Towards nano-physiology of insects with atomic force microscopy

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1. Introduction

Recent studies in insect physiology continue to reveal new mechanisms for examining respiration (Hetz and Bradley, 2005; Westneat et al., 2003), communication (Cator et al., 2009) and other aspects of insect behavior and function. However, little exploration has begun in insect physiology with modern nanotechnology tools, which could lead us to yet unexplored areas. Atomic force microscopy (AFM) is one of the major techniques being used in modern nanotechnology. The AFM technique has become popular in the study of biological materials at the nanoscale, ranging from simple imaging (Firtel et al., 2004; Sokolov et al., 1996) to the measurements of biomechanics (Berdyyeva et al., 2005; Iyer et al., 2009; Pelling et al., 2004; Radmacher et al., 1994). AFM is based on detection of forces acting between a sharp AFM probe and the sample surface. The probe is attached to a flexible AFM cantilever. The motion of the cantilever is detected by various methods. The most popular detection method is an optical system, in which laser light is reflected from a photodiode, and subsequently translated into information about the deflection of the cantilever, i.e., force. When the probe makes contact with the surface of interest, the AFM system records deflection of the cantilever with nanometer precision. Scanning over a sample surface, one can record information about the topography of the surface (for example, bumps under the probe result in a higher deflection of the cantilever). If the surface itself is oscillating, the oscillations can be recorded when scanning over the surface is switched off.

As was shown, AFM is capable of measuring motion of the surface of small biological objects, such as cells at the level of several nanometers (Domke et al., 1999; Maksym et al., 2000; Pelling et al., 2004; Radmacher et al., 1994; Szabo et al., 2002). Contrary to common expectations, it is much harder to use the AFM technique for studying larger objects. AFM allows for ease of detection of oscillations of smaller objects down to the molecular scale with no need in any technical modification. Expansion of this technique to study larger objects, such as insects has been restricted by the limit of vertical motion of the AFM probe (typically within 50 μm). A technical solution was suggested (Dokukin et al., 2010) to keep insect motion partially restricted while recording the AFM signal with the help of a special stage. It was shown that the method allowed for recording of information from the internal live processes of the insect at sub nanometer scale.

Here we use an improved version of that AFM method to demonstrate measuring surface oscillations of a model insect, the ladybird beetle (Hippodamia convergens), by measuring surface oscillations at the sub-Angstrom (picometer) level (Guz et al., 2010). This allows recording a broader spectral range of oscillations (up to several kHz). These data show not only known breathing, heartbeat cycles (Slama, 2003; Slama and Miller, 2001; Slama and Farkas, 2005; Tartes et al., 2000), coelopulses (Slama,
1999, 2000), etc., but also a variety of new high frequency and small amplitude spectral peaks.

As was shown (Dokukin et al., 2010), the recorded signals demonstrate spectral peaks that are associated with internal processes of the beetle. (Oscillations, noise due to the surface motion of elytra, wings, hair, and keratin plates were identified as separate spectral futures.) An integral of the recorded spectra was used to study vision sensitivity of ladybird beetles to different wavelengths of light, the speed of the beetle’s adaptation to repetitive flashing light and its relaxation back to the initial stage (Guz et al., 2010). However, the paramount question about the nature of spectral peaks in the observed spectra remains unsolved.

In the present work, we demonstrate how one could identify the nature of the peaks, or at least relate them to a specific organ, by using various physical, chemical, and biological stimuli. As such, the developed methodology is a relatively non-invasive technique, which can provide new information about the work of individual organs and muscles of insects, to correlate the physiological response of organs with various external and internal stimuli. This may provide new information about the internal machinery of insects, and lay the ground-work for development of the area of “insect nanophysiology”.

2. Material and methods

2.1. Atomic force microscopy (AFM) and data acquisition system

All vibrations of the insect’s surface were recorded by means of a Veeco Dimension 3100 AFM equipped with a NanoScope V controller (all other versions of the controller could also be used). Veeco Silicone Nitride integrate DNP tips with spring constant $k \sim 0.06$ N/m were used in this study. The signal, the vertical position of the AFM cantilever was recorded. This signal was directly sampled using a National Instruments ADC 24-bit card (NI PCI-5922) at 50 kHz. Simultaneously, the external sound was recorded using a wideband microphone, RadioShack Supercardioid Dynamic Microphone 33-3042. The microphone has a frequency response of 50–15000 Hz at $-72 \pm 3$ dB sensitivity. The sound was sampled using the second input channel of the same ADC card. LabView (by National Instruments) version 8.2 was used as the data collection interface.

