Video Article

Identification of Olfactory Volatiles using Gas Chromatography-Multi-unit Recordings (GCMR) in the Insect Antennal Lobe

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URL: http://www.jove.com/video/4381
DOI: doi:10.3791/4381

Keywords: Neuroscience, Issue 72, Neurobiology, Physiology, Biochemistry, Chemistry, Entomlogy, Behavior, electrophysiology, olfaction, olfactory system, insect, multi-channel recording, gas chromatography, pollination, bees, *Bombus impatiens*, antennae, brain, animal model

Date Published: 2/24/2013

Citation: Byers, K.J.R.P., Sanders, E., Riffell, J.A. Identification of Olfactory Volatiles using Gas Chromatography-Multi-unit Recordings (GCMR) in the Insect Antennal Lobe. *J. Vis. Exp.* (72), e4381, doi:10.3791/4381 (2013).

Abstract

All organisms inhabit a world full of sensory stimuli that determine their behavioral and physiological response to their environment. Olfaction is especially important in insects, which use their olfactory systems to respond to, and discriminate amongst, complex odor stimuli. These odors elicit behaviors that mediate processes such as reproduction and habitat selection[1-3]. Additionally, chemical sensing by insects mediates behaviors that are highly significant for agriculture and human health, including pollination[4-6], herbivory of food crops[7], and transmission of disease[8,9]. Identification of olfactory signals and their role in insect behavior is thus important for understanding both ecological processes and human food resources and well-being.

To date, the identification of volatiles that drive insect behavior has been difficult and often tedious. Current techniques include gas chromatography-coupled electroantennogram recording (GC-EAG), and gas chromatography-coupled single sensillum recordings (GC-SSR)[10-12]. These techniques proved to be vital in the identification of bioactive compounds. We have developed a method that uses gas chromatography coupled to multi-channel electrophysiological recordings (termed 'GCMR') from neurons in the antennal lobe (AL; the insect's primary olfactory center)[13,14]. This state-of-the-art technique allows us to probe how odor information is represented in the insect brain. Moreover, because neural responses to odors at this level of olfactory processing are highly sensitive owing to the degree of convergence of the antenna's receptor neurons into AL neurons, AL recordings will allow the detection of active constituents of natural odors efficiently and with high sensitivity. Here we describe GCMR and give an example of its use.

Several general steps are involved in the detection of bioactive volatiles and insect response. Volatiles first need to be collected from sources of interest (in this example we use flowers from the genus *Mimulus* (Phyrmaceae)) and characterized as needed using standard GC-MS techniques[14-16]. Insects are prepared for study using minimal dissection, after which a recording electrode is inserted into the antennal lobe and multi-channel neural recording begins. Post-processing of the neural data then reveals which particular odorants cause significant neural responses by the insect nervous system.

Although the example we present here is specific to pollination studies, GCMR can be expanded to a wide range of study organisms and volatile sources. For instance, this method can be used in the identification of odorants attracting or repelling vector insects and crop pests. Moreover, GCMR can also be used to identify attractants for beneficial insects, such as pollinators. The technique may be expanded to non-insect subjects as well.

Video Link

The video component of this article can be found at http://www.jove.com/video/4381/

Protocol

1. Volatile Follection

1. In this example, we use volatile samples from *M. lewisii* flowers - an alpine wildflower native to California. Volatiles are collected using dynamic sorption methods according to Riffell *et al.*[14]. Briefly, this method employs a closed loop trapping system where flowers are enclosed in a Teflon bag. Using an inert vacuum pump, the air around the flowers is sucked through a "trap" comprised of a Pasteur pipette filled with Porapak Q matrix. The return air from the pump is filtered by activated charcoal. After a prescribed time period, in our case 24 h, the Porapak Q matrix is eluted with a non-polar solvent, typically hexane, to collect the concentrated extract. The extract is then stored in -80 °C until analysis. If necessary, samples can be concentrated prior to analysis under a stream of nitrogen gas. Unless the sample is already well-characterized, run an aliquot of it through a gas chromatograph-mass spectrometer (GC-MS) to identify volatile components prior to using the sample.

