Video Article

Early Metamorphic Insertion Technology for Insect Flight Behavior Monitoring

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Abstract

Early Metamorphosis Insertion Technology (EMIT) is a novel methodology for integrating microfabricated neuromuscular recording and actuation platforms on insects during their metamorphic development. Here, the implants are fused within the structure and function of the neuromuscular system as a result of metamorphic tissue remaking. The implants emerge with the insect where the development of tissue around the electronics during pupal development results in a bioelectrically and biomechanically enhanced tissue interface. This relatively more reliable and stable interface would be beneficial for many researchers exploring the neural basis of the insect locomotion with alleviated traumatic effects caused during adult stage insertions. In this article, we implant our electrodes into the indirect flight muscles of *Manduca sexta*. Located in the dorsal-thorax, these main flight powering dorsoventral and dorsolongitudinal muscles actuate the wings and supply the mechanical power for up and down strokes. Relative contraction of these two muscle groups has been under investigation to explore how the yaw maneuver is neurophysiologically coordinated. To characterize the flight dynamics, insects are often tethered with wires and their flight is recorded with digital cameras. We also developed a novel way to tether *Manduca sexta* on a magnetically levitating frame where the insect is connected to a commercially available wireless neural amplifier. This set up can be used to limit the degree of freedom to yawing “only” while transmitting the related electromyography signals from dorsoventral and dorsolongitudinal muscle groups.

Video Link

The video component of this article can be found at http://www.jove.com/video/50901/

Introduction

Inserting electrodes, even with attached electronic systems to insects for telemetric recording applications, has been a major method to understand how neural systems function during natural flight [1]. Attaching or implanting artificial systems in insects has posed many challenges involving the potential to disturb the natural flight of the insect. Superficial attachment or surgical insertion of artificial platforms on the adult insect is unreliable due to possible shifting of the inserted devices caused by body-induced inertial and stress forces. Superficially attached or surgically inserted electrodes are also prone to be rejected by the insects as a foreign body. Furthermore, the implantation operation requires the removal of scales and piles around the exoskeleton. The thick cuticle layer also needs to be penetrated for surgical innervations which could cause collateral tissue damage, thereby interfering with the natural flight of the insect. All these factors can make a surgical or superficial implantation operation a challenging and delicate task. In order to alleviate these concerns involved in externally attaching control and sensing systems to the insects, a novel methodology involving metamorphic growth will be described in this article.

The metamorphic development of holometabolic insects starts with the transformation of the larva (or nymph) into an adult with an intermediate pupal stage (**Figure 1**). The metamorphosis process involves an extensive tissue reprogramming including degeneration followed by remodeling. This transformation turns a terrestrial larva to an adult insect demonstrating several complex behaviors[2,3].

The survival of insects after extreme parabiotic surgeries has been demonstrated where the surgeries were performed during the early metamorphic stages[4,5]. In these surgeries, the developmental histogenesis caused surgical wounds to be repaired in shorter durations. Following these observations, a new technique has been developed where the implantation of electrically conductive electrodes was performed during the earlier stages of metamorphic growth (Figure 1). This enables a biomechanically secure attachment on the insect[6]. A highly reliable interface is also secured with the insect’s neural and neuromuscular systems[7]. This technique is known as “Early Metamorphosis Insertion Technology” (EMIT) [8].

After the rebuilding of the entire tissue system, structures inserted in the pupa emerge with the adult insect. Flight muscle groups make up to 65% of the total thoracic body mass and, thus, is a relatively convenient target for the EMIT procedure[9]. During the basic wing beat, the changes in the morphology of the flight powering dorsolongitudinal (dl) and the dorsoventral (dv) muscles cause the wing articulation geometry to generate lift[10]. Therefore the functional coordination of *dl* and *dv* muscles has been an active research topic under flight neurophysiology. Tethering insects in electronically programmed visual environments has been the most common method for studying the neurophysiology of complex locomotory behaviors[11,12]. Cylindrical arenas composed of light emitting diode panels have been used for these virtual-reality environments, where flying insects are tethered in the middle and the motion is simulated by dynamically updating the surrounding panoramic

