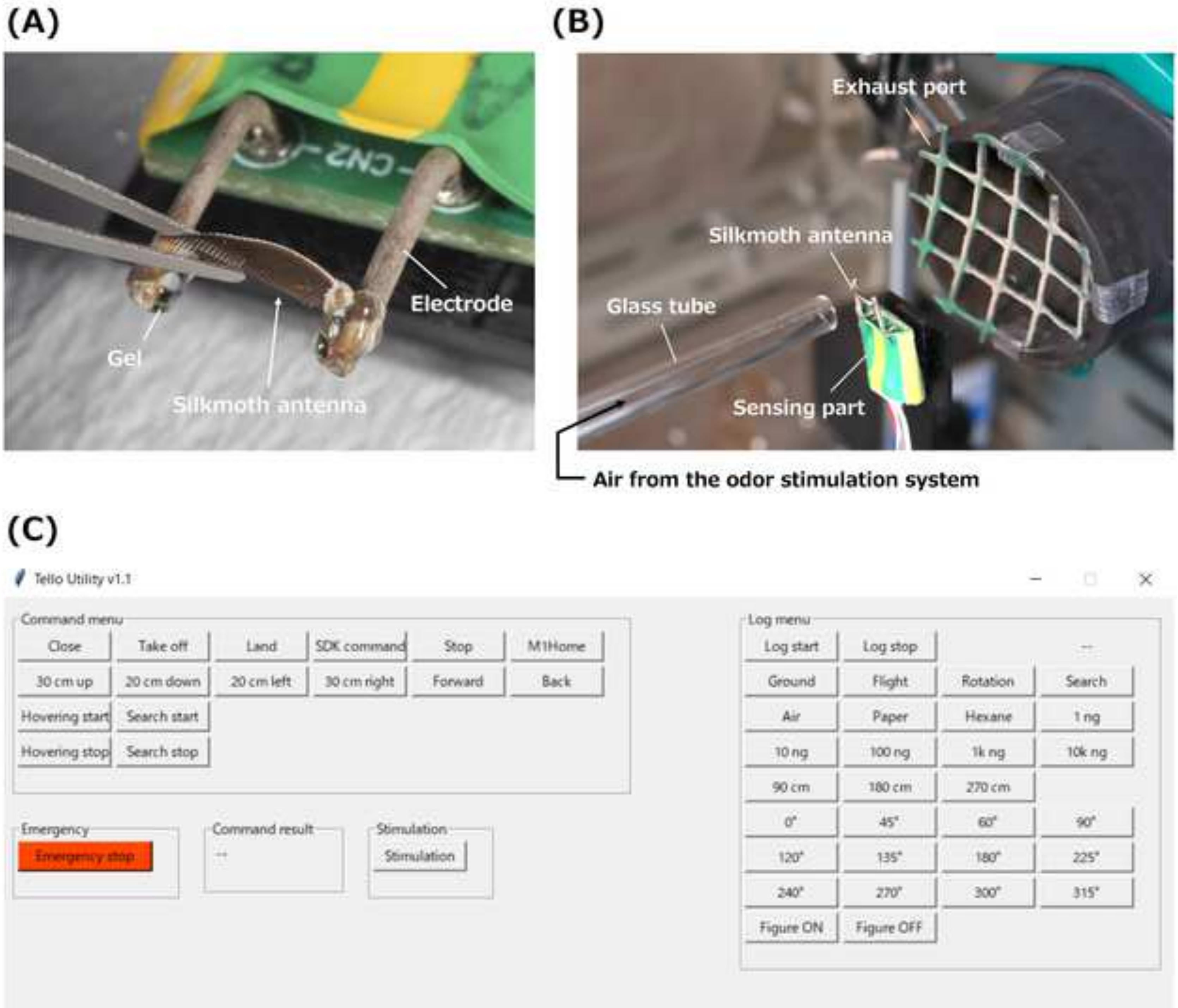

(A)
Electrode
Gel
Silkmoth antenna
(B)
Exhaust port
Silkmoth antenna
Glass tube
Sensing part
Air from the odor stimulation system
(C)
Tello Utility v1.1
Command menu
Close
Take off
Land
SDK command
Stop
M1Home
30 cm up
20 cm down
20 cm left
30 cm right
Forward
Back
Hovering start
Search start
Hovering stop
Search stop
Emergency
Emergency stop
Command result
--
Stimulation
Stimulation
Log menu
Log start
Log stop
--
Ground
Flight
Rotation
Search
Air
Paper
Hexane
1 ng
10 ng
100 ng
1k ng
10k ng
90 cm
180 cm
270 cm
0°
45°
60°
90°
120°
135°
180°
225°
240°
270°
300°
315°
Figure ON
Figure OFF