2. Electrophysiological Preparation

1. Cut approximately 1 cm from the end of a 1,000 µl pipette tip. Place a bumble bee (*Bombus impatiens*) into the base of the pipette tip and gently push towards the opposite end of the tip until only the head is exposed.
2. Melt dental wax and mold it around the exposed head, making sure that the wax adheres onto the compound eyes for security and to make the bee's head completely immobile. Be sure not to get any wax onto the antenna of the bee.
3. Once the head is secure, make a square, window-like, incision into the head capsule using a razor blade-breaker or the appropriate sized scalpel to cut the cuticle. Using the blade-breaker, start from the dorsal side of the head capsule, immediately behind the antenna and adjacent to one of the compound eyes. Cut a straight line, from one compound eye to the contralateral compound eye. After cutting a straight line to the opposite compound eye, begin making an incision dorsally until the head capsule curves and ends near its thorax. At this point, begin to cut towards the opposite end of the head capsule. Finally, once a line has been cut to the opposite end, cut a line back up to the starting position of the initial incision. It is important to remove the cuticle that is adjacent to the antenna, as this will impede the insertion of the electrode.
4. Once the cuticle is cut, use a pair of forceps to remove the bee cuticle, which should then expose the bee's brain and, more importantly, the antennal lobes. Immediately begin superfusing the brain with insect saline, so that the brain does not become dessicated. After the brain is exposed, carefully use a pair of very fine forceps to remove the perineural sheath immediately above the antennal lobes. Be very careful not to puncture the bee's brain with the forceps.

3. Gas Chromatography with Multi-channel Recording

1. The bee "preparation" - fixed in a tube with its brain exposed - is now ready for electrophysiological recording. Place the bee in a clamp, fixed to a magnetic base that is located on an air table.
2. Arrange an IV bag, flow controller, and tubing (filled with insect saline) so that the saline continuously superfuses the brain.
3. Using a micromanipulator, insertareference electrode, made of tungsten wire, into the bee's eye.
4. Using a separate micromanipulator, insert the multi-channel electrode, such as a coiled wire tetrode, or silicon multi-channel electrode (Neuronexus Technologies), into the antennal lobes of the bee. This electrode is connected to a pre-amplifier such as the TDT system's S-3 Z series to the Z-bus bioamp processor of the TDT system. Output from the Gas Chromatogram's detector via a shielded BNC cable can be interfaced with the amplifier and data acquisition system such that both the neural and GC signal is synchronized.
5. Wait approximately 30-60 minutes for the neural recordings to stabilize. Once the spontaneous activity and waveform shape of units in the recording channels have become consistent, use an odor syringe to stimulate the bee and observe the response of the recording channels to the odor.
6. Record extracellular spikes from neurons by auto-thresholding the recording channels by 3.5 to 5 sigma of the signal on the individual recording channels. Manual thresholding may be required for some channels to avoid contamination from electrical noise. The action potentials from neurons will appear as voltage spikes in the recording channel. When the channel's voltage exceeds the threshold, the system buffers and saves the few milliseconds before and after the crossing of the threshold, thereby taking a snap-shot of the waveform, or spike.
7. Immediately next to the air table is the GC. Before injecting the floral extract into the GC, make sure that the method for the temperature ramp of the GC run is correct. In our example, we use a temperature method starting at 50 °C for 4 min followed by an increase of temperature at a rate of 10 °C/min. to 220 °C, at which time we hold the GC for an additional 6 min. We use a DB-5 GC column (J&W Scientific, Folsom, CA, USA), with helium as the carrier gas. The inlet is splitless, with a temperature set to 200 °C. The flame ionization detector temperature is set to 230 °C.
8. Inject the extract sample of the floral headspace into the heated injection port of the GC to release the adsorbed volatiles into the GC column. The effluent from the column is split 1:1 between the Flame Ionization Detector (FID) and the bee antenna using a glass "Y" connector (J&W Scientific). Begin recording from the electrode as you inject a sample into the GC.
9. After the GC run has finished, let the preparation rest for 5 to 15 min. Then either inject another sample into the GC or stimulate the preparation using single volatile compounds or mixtures of compounds. In this latter method of stimulating the preparation, pulses of air from a constant air stream are diverted through a glass syringe containing a piece of filter paper on which the compounds have been deposited. The odor stimulus was pulsed by means of a solenoid-activated valve controlled by the software.
10. If unit activity suddenly stops or changes, check the saline drip and let the preparation rest for 15 min. If the spontaneous activity does not regain its previous level then the preparation should be discarded and another bee used if available.
11. After the experiment, fix the brain with 5% formalin for 20 min with the probe still in the tissue. Next, excise the brain and place it in 2% glutaraldehyde for 4 hr, and subsequently do a graded ethanol dehydration series and clear the brain with methyl salicylate. Based on the fixing and clearing of the tissue, the locations in the AL where the electrodes punctured the tissue should be clearly discernible by confocal microscopy.