visual display. In the case of smaller insects, such as fruit fly Drosophila, tethering is achieved by attaching a metal pin to the dorsal thorax of the insect and placing the pin under a permanent magnet[13,14]. This method only allows quantification of motor responses through visual observations with high speed cameras without any electrophysiological analysis. Moreover, this method has been inefficient to suspend the larger and heavier body of *Manduca sexta*. To solve this problem, we benefitted from magnetically levitating frames where light weight frames with magnets attached to their bottom are levitated through electromagnetic forces. When combined with commercially available neural amplifiers and LED arrays, this provides a platform to control flight-motor output and record the related electrophysiology of *Manduca sexta*.

Protocol

NOTE: The source of the materials and reagents required to follow the protocol is provided in the “Reagents” Table below.

1. Preparing Printed Circuit Boards (PCBs) for Recording Electrode Connection

NOTE: In order to provide a practical experimental procedure, wire electrodes are soldered to a PCB to insert these electrodes into an FFC (flexible flat cable) connector.

1. Cut a 0.5x5 cm^2 piece of copper clad laminate.
2. Using a fine tip marker, draw three 0.1x5cm^2 rectangle pads as etching mask patterns.
3. Etch the exposed laminate using a PCB etchant inside a ventilated area or fume hood. Cover about 1 cm of the laminate cutout’s length with non-reactive tape. Fill a graduated beaker with at least 100 ml of PCB etchant and tape the copper laminate cutout to the inside of the graduated beaker with scotch tape. Half of the copper laminate cutout should be submerged in the PCB etchant.
4. Place the beaker on a rotating platform for 20 min.
5. Remove the cutout from the etchant and place it in a beaker filled with water for 10 min.
6. Using a tissue paper, apply isopropyl alcohol and remove the markings to expose the non-etched copper pads.
7. Cut the printed circuit board into smaller squares approximately 1 cm long.
8. Cut two pieces of coated, annealed, stainless steel wire (0.11” coated, 0.008” bare) using a sharp blade to lengths of 3 cm each. These pieces of stainless steel wire are the active electrodes that will be inserted into the thorax of the insect.
9. Using a blade, remove 4-5 mm of the plastic coating from each end of each wire. Use of a microscope is recommended.
10. Cut one 0.7 cm piece of insulated stainless steel wire to create a tip extension for the ground electrode. Gently remove the coating with a blade or melt it with the heat of a soldering iron as performed in step 1.9.
11. For the ground connection, cut one piece of flexible (litz or inductor) wire to a length of 4.5 cm.
12. Solder the 0.7 cm piece of stainless steel prepared in step 1.10 to the ground connection wire prepared in step 1.11. An exposed stainless steel tip should be at the end of the ground connection.
13. Tape the prepared electrode board firmly to the soldering workspace using a non-reactive tape. Use the tape to mask all but 1-2 mm of the pads on the board where the electrodes will be soldered. This masked, solder-free end of the pads will be inserted into the FFC connector described in step 4.1.
14. Align the three electrode wires such that one end of each can be soldered to the corresponding pads on the electrode board. Apply stainless steel flux across the electrode pads for easier soldering.
15. Solder each of the exposed electrodes on the pads.
16. Immerse the electrodes in acetone and isopropyl alcohol for 10 min each to clean the solder residues. Use of an ultrasonic bath improves the cleaning performance.

2. Surgical Insertion to the *Manduca Sexta* Pupae

NOTE: The insects will be most active during the transitions between day and night. Therefore, an artificial day/night cycle should be established within an insect chamber using automatic outlet timers. These should be set to simulate a 7 hr dark and 17 hr light cycle.

