

A LED Light for Photo-Inducible Cell Ablation by miniSOG

Xiao Li

Hubei University of Technology

Bo Kuai (✉ qiao4424577dixi@163.com)

Hubei University of Technology <https://orcid.org/0000-0003-2281-100X>

Bin Yu

Huazhong University of Science and Technology

Xikai Tu

Hubei University of Technology

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Abstract

Photosensitizer MiniSOG (mini-singlet oxygen generator), a green fluorescent flavoprotein, can be used to kill cells in a spatially and temporally regulated manner according to a chosen promoter when it is fused with the target cells. However, it needs 470nm blue light excitation to release reactive oxygen species (ROS) to work. In the past, researchers usually used fluorescence microscope to generate light, which wasted the lab resources and influenced other experiments. Here, we developed a homemade light emitting diode (LED) light for photo-inducible cell ablation by photosensitizers. The LED light consisted of six groups of lamps, and it could illuminate a plurality of culture dish drawers simultaneously with different light intensities. In order to watch the efficacy of cell ablation, confocal imaging, behavioral, calcium imaging and electrophysiology experiments have been performed on *C. elegans*. Most of neurons related with forward movements have been ablated after dozens of minutes light irradiation. The worms can only move backward with wriggled body, and the calcium oscillation increases in reverse movement motor neurons. The whole frequency and amplitude of miniature postsynaptic currents (PSCs) in body wall muscle decrease because of death of some neurons. These experimental results verified that the LED light was useful and convenient. This study provides a method for high-throughput cell ablation experiments.

Introduction

In order to identify neuronal functions in multicellular organisms, we need to select killing groups of neurons in a precise manner. At present, the commonly used methods of killing cells mainly include physical methods, chemical blocking and genetic means. Physical methods usually use laser to irradiate cells, which leads to cell death through the thermal effect (Frenster et al. 2020). Limited by the size of the laser spot, it is generally unable to kill cells accurately. Chemical blocking, such as tetrodotoxin, is too toxic to cause safety accidents. The genetic method is to transfer the genes that can lead to apoptosis into specific cells.

The methods expressing the enzymes related to the programmed cell-death are not universal, because it varies with the cell types and physiological states (Bedoui et al. 2020). Recently, some conditional cell ablation methods have been developed. For example, transgenic expression of *Escherichia coli* nitrofurantoin or ion channel in zebrafish can specifically ablate cells of interest (Mruk et al. 2020). Targeted expression of the conserved genes *Arf1* in mice cancer cells can cause inducible cell killing by mitochondrial defects with injection of tamoxifen (Wang et al. 2020). Clinically, photosensitizing reagents can also be used to kill malignant cells in photodynamic therapy. Upon light excitation, the reactive oxygen species (ROS) will be released from photosensitizers, which will cause cell death according to the proteins accumulation from the intermembrane space of mitochondria (Orrenius et al. 2003).

The target cells can be located by attaching the mark proteins to the deoxyribonucleic Acid (DNA) sequence, so these photosensitizers can be encoded to provide a special method to decode cellular function in a spatially and temporally regulated manner (McFarland et al. 2020). Generally speaking, light with wavelength more than 800 nm cannot excite singlet state oxygen, so the strong absorption peaks of photosensitizers are usually less than 800 nm (Abrahamse and Hamblin 2016). The first fully genetically encoded photosensitizer is named KillerRed, which is a red fluorescent protein (Bulina et al. 2006). When KillerRed protein is irradiated with 520–590 nm green-orange light, ROS is ejected, which will ablate cells lying in membranes (Liao et al. 2014), lysosomes (Serebrovskaya et al. 2013), mitochondria (X. Li et al. 2019) and nucleus (Yuan et al. 2019). As an optogenetic tool, KillerRed has extensively been used to kill specific neurons in *Caenorhabditis elegans* (Lee et al. 2014), in transgenic zebrafish (Buckley et al. 2017), in *Xenopus laevis* embryos (Riani et al. 2021) and in the mice (Cao et al. 2020). However, KillerRed has not yet become a universal tool because of its dimerization tendency, which makes it difficult to fuse with proteins of interest (Takemoto et al. 2013). In addition, KillerRed is type I photosensitizer, which forms ROS need a series of intermediate reactants including radical cation, radical anion, hydrogen peroxide, etc (Vegh et al. 2011).