4. Data Analysis

1. Analyze collected data after the experiment to separate and identify the recorded neural units. Use typical software programs (Offline Sorter, MClust, and SClust) to separate waveforms, or "spikes", based on spike shapes, such as peak or valley amplitude, peak half-width, etc., or reduced measures (principal components)[17,18] (**Figure 2**). Use only those clusters of spikes that separated in three-dimensional space (PC1-PC3) and are statistically different from one another (multivariate ANOVA; $p < 0.05$) (**Figure 2**) for further analysis. Please refer to citation #s 17-19 for full description of tetrode recording and spike-sorting methodologies.
2. Time-stamp spikes in each cluster, and export these data for analysis using MATLAB or Neuroexplorer (Nex Technologies, Winston-Salem, NC) to create raster plots and firing rate responses (**Figures 2, 3A**).
3. Identify the retention times of volatiles using the simultaneously recorded GC data. Use the retention times of the volatiles, determined by the apex of the peak from the chromatogram, to examine unit responses at those time points.

4. To examine individual unit responses through the GC run, bin the number of spikes in 100 msec intervals and examine the time-course of firing rate responses with reference to the retention time of eluting volatiles. The binning of spikes in 100 msec intervals provides enough detail, or signal, about the time-course of the neuronal response to an eluting odorant from the GC.
5. To examine the population responses to the different eluting volatiles, integrate the firing rate responses of individual units over a 3 sec sampling window, 1.5 sec before, and 1.5 sec after, the retention time of the volatile (**Figure 3**). This time period is typical of the duration of an eluting volatile from the GC. We show firing rate responses of the units by color-coding them (red is a high firing rate response; blue is a low response) and arranging them as an activity matrix with each row representing the ensemble response to the GC effluent (columns) (**Figure 3**).

Representative Results

In the GCMR assay using the *M. lewisii* floral scent, we inject 3 µl of the extract into the GC. The total number of volatiles eluting through the GC is typically 60-70 volatiles. The scent of *M. lewisii* is predominantly composed of monoterpenoids, including *β*-myrcene (acyclic) and *α*-pinene, with the remainder of the scent composed of six-carbon volatiles, such as 2-hexanol, and sesquiterpenoids that comprise < 1% of the headspace.

GCMR takes advantage of the sensitivity of antennal lobe neurons as well as the neuronal processing of biologically important volatiles. However, multi-channel recordings of this nature, in effect, take a random sample of neurons in the antennal lobe. This is because slight changes in the position of the probe position between different preparations can cause the recording array to sample different neurons. Moreover, the exact positions and morphologies of the recorded neurons are unknown because the recording is extracellular. To accommodate for these effects, we typically run GCMR experiments with 8 to 16 preparations, with 8 to 18 neural units in each preparation. For purposes of illustration, however, we will use data from only one preparation (8 units).