Fig.4

Click here to access/download;Figure;Fig.4.tif

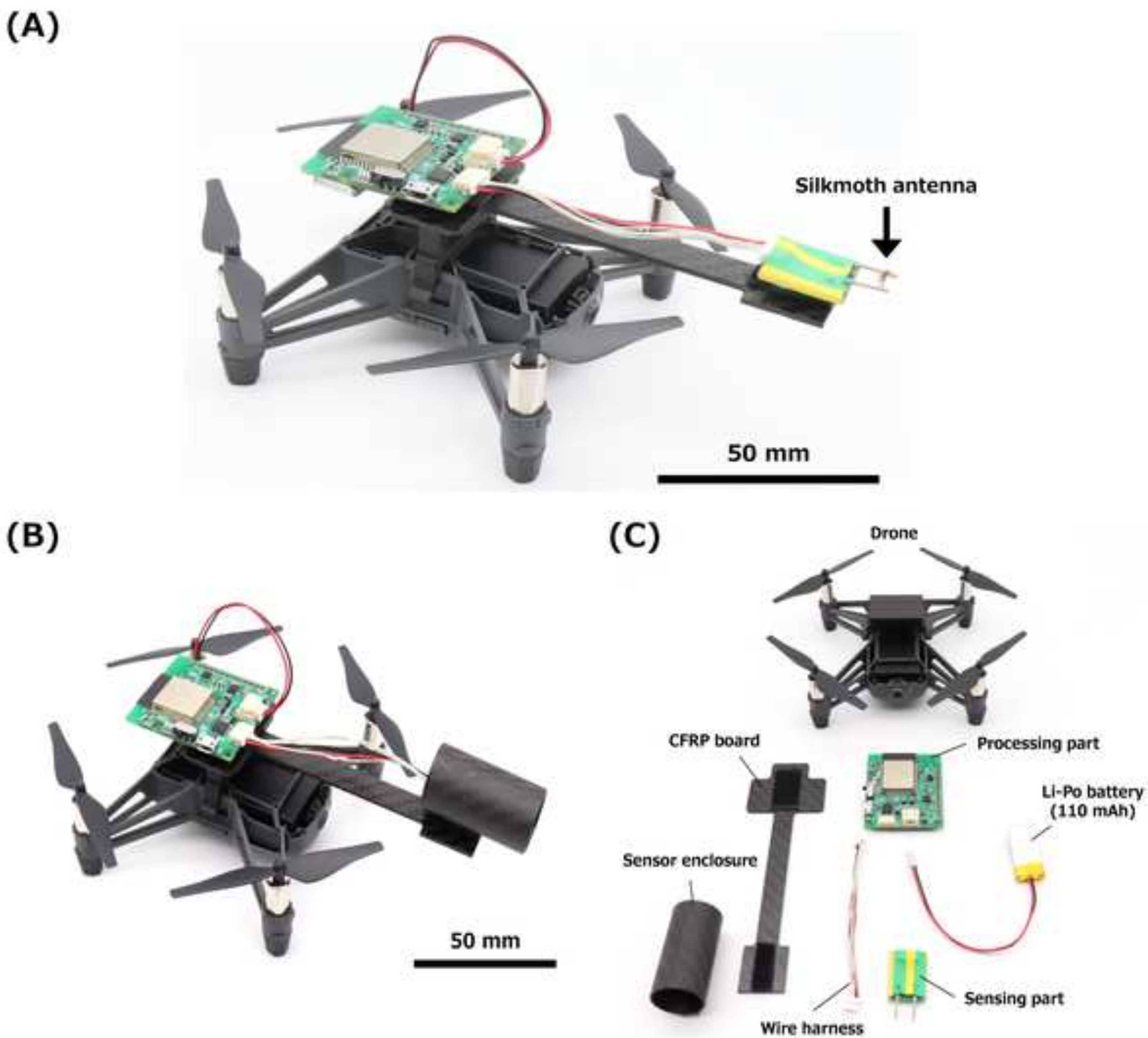


Fig.5

Click here to access/download;Figure;Fig.5.tif

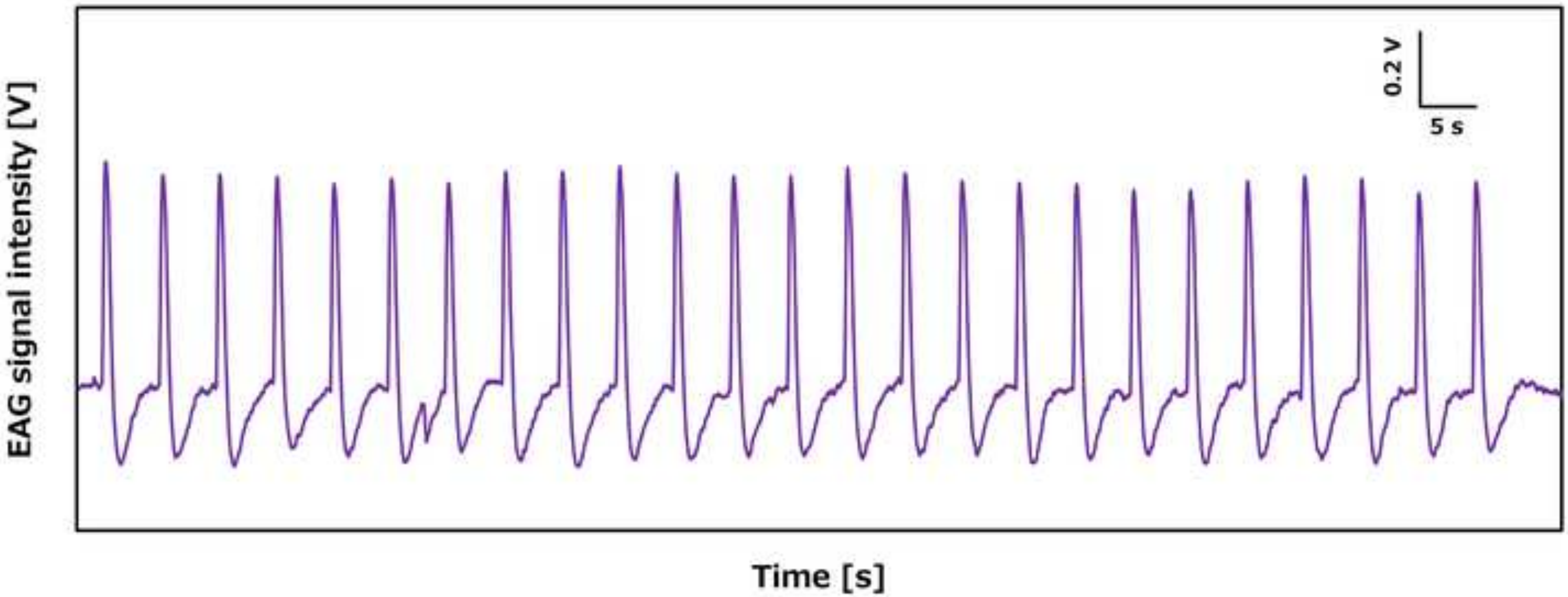


Fig.6

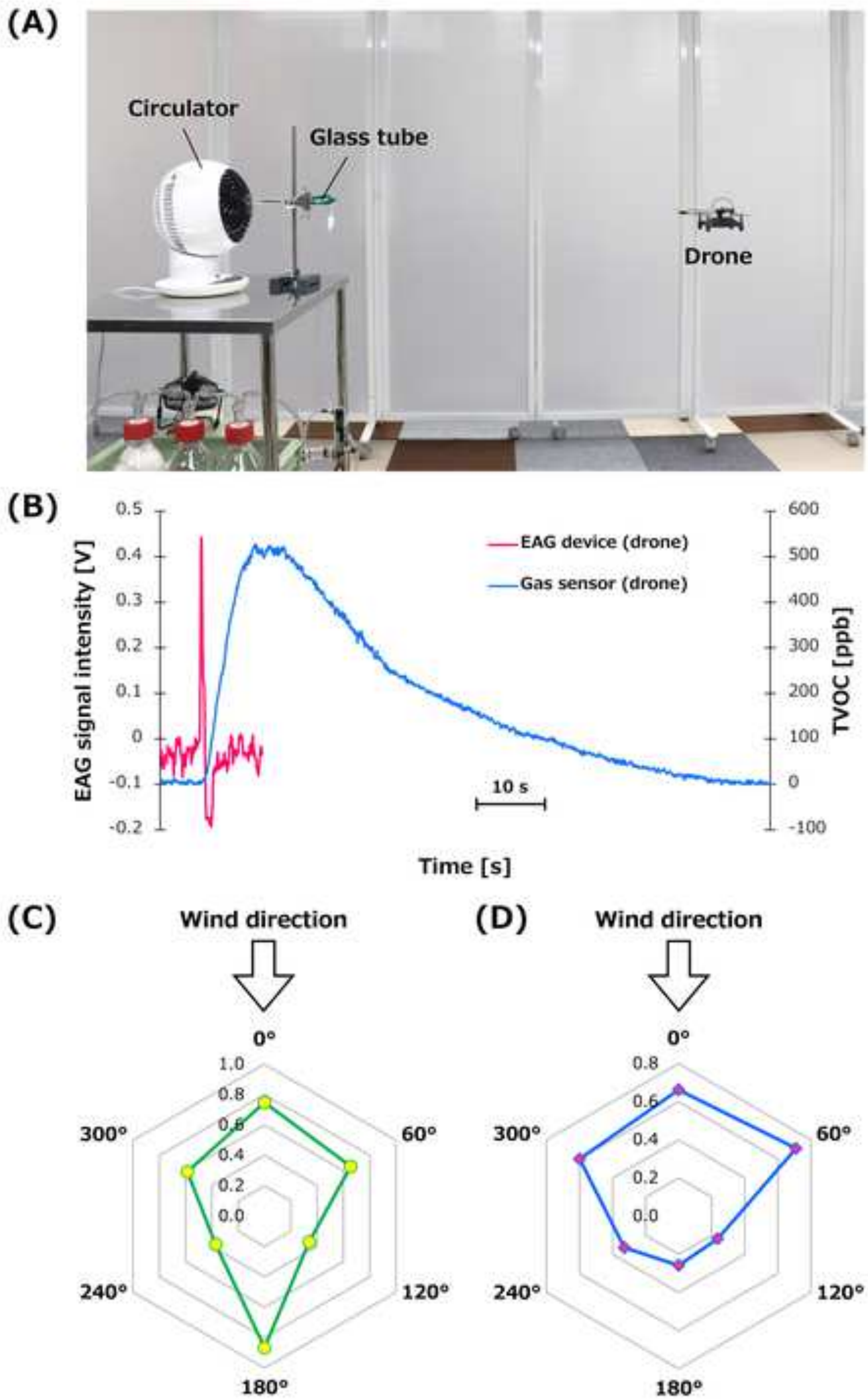

(A)
Circulator
Glass tube
Drone
(B)
EAG signal intensity [V]
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
EAG device (drone)
Gas sensor (drone)
600
500
400
300
200
100
0
-100
TVOC [ppb]
10 s
Time [s]
(C)
Wind direction
0°
1.0
0.8
0.6
0.4
0.2
0.0
60°
120°
180°
240°
300°
(D)
Wind direction
0°
0.8
0.6
0.4
0.2
0.0
60°
120°
180°
240°
300°