Recent studies demonstrated that flavin mononucleotide based fluorescent proteins could also be achieved from bacterial and plant species by lightoxygen-voltage-sensing (LOV) domains gene mutation (Chapman et al. 2008; Drepper et al. 2007). By this method, a protein named miniSOG is developed. As a fluorescent flavoprotein, miniSOG consists of 106 amino acids engineered from *Arabidopsis phototropin 2* (Qi et al. 2012; Shu et al. 2011b). Illumination of miniSOG with blue light can generate sufficient singlet oxygen, which will generate ROS to ablate cells. Because miniSOG is encoded by one gene, and it can be easily fused to many specific functional proteins, which localize accurately in mammalian cells (Gao et al. 2020), intact nematodes (S. Gao et al. 2018), and *drosophila* (Ng et al. 2016), without the assistance from the exogenous ligands and probes (Souslova et al. 2017). When fused to a certain protein, miniSOG can be used to inactivate a protein molecule (Lin et al. 2013) or kill a whole cell when targeted to mitochondria or lysosomes (Y. Li and Cui 2020a) or plasma membrane or fused with the histone H2B (Yuzhakova et al. 2020). Furthermore, miniSOG is a promising photosensitizer, which can reduce levels of flavin mononucleotide in tumors with low penetrating blue light (Y. Li and Cui 2020b; Shcherbakova et al. 2015).

MiniSOG can cause rapid and effective death of cells in a spatially and temporally regulated manner. As a promising photosensitizer, but it needs 470nm blue light excitation to release ROS. In the past researches, the blue light was usually generated by fluorescence microscope (Bulina et al. 2006; Qi et al. 2012; Shu et al. 2011), which wasted the lab resources and influenced other experiments. Here, we developed a LED light for photo-inducible cell ablation by photosensitizers and verified that it is useful and convenient according to fluorescent flavoprotein miniSOG in model organism *Caenorhabditis elegans*.

Materials And Methods

Principles of miniSOG

As an engineered fluorescent protein, miniSOG will generate singlet oxygen by blue light illumination. Under blue light irradiation, miniSOG absorbs light energy and makes it transition from the ground state (S_0) to the first excited state (S_1). S_1 is excited to the second excited state (S_2) through internal transition energy. Based on S_2 , miniSOG is excited into a singlet state. The excited singlet state experiences internal energy transition and forms a more stable excited triplet state. The triplet state miniSOG can also decay back to the ground state by emitting a phosphorescent photon. This energy transfer step leads to the formation of singlet oxygen. This photochemical reaction process finally forms ROS, which destroys the amino acids, fats and unsaturated fatty acids in cells, and then causes cell death. The reaction is commonly known as a Type II photochemical process (Abrahamse and Hamblin 2016), which generate ROS more easily than Type I photosensitizer. Furthermore, miniSOG can kill neurons rapidly and effectively without detectable damages to surrounding tissues, and it is also reported ablating neurons at different developmental stages (Qi et al. 2012).

Constructs and molecular biology

The strain *hpls603*, *hpls459*, *hpls603; hpls459* (Table 1) were used to detect neuronal activity in this paper. The construction method of miniSOG nematode strain was described in the previous research (Kawano et al., 2011). Briefly, as is shown in Fig. 1: firstly, the DNA fragment included target cell promoter, mito-miniSOG and marker protein, which was achieved by the coupled reaction. Then the DNA fragment was microinjected into the L4 stage nematode. Finally, the miniSOG nematode strain could be produced by passage culture. Three kinds of promoters used in this paper were from N2 *C. elegans* genomic DNA, including 5.1 kb Pnmr-1, 2.5 kb Punc-4, and 0.86 kb Plgc-55B genomic sequence with ATG start codon. The pnmr-1 used in this paper excludes the 2 kb internal fragment encoding *cex-1*, which interferes with the expression of the reporter gene. All *C. elegans* are cultured on standard nematode growth medium plates seeded with OP50 and are maintained at 22 °C incubators.