2.2. AFM stage and data collection method

The key element of the stage is a thin metallic membrane with an opening of a few millimeters in diameter. Specific diameter

depends on the type of the insect of interest. In this work we used a 5 mm opening. The insect was attached from underneath the membrane, Fig. 1(a), with the help of Easy Removal Scotch tape. An important part of the method was the double-stick tape surrounding the aperture of the membrane, Fig. 1(a). This minimized the insect’s motion in the vertical direction while keeping it relatively freely with the Scotch tape (which might otherwise be damaging for the insect). It also eliminated any movement in the lateral directions. The other important part of the insect attachment was the sticky tape applied on the top of the membrane to further restrict vertical motion. This is particularly important when one deals with a soft part of the insect, which may otherwise move over a substantial distance (and break the AFM cantilever).

An AFM tip was positioned on the top of the insect through the aperture in the holder membrane, Fig. 1(a). The scan size was set at 0 nm and scan rate was set at 0.1 Hz. To minimize noise coming from feedback (which is used to keep load force constant during scanning), the feedback gain parameters were set between 0 and 0.01 for the integral, and to 0 for the proportional gain. The spectral signals shown in this work were collected with both integral and proportional gains equal to 0.

2.3. Insects

H. convergens ladybird beetles (Hirt’s Gardens, Granger, OH) were used in this work. H. convergens is a widespread predator throughout North American agroecosystems, and is used in the management of cropland pests such as aphids and the Colorado potato beetle.

3. Experimental

A schematic of the experimental method is shown in Fig. 1(a). The AFM probe, attached to a flexible AFM cantilever, gently touches the insect surface. Because the stiffness of the cantilever is typically less than that of the insect surface, the recorded cantilever position equals the motion of the insect surface. A typical signal collected from the elytra of a beetle is shown in Fig. 1(b). To study the recorded signals quantitatively, one can analyze their Fourier spectra, shown in Fig. 1(c). To separate oscillations coming from the insect from oscillation coming from room noise, and to monitor the constancy of this noise during the course of experiments, a broad band microphone was used to record room noise. A baseline noise was found as the spectra recorded on a dead beetle (this included both the room and

![Fig. 1. The AFM setup used to detect surface oscillations. (a) A special insect holder that restricts the motion of the insect. Laser light reflected from the AFM cantilever is translated into the beetle’s vertical surface position. (b) An example of the recorded signal, and (c) its spectra; curve 1 is the signal coming from a live beetle, curve 2 is the baseline signal recorded on a dead beetle. The room noise was recorded (curve “mic”) with a wide-band microphone in the vicinity of the holder.](image-url)
instrumental noise). Curve 1 shown in Fig. 1(c) is the signal coming from a live beetle, whereas curve 2 is the baseline signal recorded on a dead beetle. The room noise was recorded (curve “mic” in Fig. 1(c)) with the microphone positioned in the vicinity of the holder.

One can see in Fig. 1(c) that a number of spectral peaks are caused by either the room noise or the apparatus noise (the peaks specific to the dead beetle spectrum). After excluding those peaks from consideration, one can identify in curve 1 the frequencies typical for living ladybird beetles. From an analysis of the low frequency part of the spectra (0.0001 Hz), one can see well-known breathing, heartbeat cycles (Slama, 2003; Slama and Miller, 2001; Slama and Farkas, 2005; Tartes et al., 2000), and presumably coelopulses (Slama, 1999, 2000). As one can see from Fig. 1(c), this approach allows collecting mechanical oscillations of the insect’s surface down to a single picometer level (the baseline signal, curve 2). Ability to detect such small amplitudes allows recording this signal up to several KHz (starting from ~0.1 Hz). For a technical comparison, a recently described rather sensitive system of optical detection (Pelling et al., 2009) allows for detection of the surface oscillations of an area of ~500 μm² with a noise level of 0.5 ± 0.2 nm root-mean-squared (r.m.s.). The AFM method used here allows for the possibility to address areas as small as ~100 nm² (0.0001 μm²) with a noise level of 2 ± 0.2 × 10⁻³ nm r.m.s. at the range of frequencies of 60–120 Hz. Signals corresponding to the higher frequencies, above 5 Hz and up to tens of KHz apparently have not been detected when using previous methods due to low sensitivity to the high frequency oscillations. Such high frequency sensitivity was intrinsically limited in those methods because of the large mass of the used sensor (reflector), which required a substantial force to noticeably move the sensor at a high frequency. The frequencies of many peaks seen in Fig. 1(c) are substantially higher than frequencies associated with previously known breathing, gut peristalsis, coelopulses, or heart beating.