From the GCMR experiments, we have found that units are surprisingly selective in their response to volatiles, as depicted in **Figure 3A**. The lower trace (in black) denotes the ion chromatogram, where each peak corresponds to a given volatile that is arriving at the detector over time. The upper trace (blue) shows the firing rate responses of a unit. Unit responses were calculated by binning the numbers of spikes produced in a 100 msec interval, and dividing by that timeframe to produce the rate. In the example, the neural unit is selective in response to D-limonene. Note that, in this unit, the spontaneous firing rate can still be variable and subject to random fluctuations. Nonetheless, responses to D-limonene were well above the 95% confidence interval, as calculated by the variance in the firing rate responses through time.

Not all units, however, responded to the volatiles eluting from the GC. In fact, on average approximately 50% of recorded units in each ensemble were unresponsive (**Figure 3B**). This is surprising given the diversity of volatile compounds in the floral headspace that are eluting from the GC. However, the proportion of non-responsive units in an ensemble is surprisingly consistent between preparations, as found in previous studies[13,14].

Beyond the responses of single units, the GCMR system also enables the analysis of population-level responses to the odorants eluting from the GC. In the example shown here, there is strong ensemble selectivity for a group of several odorants (**Figure 3B**). The top plot shows the chromatogram with each peak corresponding to an odorant (numbered on the x-axis). Odorants 33 and 35 (D-limonene, and *trans-β*-ocimene, respectively) produced robust responses in the ensemble, as represented by the normalized firing rate of each unit in the ensemble (color scale).

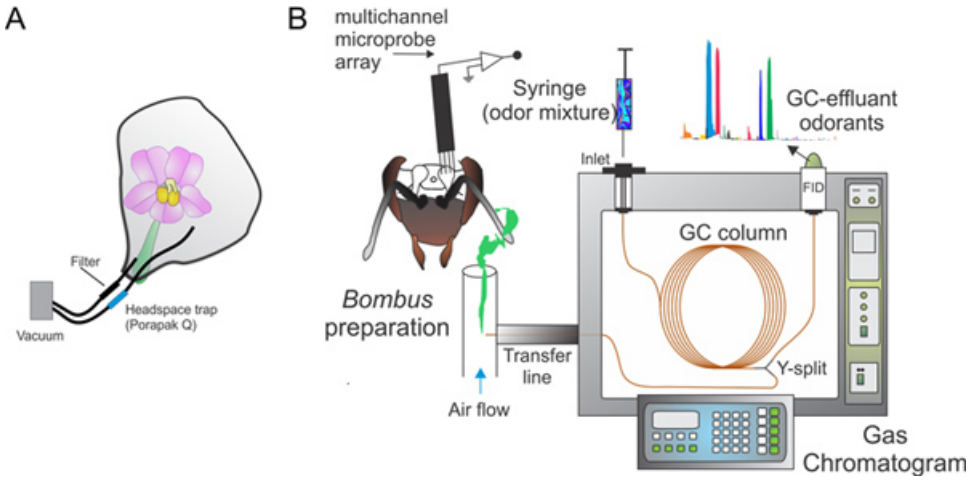

Figure 1. Schematic diagram of headspace sorption and the GCMR system. (A) In the schematic, a flower is enclosed within a Teflon bag, and using a vacuum pump, the air from the bag is sucked through a volatile trap (Porapak Q) to concentrate the emitted volatiles. The air is filtered and returned to the enclosed flower. **(B)** The extract sample of the floral headspace is injected in the GC and the effluent from the column is split such that half of the flow enters either the GC's flame ionization detector, and the other half of the effluent is carried by a heated transfer line and arrives simultaneously at the bee's antenna. Action potentials from the AL neural ensemble are continuously recorded extracellularly during the 20 min of odor delivery via GC. Click here to view larger figure.