1. Examine the *Manduca sexta* pupae daily to determine an appropriate insertion time. The pupae are ready for insertion approximately one day after the wings exhibit dark spots.
2. To anesthetize the pupae, place them in the refrigerator (4C) for around 6 hr.
3. Prepare the insertion workspace. The workspace should include isopropyl alcohol, sharp tweezers, blades, and a 30 G hypodermic needle. As an option, cyanoacrylate adhesive may be used to enhance the electrode fixation.
4. Sterilize the needle, tweezers, and the electrodes by dipping them into or wiping with isopropyl alcohol.
5. Remove the pupa from the refrigerator and transfer it to the workspace.
6. Determine the location on the thorax that corresponds to the muscle group of interest. The focus of the work in this example is the dorsoventral muscles responsible for wing upstroke movement.
7. Using a sharp blade, gently scratch a 1x1 cm^2 rectangle through the exocutical layer. Using the tweezers, slowly peel off these pieces.
8. (Optional) Use a vacuum to remove wing hair from the exposed region of the thorax.
9. Slowly insert the needle about 5mm into the mesothorax where the wings attach to the thorax to create two insertion points targeting the muscle group.
10. Using tweezers, guide the two recording electrodes into the two insertion points.
11. (Optional) To enhance the mechanical durability, clean the hair around the electrodes and generously apply cyanoacrylate adhesive around each insertion point on the thorax with a wire applicator.
12. Prepare a cage for emergence with proper material (rough and textured) covering the walls and ceiling so that the insect may climb upon emergence. Perforated cardboard boxes or packing paper may be used.
13. Prepare a rigid fixation stick with around 6 cm length and 2 mm diameter. Plastic stirrers, a cotton swab, or metal wires can be used for this step.
14. Carefully slide this stick through the hole underneath the protruding proboscis.

15. Fix both sides of the stick on the cage surface such that the pupa cannot roll around. Position the pupa inside of the cage such that the mesothorax is facing up. Extensive movement may cause damage to the electrode, loss of hemolymph, or render the insertion useless.

3. Inserting the Ground Electrode into *Manduca Sexta*

NOTE: The ground (reference) electrode should be inserted into the abdomen or distal parts of the thorax to avoid signal coupling. This insertion can be done either during the later stages of the pupal development or after the insect emerges. The window for the ground electrode has to be prepared in the pupal stage for either a pupal or adult stage ground electrode insertion.

1. For pupal stage insertion: after peeling of the mesothoracic cuticle around the active electrode (see step 2.7), scratch another rectangle through the exocutical layer (around 0.5x0.5 cm^2) on the dorsal abdomen close to the thorax using the 30 G hypodermic needle. Insert the ground electrode into this window using the technique described in section 2.
2. For adult stage ground electrode insertion: once the insect has emerged, place it in the refrigerator at 4 °C for 6 to 24 hr to immobilize. The remaining steps are the same for both pupal and adult stage insertions.
3. Prepare the insertion workspace including isopropyl alcohol, sharp tweezers, a 30 G hypodermic needle, cyanoacrylate adhesive, a piece of wire for application of glue, a thermal cauterizer (optional), and a dental wax stick (optional).
4. Locate an insertion point approximately 1-2 cm away from the recording electrodes along the posterior abdomen.
5. Slowly insert the needle to puncture the abdomen and provide an insertion site.
6. Using tweezers carefully insert the ground electrode into the insertion site and apply pressure until it is 3-4 mm deep. Hold the electrode in place and use a wire to apply glue around the insertion site.
7. (Optional) To enhance the mechanical strength, use the thermal cauterizer and collect a small (2-3 mm) bead of wax at the tip. Place the tip close to the insertion site and apply heat such that the wax surrounds the electrode and holds it firmly in place.

4. Preparation of the Adapter Board

NOTE: An adapter board is required to connect the electrode board to the wireless recording headstage through an FFC (Flat Flexible Cable) connector. For this, a board similar to the electrode board needs to be prepared by following steps 1.1 to 1.7.

1. Solder a FFC connector to one end of the prepared board.
2. Solder three 30 AWG (American Wire Gauge) hook up wires to three pads on the other end.
3. Solder three mini connectors to the three pads on the adapter board for oscilloscope readings as described in the next stage.
4. Solder the other end of these three wires to the headstage connector.
5. Secure the headstage circuit board on top of the levitation frame.