Fabrication of LED light

As is shown in the Fig. 2, the homemade LED light includes control box, lamp box, light-emitting part, power supply, switch and controller. Switch and controller are fastened in the control box. Culture dishes, shelf, little drawers, transparent heat insulation plate and LED lamp group are put in the lamp box. This homemade LED light has some advantages. First, a plurality of culture dish drawers can be inserted into the shelf of different heights, and the drawer handle is convenient to be arranged outside the culture dish drawer. Second, electric fans, radiators and heat shields are installed to ensure that the worms will not be heated to death. Third, there are six groups of lamps, and each group of lamps is composed of six 1W lamp beads, which can emit 470 nm blue light. More information can be found in Chinese patents (No. CN201910801243.8).

Neuron ablation

All members of motor neurons (MNs) and premotor interneurons (INs) were performed ablation on the plate using the homemade LED box. Worms were put on the standard NGM culture plates with a transparent lid, which could prevent dust in the air from entering into the medium. Then these whole plates with worms were illuminated under the homemade 470 nm blue LED light box for 30-45 minutes. Ablation was performed when the animals grow up to L1 or L2 stage. Later L4 stage animals were recorded for behavioral or calcium imaging analyses. Day 1 animals were recorded for behavioral analyses. Before the behavioral and calcium activity detection, the animals were examined for the RFP signals under the microscope.

Confocal Imaging

The later L4 stage *hpls603* nematode were anesthetized by 2.5 mM levamisole (Sigma-Aldrich) during M9 buffer. Fluorescence signals were captured when the worms alive by a Plan-Apochromatic 60×objective on a confocal microscope (FV3000, Olympus). Ten animals were recorded both in the control group (without the ablation of premotor INs and B-MNs) and ablated group (with the ablation of premotor INs and B-MNs) in the same conditions.

Behavioral analysis

The day 1 stage *hpls603* worms, maintained in standard culture conditions, was transferred to a 60 mm imaging plate seeded with a thin layer of OP50 with or without neurons ablation. Then these crawling worms were recorded for 3 minutes by a stereoscopic fluorescence microscope (Axio Zoom V16, Zeiss) equipped with a digital camera (acA2500-60um, Basler). All videos were captured with a 10X objective, at 10 frames every second. The worm images were skeletonized and divided into 33 segments (Fig. 4 & Fig. 5) by in-house written MATLAB scripts. In addition, we tracked the mid-point of the animals and calculated the velocity and direction of the animal's movement between each frame. Finally, the curvatures of the whole worm, the angle between three joint points defined as the curvature of the middle point, were calculated and shown as color map(S. Gao et al. 2018).

Calcium imaging

DA9 MN activity recording was carried out as previous paper(S. Gao et al. 2018). In the ablated group, the worms were illuminated by blue light LED for 40 min in L2 stage and recorded after 20-24h. These worms (*hpls603;hpls459*) of an integrated strain expressing GCaMP6 in body-wall muscle cells were glued on a 2% agarose pad on a slide, suspended in the M9 buffer and imaged with a 60X objective CCD camera at 100 ms per frame. Data were collected by MicroManager and analyzed by ImageJ. The animals (*hpls603;hpls459*) of the control group weren't illuminated by blue light LED and were carried out the same operation like the ablated group.