Fig.7

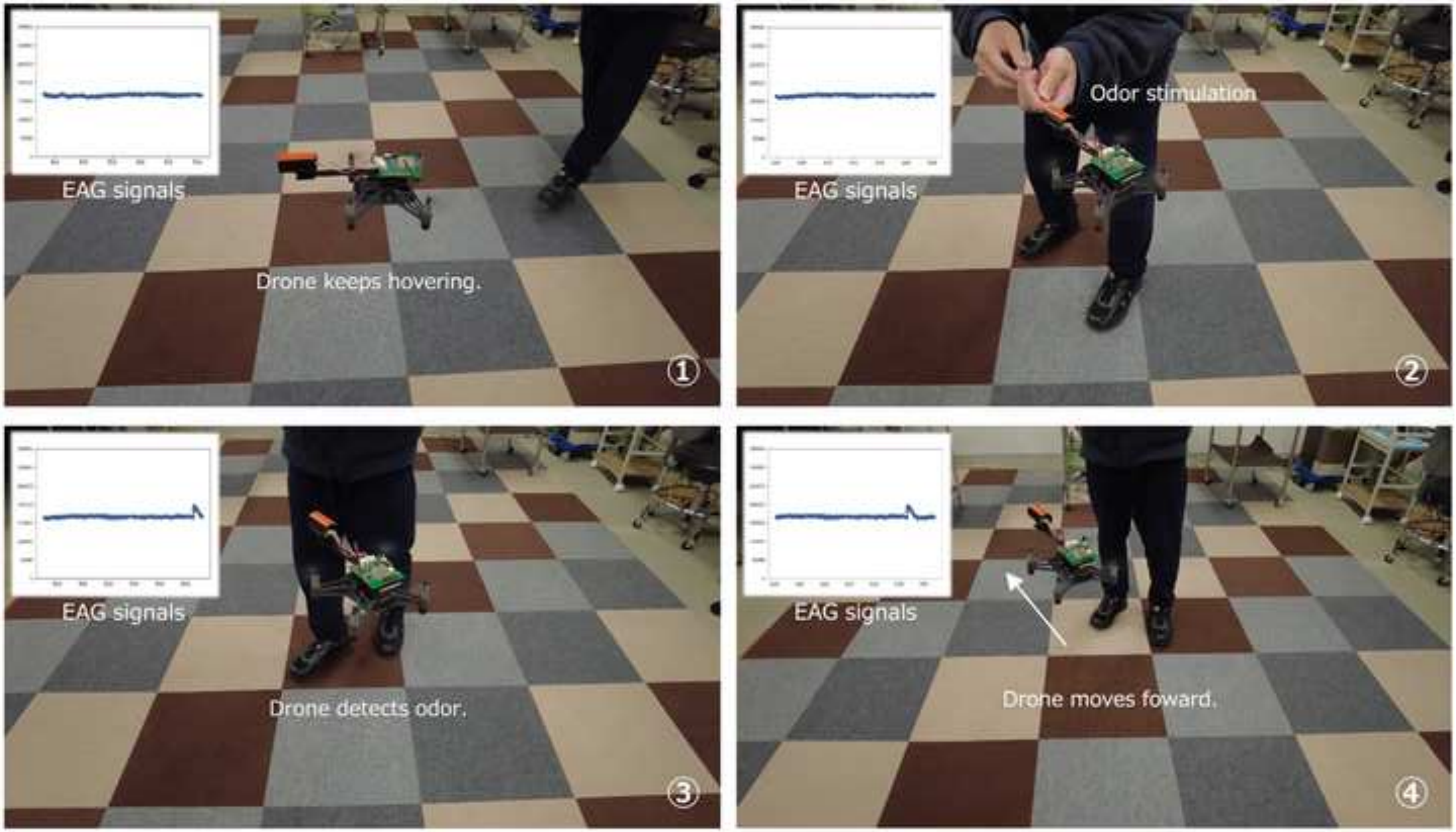
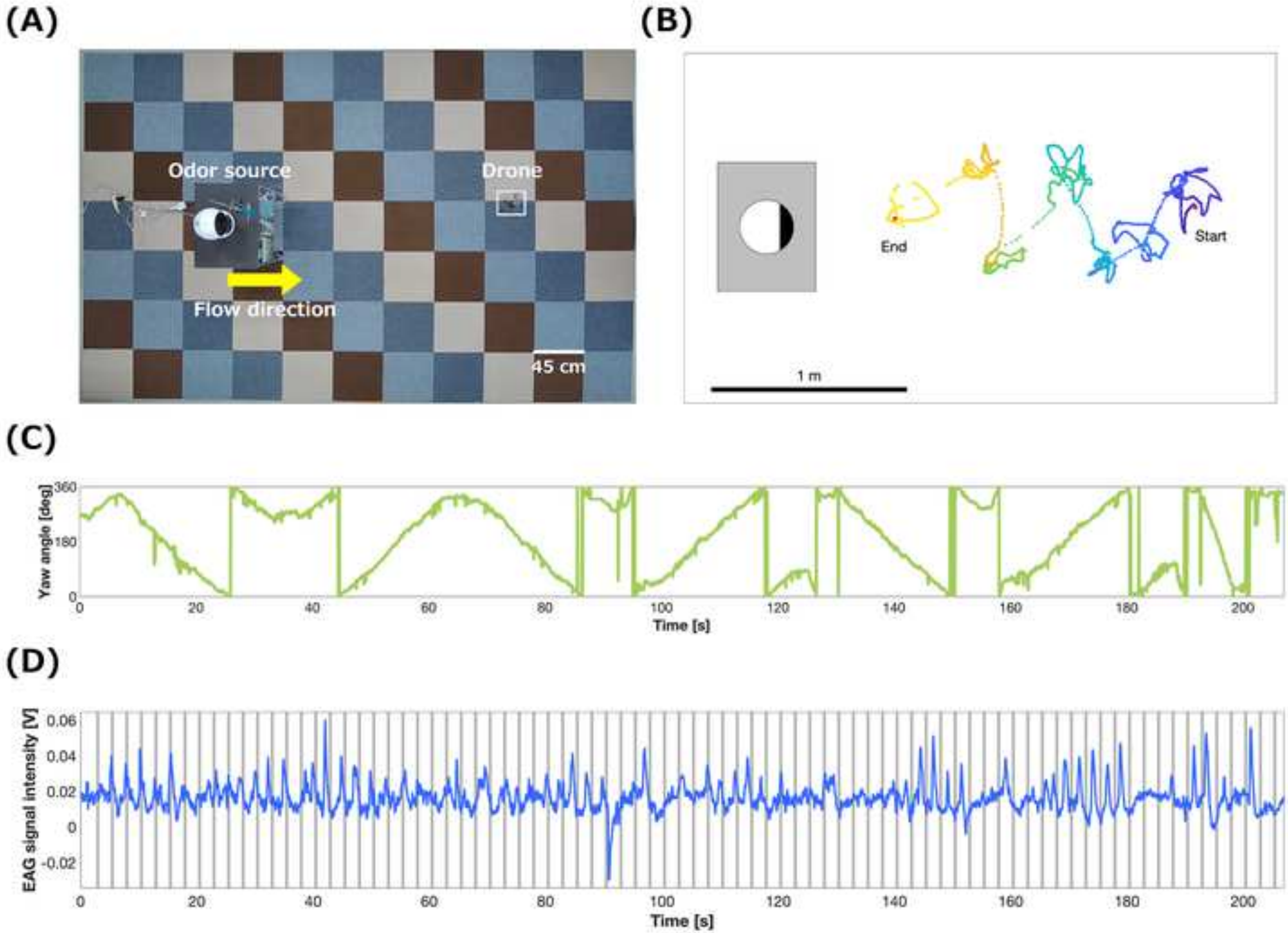

EAG signals
Drone keeps hovering.
①
EAG signals
Odor stimulation
②
EAG signals
Drone detects odor.
③
EAG signals
Drone moves foward.
④

Fig.8 Click here to access/download;Figure;Fig.8.tif



Movie

Click here to access/download
Video or Animated Figure
Movie.mp4

Click here to access/download
Table of Materials
Materials.xlsx

Responses to Reviewers

Editorial comments:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
- ***Response:*** We have modified the typos, and the revised manuscript has undergone English language editing.

- Please provide an email address for each author.
- ***Response:*** All authors' addresses have been added on the first page.

- Please revise the following lines to avoid overlap with previously published work: please check protocol text in attached iThenticate report after converting the tense to imperative; lines 260-262.
- ***Response:*** We modified the sentences of lines 260–262 in the original manuscript to avoid overlap with previous our paper (**lines 357–359**).

- JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
- ***Response:*** We have removed commercial language and added commercial products in the Table of Materials.

- JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Tello EDU" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.
- ***Response:*** We removed the word "Tello EDU" in the manuscript and added the detail in the Table of Materials.

- Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
- ***Response:*** We removed personal pronouns in the protocol.

- Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
- ***Response:*** We used imperative tense and removed the word "should be" in the protocol. We add the safety measures in NOTE (**lines 229–231**).

- Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. Please be sure to include specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
- ***Response:*** We have modified the procedures in the revised manuscript, especially Procedure 6 (drone experiments, **lines 243–258**), to explain the details.

- Step 2.2: How much was withdrawn and injected into the vial?
- ***Response:*** We modified the sentence for correct expression about the amount of injection into the vial in the step 2.1 and 2.2 in the revised manuscript (**lines 133–136**).

- Note B after 5: Do you mean the Tello EDU could not move on the floor?
- ***Response:*** The drone used in this study drifted during hovering on smooth floor such as tile, and this may have been caused by the instability of an infrared sensor under the body of the drone. We have added the relevant information as highlighted text in the revised manuscript to clarify our intention and discussed this problem as a troubleshooting in the discussion (**lines 456–463**).

- Do the EAG device and the Tello EDU have their own software? Specify in Table of Materials.
- ***Response:*** Tello EDU has a software development kit (SDK) and it can be used the website of Ryze Tech. The EAG device has custom software to measure signals and communicate with the PC. We have added the relevant information in the Table of Materials.

- Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section.
- ***Response:*** We have confirmed the paragraph indentation, line spacings, and included a single space between each step, sub-step and note in the protocols.

- Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
- ***Response:*** We have confirmed that Calibri 12-point font is used along with one-inch margins on all sides. We have included a one-line space between each protocol step.

- Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
- ***Response:*** We added a scale bar in **Figure 2C** in the revised figures.

- As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: Please sort the Materials Table alphabetically by the name of the material.

  a) Critical steps within the protocol

  b) Any modifications and troubleshooting of the technique

  c) Any limitations of the technique

  d) The significance with respect to existing methods

  e) Any future applications of the technique

- ***Response:*** We modified the discussion to add details of critical steps (**lines 448–456**), modifications and troubleshooting (**lines 456–443**), limitations (**lines 481–487**), significance (**lines 465–480**), and future applications (**lines 499–513**) of the bio-hybrid odor-detecting drone in discussion. We also sorted the Table of Materials alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The JOVE paper by Terutsuki et al, titled: Electroantennography-based bio-hybrid odor-detecting drone for odor sensing and discriminating using silkmoth antennae is a method article demonstrating the use of bio hybrid odor detecting drone in locating odorant source. The signals were acquired from Moth antenna using EAG, later transformed by Wi-Fi to a PC for analyzation. The navigation itself conducted by spiral-surge algorithm and an improvement of the EAG device was suggested in the form of sensor enclosure created by the author. This paper is based on the paper that was published in Sensors and Actuators B: Chemical (2021): 129770.

While we find this protocol very helpful and significant for the field of bio-hybrid odor-detecting drone we found number of points that should be addressed to make this paper stronger, clearer, easier to follow, and reproduce.

Major Concerns:

1. The use of the words "odor discriminating" in the title is misleading since it indicates the ability to distinguish between different odorants (as in the references Myrick and Park, 12 &13) whereas this manuscript deals with odor localization only.

- ***Response:*** We have removed the word "discriminating" and modified the title.

2. As the goal of this paper is to present a protocol for others to follow and repeat, we find that many of the steps are not clear enough to follow and in fact are more coherent in the original paper methods section (Terutsuki et al., Sensors and Actuators B: Chemicals 339, 129770 (2021)). Here are number of examples, but it should be pointed out that there are more:

a. Step 2.2: "nearly 1mL…", why there is not an exact number here? Was there a problem to withdraw an exact amount?

- ***Response:*** We have modified the sentence for correct expression about the amount of injection into the vial in the steps 2.1 and 2.2 in the revised manuscript (**lines 133–136**).

b. Step 3A: a photo or scheme of the system is needed. Where are the cotton and the activated carbon granules located, is it already part of the pump or should be added by the experimenter?

- ***Response:*** We have added the picture and text of the odor stimulation system in **Figure 1C** and in the revised manuscript (**lines 157–159**). We indicated the place of cotton and activated carbon granules.

c. Step 3C: Is this the flow rate for the "desk" system or for the drone? Isn't too strong of an air flow add noise to the recordings and change the signal?

- ***Response:*** This flow rate (5 L min^{-1}) was used stimulations on the desk. We previously confirmed that air stimulation at this flow rate has no effect on signal detection of the EAG device (Terutsuki et al., *Sensors and Actuators B: Chemical*, 2021). We have added the relevant information as highlighted text in the revised manuscript (**lines 161–164**).

d. Step 3F: The solenoid valve is activated automatically or manually? The stimulus application is joint with the general air flow or separated? What is the stimulus air flow? Etc.