5. Prerecording with Oscilloscope (Optional)

NOTE: In order to assess the reliability of the electrodes and observe the signal to noise ratio, tethered oscilloscope recordings can be obtained before deploying the wireless recording system. The mini wire connectors on the adapter board should be used for this.

1. Connect the oscilloscope to an extracellular neural recording amplifier. Set the amplifier parameters to a high-pass cut-off frequency of 1 Hz, a low-pass cut-off frequency of 20 kHz, and a gain of 100.
2. Connect each of the female mini wire connectors on the adapter board to the amplifier input channels.
3. Remove the insect with the implanted electrode board from the cage when it is in an active state (during its dawn time). Place a piece of tissue paper under the insect for it to rest on before measurements are taken.
4. Using tweezers, slide the electrode board into the FFC receptor on the adapter board. Observe a flat and low voltage baseline when the insect is resting and the generation of electromyogram (EMG) spikes as the insect flaps its wings.
   NOTE: Refer to Section 6: Observing insect flight with the Wireless Recording System for representative oscilloscope results.
5. Adjust the viewing parameters of the oscilloscope as needed. Capture the data on the oscilloscope and save the data.

6. Observing Insect Flight with the Wireless Recording System

NOTE: An electromagnetic levitation platform can be built for wireless recording of EMG signals during tethered *Manduca sexta* flight. The levitation platform consists of a frame designed to balance a tethering mechanism. The levitation allows the frame, and therefore the insect, to yaw during testing without the constraint of tethering wires. The frame can be rapid-prototyped using a fused deposition modeling (FDM) machine. A magnet needs to be attached to the bottom of this frame to be levitated by a series of magnets in the base platform. The insect is connected to the FFC connector suspended from the top of the frame. This levitating platform is located inside of the LED Arena which was constructed using 60 panels composed of an array of 5x7 individual LEDs. This system was based on established methods for developing an environment for visual stimulation of fruit flies[15,16,17]. The arena is controlled by a microcontroller allowing simulation of both clockwise and counterclockwise rotation as well as control of the rotational speed.

1. Set up the wireless recording system by connecting the headstage to the adapter board connector on the levitation platform.
2. Remove the insect from the cage when it is in an active state preferably during its dawn time.
3. Using tweezers, carefully insert the electrode board into the FFC receptor on the levitating frame such that the insect is suspended firmly within the setup.
4. Place the magnetic wand near the magnetic switch on the headstage to activate wireless data transmission. A blue light will come on indicating that the headstage is active.
5. Turn off the lights in the room for complete darkness. A red lamp can be used to add lighting to the room. Open the telemetry data collection software on a computer and select the appropriate preloaded configuration file if provided. Start the data acquisition to begin viewing signals.

6. Select the relevant user interface for observation of EMG signals on the wireless recording system to ensure a reliable wireless connection and electrode operation.
7. Turn on all the LED Arena components: Regulated DC power supply and microcontroller. The microcontroller can adjust the rotations per minute of the cyclic light pattern and can also control the direction of the light rotation.
8. Slowly balance the levitation platform within the arena. Align the frame above the center of the levitation base carefully, otherwise the frame will be pulled quickly to the ground possibly injuring the insect.
9. Initiate the video recording system.
10. Select the relevant recording tab of the software interface. Designate the recording time and file save destination. Choose the appropriate output settings to save the data. Click the start button to initiate a recording session within the software. This will save the data file which can be imported into numerical computing environments.
11. Observe as the insect flies in the direction that corresponds with the movement of the LEDs. Reverse the direction of the LEDs and confirm that the insect reverses direction. Perform this as many times as desired.

Representative Results

A schematic of the overall EMIT procedure is presented in **Figure 1**, showing the major stages in the hawkmoth's metamorphic cycle and the corresponding electrode insertion steps. The electrode insertion should be performed in the late pupal stage 4 to 7 days before eclosion. This allows the muscle fibers to develop around the electrodes and secure the implant in the insect.