4. Results and discussion

Because of the wealth of new information observed in the high frequency spectrum, the important question to answer is to identify the nature of the observed spectral peaks. The recorded signals do not depend on the insect’s condition alone; they also vary for different parts of the insect. It is possible to identify signals that are present only in live insects and thus come from internal processes (Dokukin et al., 2010). These signals can originate either from contraction of insect muscles or the hydrodynamic motion of its internal fluids. While the exact identification of the nature of each peak will be the subject of a series of separate works, here we demonstrate several examples of such identification (not necessarily unambiguous every time). This identification is made using various physical, chemical, and biological stimuli.

First, we demonstrate a physical approach to identify specific high frequency spectral peaks induced by the beetle’s heart. In this approach, an AFM tip was physically located in the vicinity of the organ of interest. The recorded spectra are compared with the locations far from the organ. The organ’s specific spectral peaks should be of high intensity in the vicinity of the organ. This is because the square of the peak amplitude is proportional to the energy of the oscillations, which decreases as the distance from the organ increases (due to energy dissipation in the insect’s tissue).

To identify spectral features related to heart activity, we collected signals shown in Fig. 2. The AFM probe was physically located in the vicinity of the heart (curve 2). This signal was compared with the spectrum collected at the elytra near the pronotum (curve 1) and with respect to the reference curve of the dead beetle (curve 3). One can see that a low-frequency peak of 0.6 Hz and high frequency 293 Hz peaks are well correlated with the heart location. The low-frequency feature is in agreement with expected values reported before (Slama, 2003; Slama and Miller, 2001; Slama and Farkas, 2005; Tartes et al., 2000). The high frequency 293 Hz peak is new. Comparing these low and high frequency peaks, one notes that signal/noise ratio is substantially higher for the high frequency peak. Moreover, it is rather difficult to use 0.6 Hz peak to study the heart activity because of a confusing proximity of the other low frequency peaks corresponding to coelopulses, breathing, and perhaps peristaltic movements of the gut. At the same time, 293 Hz peak is a clear stand-alone feature of the spectrum, which could be unambiguously used to study heart activity.

Fig. 3 demonstrates an example of the use of a biological stimulus to learn the nature of particular peaks. Here we fed ladybird beetles with sugar water (~5 wt%). A small droplet on a fine wood stick was used to approach the beetle’s mouth until contact was made. Because the beetle did not drink for several hours previously we assumed it would drink the offered water (this was checked by observing the beetle’s natural behavior in a Petri dish). Drinking is a complex action involving muscles activation as well as the motion of the liquid. The signals were collected on the beetle elytra while drinking (curve 2 of Fig. 3). One can clearly see the following alteration of the spectra (curve 2) compared to the normal state of the beetle and a dead beetle (curves 1 and 3, respectively). The peaks around 15–17, 120 and 250 Hz became smaller or disappeared. A large peak of 280 Hz decreased by ~25% and shifted to 273 Hz. There are also a broad
Figure 4. “Chemical” approach to identifying the nature of the spectral peaks by exposing the insect to carbon dioxide gas (curve 2). Control curve (3) corresponds to dead insects. Curve (1) is the spectrum collected on live undisturbed insects. 0.01 nm added to high frequency curve 1 for better visualization. Only parts of spectra showing differences are shown.

![Figure 4](image-url)
external stimuli in a relatively non-invasive manner. This may shed light on unsolved problems of insect functions and behavior. The approach described here could lead to the emergence of "insect nanophysiology".

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References