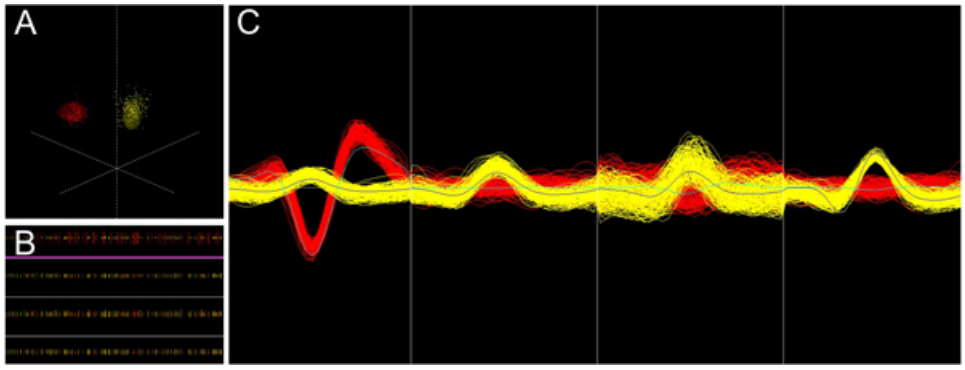

Figure 2. Sorting of units recorded using the multi-channel recording array (MR) in the bee's AL. The two shanks are spaced such that the array encompasses a large volume of the AL. **(A)** The waveform characteristic (*e.g.* amplitude, valley) of each "spike" in the tetrode recording can be plotted in three-dimensional space. In the example shown here, the height of each spike in 3 of the 4 recording channels is plotted in 3-D. Because each unit will have its own spike shape, the spikes from a given unit will cluster together, thereby allowing the units to be identified and sorted from one another. Sorted units showed clear differences in firing responses **(B; raster plot)** and waveform shape **(C)** Positioning of the four channels on each shank provided recording of broad zones within processing glomeruli and neuropil. Neural activity was recorded on each of the four channels, plotted in 3-dimensional space (as shown in **A**), and sorted according to waveform characteristics. Click here to view larger figure.

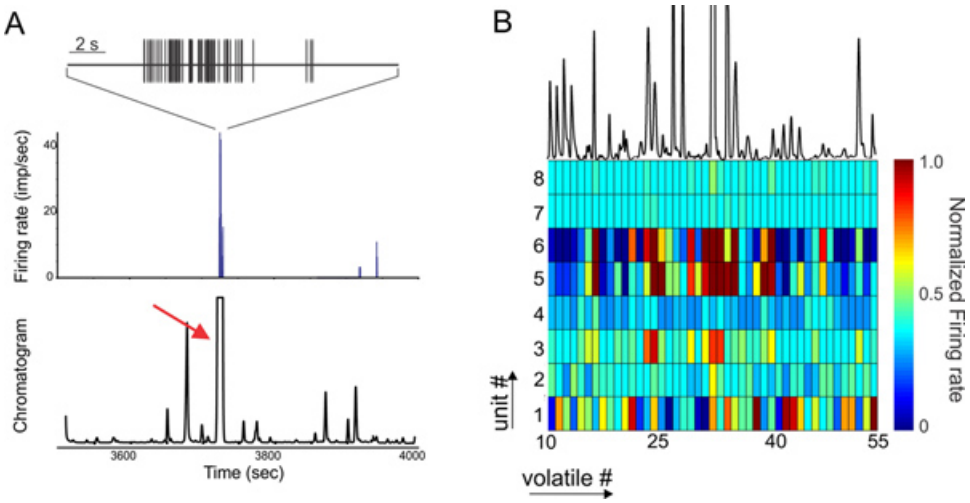

Figure 3. (A) Firing rate histograms of unit responses to the eluting compounds from the *M. lewisii* headspace extract (3 µl injection) (bottom trace, black). Certain odorants (*e.g.* D-limonene; red arrow) evoked significant responses in units. **(B)** Response of all units that were recorded from the MR to each odorant eluted from the GC. The surface plot is color-coded according to the normalized firing rate responses of the individual units. Click here to view larger figure.