The typical result of a completed late pupal stage insertion where the two active electrodes and the ground electrodes have been inserted is shown in **Figure 2**.

The typical result of a completed adult stage insertion where the two active electrodes and the ground electrode have been inserted is shown in **Figure 3**.

The LED arena used to induce turning during flight for the *Manduca sexta* is shown in **Figure 4**. A microcontroller was programmed to allow control of the rotational speed of the LED array vertical pattern. The angular velocity of the LED pattern was set to 7.3 degrees per second. The magnetic levitation platform was placed in the center of the LED arena to allow the insect to freely turn in response to the LED array.

Figure 5 shows the muscle potential signal acquired from the dorsoventral muscles with the oscilloscope before and after wing flapping. The signal has been processed with 100 times amplification and a high-pass filter of 1 Hz and a low pass filter of 20 kHz. In the quiescent period, no muscle potentials are observed. The muscle potentials during wing flapping occur at approximately 15 Hz-20Hz.

Figure 6 shows the muscle potential signal acquired with the wireless instrumentation before and after wing flapping. In the quiescent period, no muscle potentials are observed. The muscle potentials during wing flapping occur at approximately 15Hz-20 Hz.

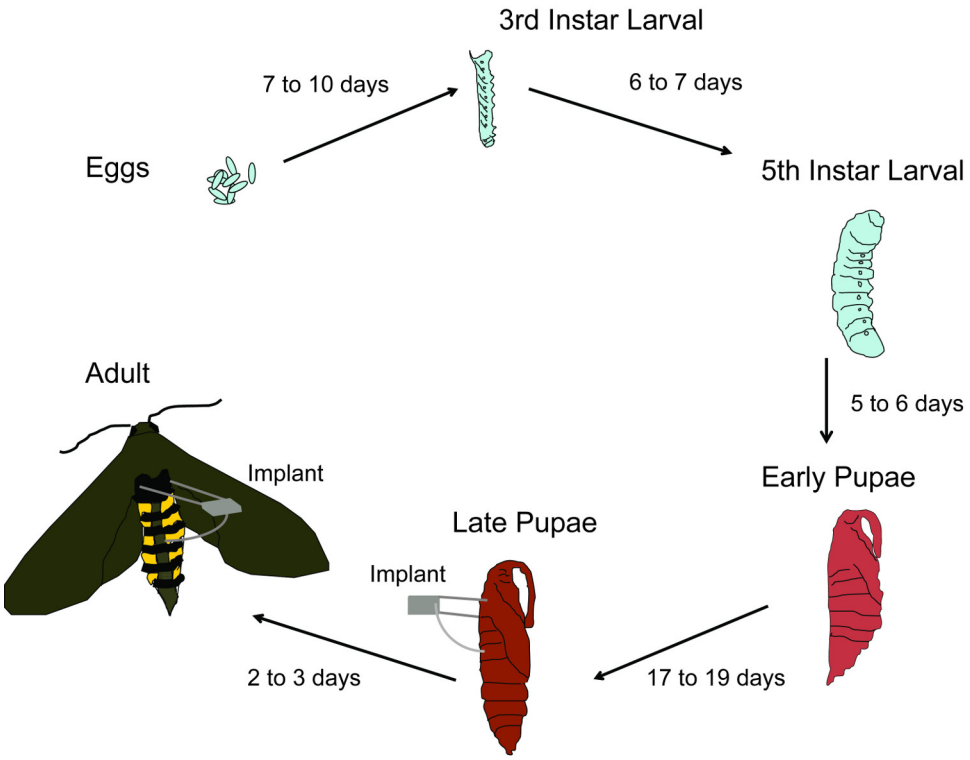

Figure 1. EMIT Procedure. A schematic diagram of the EMIT procedure performed on *Manduca sexta*, as described in the protocol.

Figure 2. Pupa Insertion. Photograph of a late stage pupa immediately after the recording electrodes were inserted using EMIT.