- ***Response:*** The solenoid valve was activated by a microcontroller automatically and we clarified this in **lines 169–170**. We have added a photograph and description of the odor stimulation system in **Figure 1C** and in the revised manuscript (**lines 157–159**). We indicated the airflow path in **Figure 1C**.

e. Step 3 procedures: What program was used for signal acquisition, what are the HPF and LPF, does the moth been through anesthesia before its antenna removed? does the antenna was cut from both sides before attaching it to the electrodes? What kind of electrodes? etc.

- ***Response:*** We developed custom software to collect EAG measurement data. We added information of filters (**lines 150–151**). Isolation of a silkmoth antenna was conducted without anesthesia (**line 179**). We cut both sides of the isolated a silkmoth antenna and attached it to the Ag/AgCl coated electrodes by electrically conductive gel (**lines 174–176**, **182–183**).

f. Step 5B: "the Tello EDU could not fly stably on the smooth floor". It is unclear why the drone should fly on the floor.

- ***Response:*** The drone in this study drifted during hovering on smooth floor such as tile, and this may have caused instability of an infrared sensor under the body of the drone. We have added the relevant information as highlighted text in the revised manuscript to clarify our intention and discussed this as a troubleshooting problem in the discussion (**lines 456–463**).

3. One of the major issues of this paper, is related to the spiral-surge algorithm. First of all, we feel that it Thishould be explained in more details as in figure 6 in "Real-time odor concentration and direction recognition for efficient odor source localization using a small bio-hybrid drone". Secondly, and even more important, is that if the authors truly intend that others will be able to reproduce their innovative work, the authors should share the code, as open source and as SI. This is curtail for this paper.

- ***Response:*** We have added the relevant information as highlighted text in the revised manuscript to explain the details of the spiral-surge algorithm in the discussion (**lines 387–392**).

- ***Response:*** The source code of Tello EDU and the EAG device cannot be released for free because it contains many people's efforts; however, Ryze Tech offers a software development kit (SDK) and sample programs (Python). Therefore, you can develop flight program for Tello EDU based on them. We added the relevant information as highlighted text in the revised manuscript (**lines 223–225**) and in the Table of Materials.

4. The sensor enclosure is a significant change from other bio-hybrid drones exist. Lines 284-286 ("The sensor enclosure did not interfere with the drone flight and enhanced the sensor directivity of the EAG device."), where can one find a proof to this statement?

- ***Response:*** We referred and the evaluation results of the sensor directivity of the EAG device from the previous paper (Terutsuki et al., *Sensors and Actuators B: Chemical*, 2021) and added them in **Figures 6C and D**. We modified the relevant information as highlighted text in the revised manuscript (**lines 374–381**).

5. Additional point is the data acquisition and data analysis. It is unclear what software was used. Was both the analysis and acquisition were performed on the platform or off platform? Was this used with costume algorithm? If yes, where can one find it? (open source). The GUI is not available. What parameters were used for the threshold? It seems that all the information related to data acquisition and data analysis is missing and not available to the readers. It is described in the paper to press "Log Start" but the GUI is blurry in the figure, and more important, is that the software is not available, and the meaning of the instructions is not relevant and unclear.

- ***Response:*** EAG data acquisition and analysis were conducted in the PC after the EAG device sent the measurement data. We added the information in the revised manuscript (**lines 151–153**). Tello EDU has SDK and it can be downloaded from the website of Ryze Tech.
- ***Response:*** In this study, the drone searched the maximum odor concentration during the spiral movements and moved the direction, and did not use a constant threshold value (**lines 387–392**).
- ***Response:*** The source code of Tello EDU and the EAG device cannot be released for free because it contains many people's efforts; however, Ryze Tech offers the SDK and sample programs (Python). Therefore, one can develop flight program for Tello EDU based on them. We added the relevant information as highlighted text in the revised manuscript (**lines 223–225**) and in the Table of Materials.

Minor Concerns:

1. The relevance of lines 64-65 to the introduction is unclear ("Previously, we developed odorant biosensor devices based on a combination of devices insect cells that express insect odorant receptors and a microscope or electronic devices")

- ***Response:*** We modified the introduction. We added bio-hybrid approaches of previous studies for robotic and sensor fields in the introduction, and our previous research were positioned as examples of bio-hybrid odorant sensors (**lines 68–80**).

2. The major difference in the algorithm used in here compared to previous odor source localization algorithms is the lack of necessity in wind-direction information. I believe this should be more emphasized as well as the limitations of the previous algorithms (lines 71-72, 81-82).

- ***Response:*** We emphasized the merit of the spiral-surge algorithm and the drone that did not require the wind direction sensor in this study in the discussion (**lines 465–479**).

3. Line 147 is unclear.

- ***Response:*** Use of electrically conductive gel can skip to insert micrometer-scale wires to both ends of a silkmoth antenna to attach it to the EAG device. We added the relevant information as highlighted text in the revised manuscript (**lines 174–176**).

4. Figure 2D: low quality, reading the details is impossible.

- ***Response:*** We enhanced the resolution of the picture of the GUI of **Figure 3C**.