Discussion

Insect olfactory-mediated behaviors drive many different processes, including reproduction, host-site selection, and the identification of appropriate food resources. The study of these processes requires the ability to identify the volatiles emitted from the source, as well as the ability to identify those compounds that are mediating the behaviors. Complicating matters is that odors are comprised of tens to hundreds of individual compounds that together create a unique scent that is perceived differently than the individual constituents[6,7,13,19,20]. Research, initially conducted in the sex pheromone system[12], and more recently in food- and oviposition-related odors[13,20], has shown that the behavioral effectiveness of the mixture resides as a function of a few, key volatiles in the mixture, and that the mixtures elicit significantly greater behavioral responses than the individual constituents. Identifying those key constituents is thus an important component in modern-day chemical ecology as well as olfactory neurobiology.

A variety of techniques have arisen in the last fifty years for the identification of bioactive compounds that drive behavior in insects. The primary technique for the detection of volatiles by the insect antenna is gas chromatography-electroantennography (GC-EAG). EAG was originally developed by Schneider[21], who recorded small voltage fluctuations generally assumed to be caused by electrical depolarisations of many olfactory neurons between the tip and base of an insect antenna during stimulation. EAG was later integrated with the GC for precise identification of the headspace volatiles that elicit antennal responses[12,22]. In addition, the recording of individual receptor neurons from the insect antenna was later developed[11,23] and coupled with the GC to provide single-sensillum recordings, or GC-SSR. Although GC-SSR is more time-intensive and difficult than GC-EAG, the recordings provide information on the individual cell responses via action potentials in response to

the GC-effluents, and allows the identification of those receptors that are specialized to particular compounds that might be missed by GC-EAG [24].

The GC-EAG and GC-SSR techniques offer identification of those volatiles that elicit responses on the periphery, and are thus involved in odorant reception. More recent techniques have begun examining responses in the central nervous system of both mammals and insects, and are thus involved in odorant perception. These techniques fall under two broad categories: imaging methods[25] termed GC-I (gas chromatography-imaging) and direct electrophysiological methods (*e.g.* GCMR). GC-I and GCMR offer several advantages because of the convergence of the sensory neurons to the projection, or output, neurons in the AL, as well as that these methods allow the determination of how odorants are represented in the insect brain. Moreover, similar methods are now being used in the mammalian brain[25].

Despite these advantages, GCMR and GC-I offer drawbacks as well. Data analysis can be time-consuming, and in the case of the GCMR, glomerular projections of the recorded neural units are unknown due to the recordings being extracellular. Moreover, the insect AL is innervated by several different neuronal types including projection neurons (PNs) and local interneurons (LNs) thus making identification of the recorded units difficult. However, in the moth, *M. sexta*, recent work has demonstrated that PNs and LNs can be identified by the spiking behavior of the neurons, thereby allowing for the identification of these neuron types by their spontaneous activity[26]. Nonetheless, GCMR and GC-I allows the identification of the odorants that activate specialized receptor neurons that may not elicit strong EAG responses, as well as determining how the population of neurons in the brain process the volatiles. A study comparing these different methodologies has not yet been conducted, although our preliminary data suggests that the GCMR may be more suitable than the GC-EAG for those compounds that are bioactive but are at trace levels in the extract (Riffell unpublished results). Future work may examine the trade-off in the sensitivity of detecting bioactive odorants with the analysis time between the different methodologies.

Although we have focused here on detailing the assay methods we use for *B. impatiens* bees and the scent from *M. lewisii*, the extract and the insect species used can be changed if certain modifications are made. Insect species can be placed into different pipette tips (10 to 200 µl) depending upon their size. Larger species, like the moth, *Manduca sexta*, can be placed into 6 ml sample vials. In such a manner, the preparation is kept alive thereby allowing stable recordings several hours in duration.

Taken together, analytical methods for isolation of compounds together with electrophysiological recordings in the insect brain present powerful tools for the identification of bioactive compounds, and when used in tandem with behavioral experiments, may present a means by which to determine important volatiles for food-related behaviors in insects[13], as well as those involved in host-site[2], and blood-host related behaviors[27] that are important for agricultural pests and vectors of disease.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by NSF grant IOS 1121692, and by the University of Washington's Research Foundation.

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