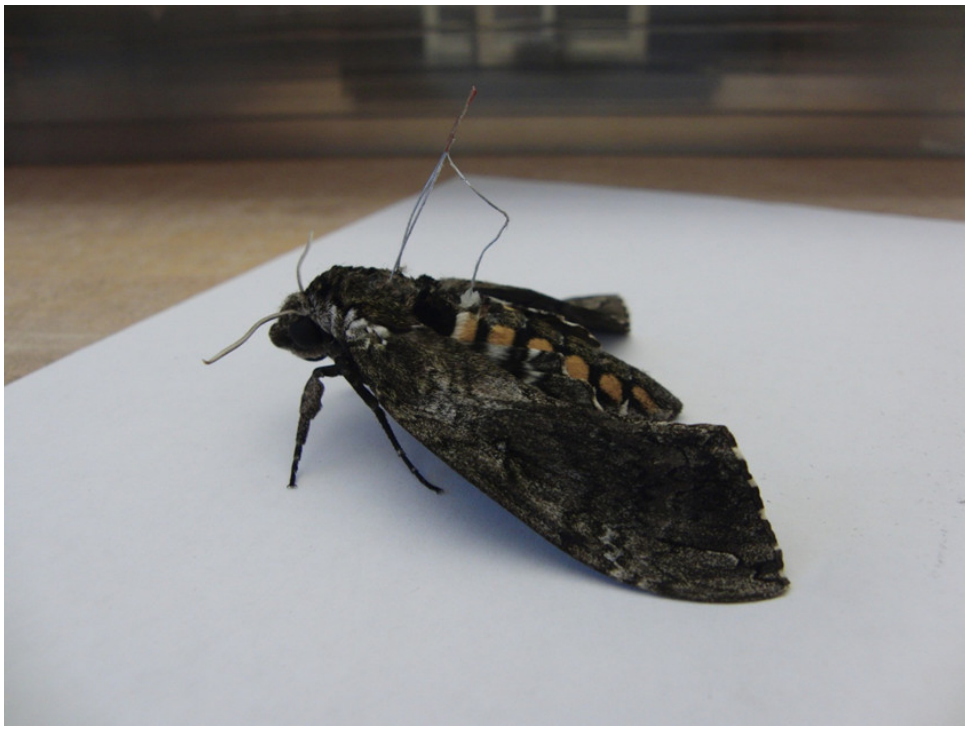

Figure 3. Moth Emergence. Photograph of an adult moth with implanted recording electrodes after eclosion.

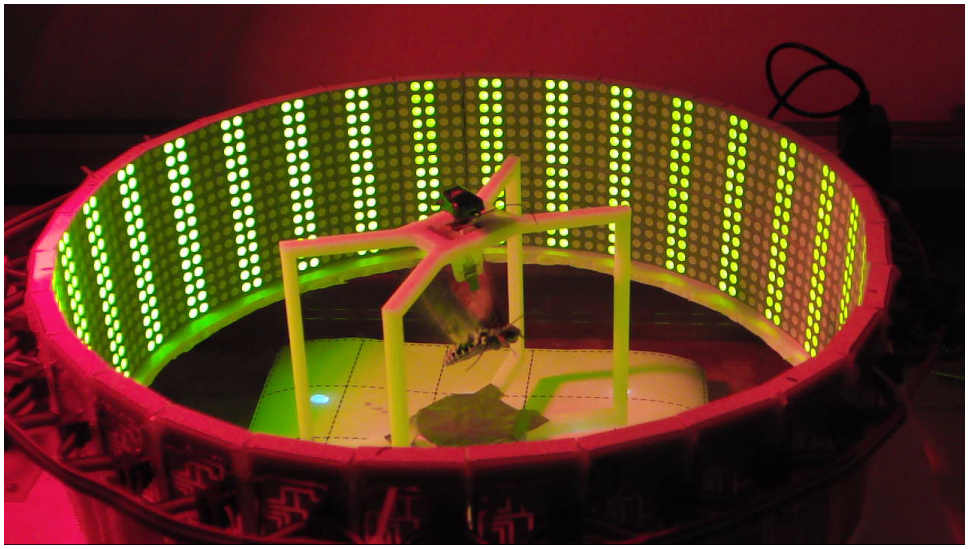

Figure 4. Recording Setup. The magnetic levitation platform and LED arena used to record EMG signals from *Manduca sexta* flight muscles. Here a *Manduca sexta* is performing a yaw maneuver in response to the revolving LE pattern.