5. Figure 3A: An arrow pointing the location of the antenna can be added for clarity.

- ***Response:*** We added the arrow to point the location of the silkmoth antenna in **Figure 4A**.

6. Step 8: A remark directing to the video is recommended. Step 8.1 is an introduction to the section and basically redundant.

- ***Response:*** We modified the remarks in the supplementary video and added the remarks in **Figure 7**. We removed step 8.1 of the original manuscript.

7. Step 9: I believe a video demonstrating step 9 will be more relevant than the video related to step 8. Since you have two of those in the main paper (Terutsuki et al., Sensors and Actuators B: Chemicals 339, 129770 (2021)) a reference to this will be satisfying.

- ***Response:*** To enhance the readability of the step 9, we have added the link of the relevant video from our previously published paper (**lines 397–399**).

8. Figure 7: lacking number of experiments (n), average time to detect the odorant. "Typical flight trajectory" representative or average?

- ***Response:*** We clarified that the typical flight trajectory, yaw angles, and EAG signal intensities were representative results in the caption of **Figure 8**. The average detection time for odor was approximately 1 s. We have added the relevant information as highlighted text in the revised manuscript (**lines 394–395**).

9. Line 342-344: "and an EAG device array on drones was not considered." This is not true: "The "Smellicopter," a bio-hybrid odor localizing nano air vehicle", Anderson et al., IEEE, 2019.

- ***Response:*** We modified the original sentence to "and an EAG device array on drones was not conducted." (**lines 493–494**).

Reviewer #2:

Manuscript Summary:

The manuscript "Electroantennography-based bio-hybrid odor-detecting drone for odor sensing and discriminating using silkmoth antennae" by Terutsuki describes small drones hybridized with insect antennae as olfactive sensors for odor source localization.

Major Concerns:

The methodological part should be expanded and make the protocol clearer. Also, were antennae obtained from mature males? This could affect the development of some sensing ability the antennae.

The state of the art on bio-hybrid systems should be increased for an added scientific value of the study.

Authors may comment the following articles on biohybrid systems using animal organs or individuals:

Authors should comment this works an others.

1. Romano, D., Donati, E., Benelli, G., & Stefanini, C. (2019). A review on animal-robot interaction: from bio-hybrid organisms to mixed societies. Biological cybernetics, 113(3), 201-225.
2. Bozkurt A, Lal A, Gilmour R (2009) Radio control of insects for biobotic domestication. In: 4th international IEEE/EMBS conference on neural engineering, 2009. NER'09. IEEE, pp 215-218
3. Bozkurt A, Lobaton E, Sichitiu M (2016) A biobotic distributed sensor network for under-rubble search and rescue. Computer 49(5):38-46
4. Brown MF, Brown AA (2017) The promise of cyborg intelligence. Learn Behav 45(1):5-6
5. Cazenille L, Chemtob Y, Bonnet F, Gribovskiy A, Mondada F, Bredeche N, Halloy J (2018) How to blend a robot within a group of zebrafish: achieving social acceptance through real-time calibration of a multi-level behavioural model. arXiv preprint arXiv:1805.11371
6. Cazenille L, Collignon B, Chemtob Y, Bonnet F, Gribovskiy A, Mondada F, Bredeche N, Halloy J (2018) How mimetic should a robotic fish be to socially integrate into zebrafish groups? Bioinspir Biomim 13(2):025001. https://doi.org/10.1088/1748-3190/aa8f6a
7. Romano, D., Benelli, G., & Stefanini, C. (2021). Opposite valence social information provided by bio-robotic demonstrators shapes selection processes in the green bottle fly. Journal of the Royal Society Interface, 18(176), 20210056.

- ***Response:*** We have modified the procedures in the revised manuscript, especially procedure 6 (drone experiments, **lines 243–258**), to explain the details. We used silkmoths within 10 days that were emerged from cocoons for experiments (**line 107**).
- ***Response:*** We picked up and added the references you mentioned (Romano et la., *Biol. Cybern.*, 2019, Bozkurt et al., *Computer*, 2016, Cazenille et la., *Bioinspir. Biomim.*, 2018) in the revised manuscript (**lines 68–74**). We also referred (Yu et al., *PLOS ONE*, 2016) that is mainly mentioned in (Brown and Brown, *Learn. Behav.*, 2017) as an original research article.

- Also, authors should clearly report how their work is different compared to previous works using insect antennae as sensors. Is just the integration of an antenna on a drone the real original aspect?
- ***Response:*** We enhanced the significance and advances of our research compared to previous works that used insect antennae (**lines 83–90**) in the discussion (**lines 465–479**). The main advances of our bio-hybrid drone are that the drone can recognize odor concentration and has sensor directivity toward odor source. Therefore, the drone can install the spiral-surge algorithm. These aspects were not considered in the previous works of bio-hybrid ground mobile robots or drones based on insect antennae.

Minor Concerns:

- Please, in the text there are several typos that should be revised. Also an English revision is needed.
- ***Response:*** We have modified the typos, and the revised manuscript has undergone English language editing.