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Automated imaging of neuronal activity in freely behaving *Caenorhabditis elegans*

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ABSTRACT

In order to understand how neuronal circuits control locomotory patterns it is necessary to record neuronal activity of freely behaving animals. Here, using a new automated system for simultaneous recording of behavior and neuronal activity in freely moving *Caenorhabditis elegans* on standard agar plates, we show that spontaneous reversals from forward to backward locomotion reflect precisely the activity of the AVA command interneurons. We also witness spontaneous activity transients in the PLM sensory neurons during free behavior of the worm in standard conditions. We show that these activity transients are coupled to short spontaneous forward accelerations of the worm.

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1. Introduction

Monitoring neuronal activity of freely moving animals in a controlled environment is a crucial issue in neuroscience in order to understand how neuronal circuits integrate sensory signals to generate behavior. Recently, genetically encoded calcium indicators (Miyawaki et al., 1997; Pologruto et al., 2004) have allowed the development of non-invasive neuronal activity recording techniques, especially in small and transparent animals like the nematode *Caenorhabditis elegans* (Kerr et al., 2000). This nematode has a compact and well described nervous system, the molecular basis of its behavior has been studied in great details and its genome is significantly conserved with vertebrates. These features have made *C. elegans* a useful model system in neuroscience.

In immobilized *C. elegans*, fluorescent calcium indicators have allowed the recording of calcium transients in neurons and muscles (Kerr et al., 2000) and the correlation of sensory stimuli and neuronal activity in sensory neurons (Suzuki et al., 2003; Kimura et al., 2004; Hilliard et al., 2005). Semi-restrained worm imaging systems have then been developed in order to correlate calcium activity to the behavior of the worm. A two-objective system has been designed to record neuronal activity of a worm glued at its head while recording the behavior of the free body (Faumont and Lockery, 2006). Behavior chips have also been developed to record neuronal activity and behavior of *C. elegans* in a microfluidic channel. These devices allow a precise control of the environment of the worm (Chronis et al., 2007; Chalasani et al., 2007). In both systems the animal is constrained, and therefore many behavioral tests cannot be implemented. Moreover, recording is frequently done in liquid medium, whereas most behavioral and genetic studies have been done previously on solid agar plates.

Another system has been described for manual tracking of freely moving worms in a thermal gradient and simultaneous recording of calcium activity in a thermosensory neuron (Clark et al., 2007). In this system the worm can move freely but is under a coverslip, which prevents access to the worm to apply mechanosensory or chemical stimuli. The tracking has to be done manually and lasts only for 2 min.

Here we report the development of a new custom-made tracking system for long-term automated recording of neuronal activity and behavior of freely behaving worms in their standard laboratory environment. We could show that spontaneous reversals from forward to backward locomotion on standard plates reflect the activity of the AVA command interneurons. We also demonstrate that spontaneous activity of the PLM mechanosensory neurons correlates with forward accelerations.

2. Materials and methods

2.1. Strains generation and C. elegans maintenance

We constructed the transgenic strain AQ2139 for AVA imaging. This strain expresses the D3cpv cameleon protein (Palmer et al., 2006) under the control of the nmr-1 promotor in a *lite-1*

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background (Edwards et al., 2008). For D3cpv expression in AVA interneurons, P*nmr-1*::D3cpv plasmid was made by a LR reaction in the Gateway System (Invitrogen) from the following two plasmids. A 4.9 kb upstream sequence of the *nmr-1* gene was amplified and cloned into NheI–XhoI sites of pDEST. The D3cpv gene was amplified and cloned into pDONR by BP reaction. An integrated transgene was obtained by biolistic transformation (Praitis et al., 2001) of the P*nmr-1*::D3cpv plasmid together with the *unc-119* gene into *unc-119(ed3)* animals followed by UV irradiation (Mello et al., 1991). The resultant transgene *ljIs109* was outcrossed eight times, and crossed with *lite-1(ce314)* to make AQ2139. Pnmr-1 promotes expression in the AVA neurons and a few other classes of neurons. However, expression of D3cpv in AQ2139 is much stronger in AVA and to a lesser extent in AVE than in other cells. In fact, in control experiments that we performed in the locomotion chip (Chronis et al., 2007) using much higher magnification (e.g. 63×), we were only able to detect a signal in the AVA/AVE neurons, and not in any of the other weakly expressing neurons (data not shown). The measured signal then comes principally from the AVA neurons but could also have a component arising from the AVE neurons, which are also supposed to control backward movement and to be strongly connected to the AVA neurons.

For yc3.60 expression in PLM interneurons, a P*mec-4*::yc3.60 plasmid was made by a LR reaction in the Multisite Gateway System (Invitrogen) from the following three plasmids and pDEST R4-R3. A 1 kb upstream sequence of the *mec-4* gene, the yc3.60 gene and 3′-UTR of the *unc-54* gene were amplified and cloned into pDONR P4-P1R, pDONR 221 and pDONR P2R-P3, respectively by BP reactions. The P*mec-4*::yc3.60 plasmid was microinjected to N2 animals (Evans, 2006) that were then crossed into *lite-1(ce314)*. Pmec-4 promotes expression in the six touch receptors. However, as these cells are far apart, the PLM neurons were identified and tracked unambiguously at 20× magnification.