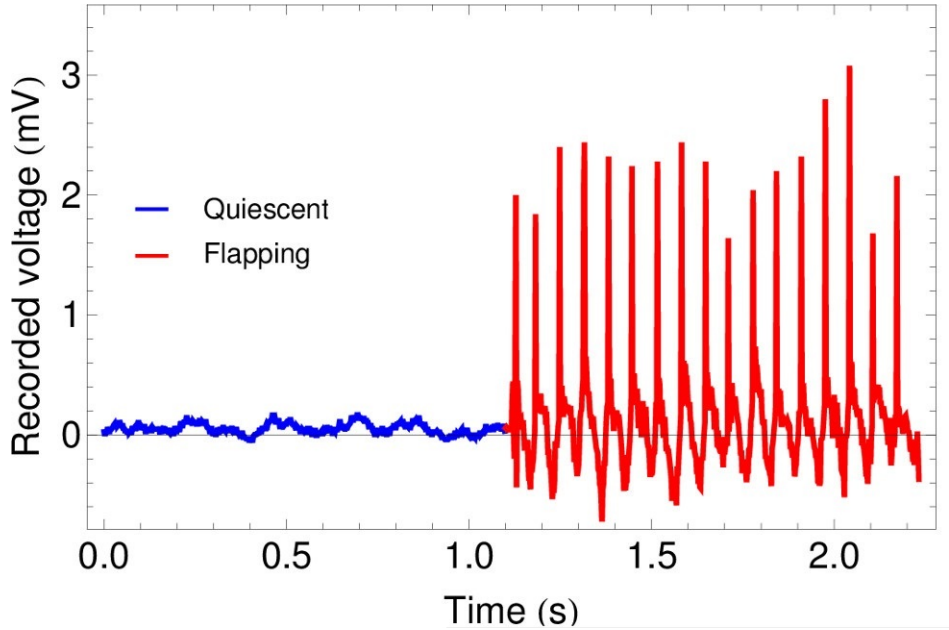

Figure 5. Oscilloscope EMG. A 2.5 sec EMG recording of a dorsoventral muscle using an amplifier and an oscilloscope.