We used *lite-1* mutants in our experiment because they are insensitive to blue light (Edwards et al., 2008). We could then minimize the behavioral perturbation induced by the fluorescence excitation illumination. Ratiometric calcium imaging using cameleon proteins was chosen in order to lower noise due to the movement of the worm. Movement artifacts are actually correlated in the CFP and the citrine channels and are then filtered out when taking the ratio of the two channels to calculate the FRET signal.

Nematodes were cultured on OP50 bacterial lawns on nematode growth medium (NGM) plates at 20 °C. We picked L4s the day before the experiment and performed all the experiments using young adults.

2.2. Imaging system

For the imaging system, we used an automated *x*–*y* stage (Marzhauser, speed 10 mm/s) holding a large 90 mm open NGM-agar plate upside-down (Fig. 1A). The plate could be seeded with OP50 bacteria. We illuminated and recorded fluorescence images of the worm's neuron under the plate through a Nikon 20× objective (working distance 5 mm, numerical aperture 0.45). The 440 nm illumination was provided by a mercury lamp (Prior) through standard excitation filters (Chroma) and an optical fiber. The fluorescence image was split by a two-wavelength splitting system (Cairn technology) equipped with dichroics and emission filters (Chroma). The two images (at 480 and 540 nm) were then projected on the two halves of a megapixel EMCCD camera (Andor Ixon). Above the plate a CCD camera (Prosilica, 1000 × 1300 pixels) was used to image the behavior of the worm through the agar with a 3× objective (Edmund). The field depth of this objective was large enough to prevent defocusing during the experiment. Optomechanical parts (custom-made and Thorlabs) were used to mount the optical parts in a compact microscopy system optimizing imaging efficiency.

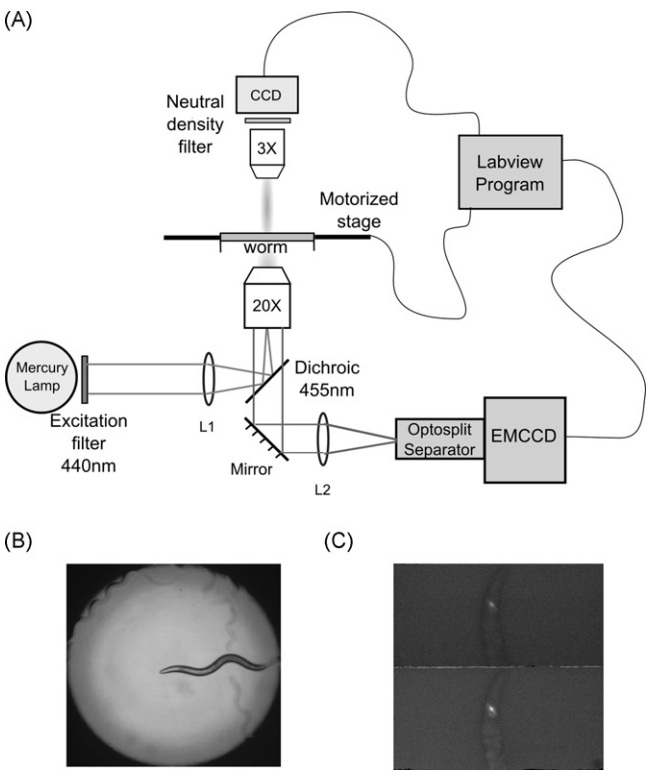

Fig. 1. Tracking system. (A) Schematic diagram of the imaging system. (B) Full worm image for behavior analysis. (C) Fluorescence ratiometric calcium imaging of AVA neurons (up: 480 nm emission, down: 540 nm emission).

2.3. Image acquisition and analysis

We transferred a worm to the experiment plate a few minutes before the experiment. The neuron of interest was then manually centered in the fluorescent image and image acquisition was started by the user. The motorized stage and both cameras were controlled via a custom-written Labview program. This program consists of a succession of 150 ms-long loops. During each loop one image is recorded on each camera, the neuron position is determined as the pixel of maximum intensity in the 540 nm emission image and the motorized stage is then moved in order to bring back the neuron to its original position in the 540 nm image. The *x*–*y* trajectory of the imaged neuron is also recorded during the whole experiment as the position of the stage at the end of each 150 ms loop.

Exposure time of the fluorescence images was set to 30 ms to avoid smearing due to the movement of the worm and the images were binned in 2 × 2 pixels. As we programmed the tracking system to keep the pixel of maximum intensity in the center of the 540 nm image via displacement of the stage at each 150 ms step, the neuron of interest was then mostly kept in the same point of the field of view of the camera during the whole experiment. Worms move mostly on an *x*–*y* plane, but slow *z*-defocusing due to unevenness of the plate was controlled by occasional manual refocusing (∼once per minute).

Fluorescence images at each of both emission wavelengths were analyzed using a MATLAB routine by averaging the 100 most intense pixels in each image. Signal to noise was high enough to confirm that these pixels actually corresponded to the neuron of interest. This method diminishes artifacts linked to slight defocus-

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