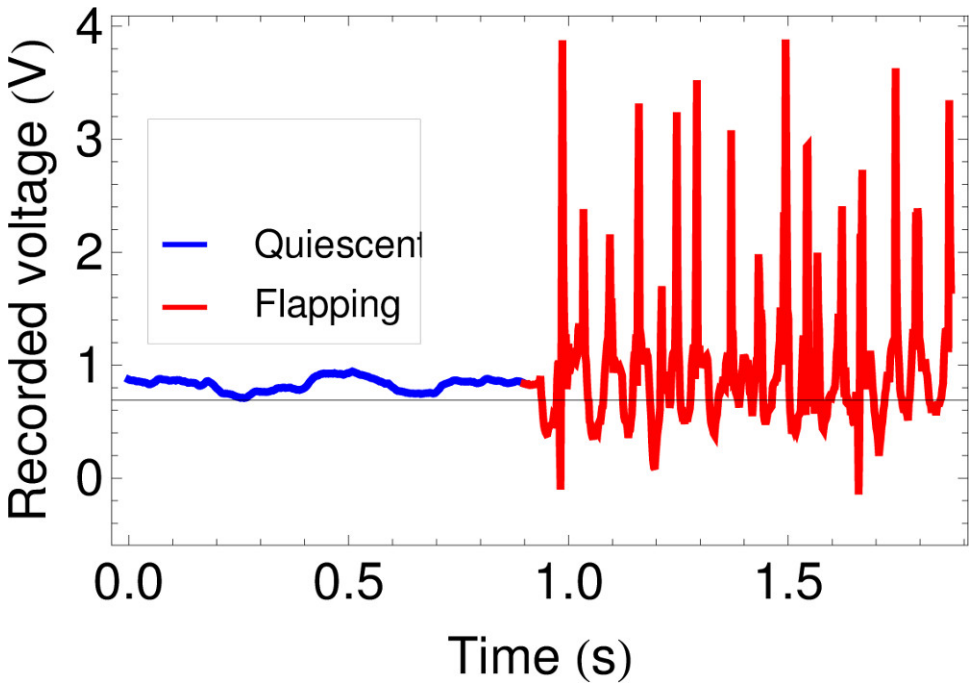

Figure 6. Wireless EMG. A 1.9 sec EMG recording of the dorsoventral muscle using the wireless headstage recording unit and data acquisition software.

Discussion

There are several critical steps during the surgical insertion of the recording electrodes that affect the ability to record data in the later steps of the protocol. The recording electrodes should be inserted into the pupa one day after exhibiting wing spots on its dorsal side. If the insertion is performed two or more days after this time, the insect's tissue will not have enough time to develop around and stabilize the inserted electrodes. This could lead to movement of the implanted electrodes and unreliable recordings in the adult stage.

It is important not to insert the recording electrodes into the pupal flight muscles at a depth of more than 5 mm. Otherwise, hemolymph will exit the insertion points and result in the development of weaker flight muscles. If hemolymph does emerge, stop the procedure and allow the pupa 24 hr to recover before attempting to insert the electrodes again. The insertion site should be cleaned thoroughly of all wing hair before the electrodes are inserted in the pupa. This prevents hair from entering the insertion holes and interfering with the electrode tissue interface.

To ensure optimal wing health in the adult moth, the insertion site should be re-cleaned of wing hair the day before eclosion using tweezers. In addition, it is recommended to use tweezers to loosen the edges of the cuticle window that was incised with the hypodermic needle to assist eclosion occurring on the following day. If any glue or hemolymph has dried near the edges of the cuticle window, the moth will not be able to inflate its wings after eclosion and this specimen will not be useful for experiments.

Although the insertion times are given in days, these may slightly vary as the timeline of the metamorphic development is a function of the rearing temperatures for poikilotherms. The provided days are for insects reared in RT. If a standard 25°C insectary incubator is used, the development will be approximately 10-20% faster and the insertion times need to be adjusted accordingly.

A limitation of this study would be the rotational inertia introduced to the setup by the rapid prototyped ABS plastic levitation frame. The mass of the frame can be up to 200 grams while the mass of a moth is about 4 grams. The benefit of using an electromagnetically levitated frame is loss of frictional contact between the frame and a supporting structure. However, the use of a relatively heavy frame causes the insect to expend more energy to complete yaw maneuvers in response to the revolving LED pattern. A modification to the tethering frame used in this study could be the use of a less dense material and/or building a thinner frame to reduce the inertial loading.

The developmental changes during metamorphosis bring new capabilities to neural engineering methods to learn how insects fly. It is a remarkable observation that the electrode insertion during the pupal stages results in alleviated tissue reactions with respect to the adult stage insertions. Therefore, EMIT based insertions ensure mechanical attachment of synthetic systems in or on an insect, while realizing a predictable neuromuscular interface with minimal short term effect on insect locomotory behavior. During the last two decades, robotists working on very small scale unmanned air vehicles have been inspired by insect flight. Beyond enabling a novel electrophysiological technique, EMIT procedure also allows for insect-machine-interfaces (IMI) that may provide access for neural engineers to the electrically excitable cells of the insect to control its sensory and behavioral physiology[8]. This has a potential to "biobotically" tame and control insect locomotion. Therefore, the specific methodology presented in this article is not only useful for studying the insect flight, but also for domesticating insects as hybrid centimeter-scale flying biobots[18]. An application of such a hybrid platform is to convert insects into mobile environmental sensing systems. These working animals can potentially assist humans in monitoring the co-shared ecosystems by gathering and storing environmental information.

Disclosures

Authors have no conflict of interest in this study.

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