Electrophysiology

All electrophysiological experiments were performed with one-day-old adult *C. elegans*. The dissection and recording protocols were the same as in previous reports(S. Gao and Zhen 2011). Before the formal experiment, glass coverslips

were coated by Sylgard 184 Silicone Elastomer (Dow Corning). The pipet solution and bath solution were prepared. The pipet solution includes (in mM): K-gluconate 115; KCl 25; CaCl₂ 0.1; MgCl₂ 5; BAPTA 1; HEPES 10; Na₂ATP 5; Na₂GTP 0.5; cAMP 0.5; cGMP 0.5, pH7.2 with KOH, ~320 mOsm. cAMP and cGMP were included to maintain the activity and longevity of the preparation. The bath solution contains (in mM): NaCl 150; KCl 5; CaCl₂ 5; MgCl₂ 1; glucose 10; sucrose 5; HEPES 15, pH7.3 with NaOH, ~330mOsm. Then worms were immobilized to the silicone surface by tissue adhesive glue (Histoacryl Blue, Braun) in M9 buffer. The cuticle on one side of the nematode was then cut to expose the internal organs. After the internal organs were sucked away with glass tube, the cuticle edge was gently glued down by WORMGLU (GluStitch Inc.) to expose the neuromuscular system. Body wall muscle cells were patched using 4–10 MΩ-resistant borosilicate pipettes (1B100F-4, World Precision Instruments). Pipettes were pulled by micropipette puller (P-1000, Sutter) and fire-polished by microforge MF-830 (Narishige). Membrane currents were recorded in the whole-cell configuration by EPC9 amplifier (HEKA, Germany), using the pulse and processed with Igor 6 (WaveMetrics) and Clampfit 10 software (Axon Instruments, Molecular Devices). Data were digitized at 10–20 kHz and filtered at 2.6 kHz. Chemicals were obtained from Sigma unless stated otherwise. Experiments were performed at room temperature.

Results And Discussion

Light intensity measurement

In order to get the right light intensity to kill neurons, the light intensity of each group of LED lamps was measured by the optical power meter (PSL-PM02) in five different positions (four corners and middle) on the bottom floor. The blue light intensity on the NGM plate was around 3.5-4.5 mW/cm^2 (Table 2). L2 stage worms were illuminated for 40 min, almost all premotor interneuron (IN) and B motor neurons (B-MN) could be ablated on the bottom floor (Fig. 3). Because the LED light consisted of six groups of lamps and the culture dish drawers have different height, it can provide different light intensities to worms simultaneously (Table 3). On the top floor, neurons can be ablated more quickly. However, the top floor is too close to light source, some worms don't look active after illumination because of the thermal effect from the high-intensity light source. In the following confocal imaging, behavioral, calcium imaging and electrophysiology experiments, all worms were exposed under the blue LED light on the bottom floor.

Locomotion analyses

There are five MN classes of neurons in *C. elegans*: A, B, D, AS, and VC. The A motor neurons (A-MN) classes contribute to the vast majority of reverse movements, and the B motor neurons (B-MN) classes contribute to the vast majority of forwarding movements. Both of them are cholinergic and excitatory, potentiating muscle contraction, while D motor neurons are GABAergic and inhibitory, assisting muscle relaxation. Some interneurons, such as AVA, AVB, PVC, etc., make electric or chemical synapses with A-MN and B-MN to partner with these locomotion. Each class is divided into subgroups that innervate dorsal or ventral muscles. In order to watch the efficacy of LED light, the miniSOG is targeted to B-MN and IN by specific promoters (Table 1). As is shown in Fig. 4, the worms can freely move forward and backward without illuminated by blue light. However, after 40 min exposed under the homemade 470 nm blue LED light box in L2 stage, the worms can only move backward (Fig. 5).

DA9 motor neuron activity recording

In order to assure that the B neurons are ablated by blue light, the DA9 motor neuron calcium signals are detected. The genetic calcium sensor GCaMP6s are used for neuronal calcium imaging. The GCaMP6s sequence is codon-optimized for expression in *hpls459* strain, then we get the *hpls603; hpls459* strain worms by cross-breeding with *hpls603* strain (Table 1). GCaMP is fused with Cherry at the C-terminus, so we can measure GFP and RFP signals simultaneously. Neurons are detected automatically in each volume by applying a circular region of interest (ROI) through the difference of Gaussian detector and then Kalman filter by ImageJ software are used to track them. For each neuron in each volume of GCaMP or

RFP channel, all the pixels within the ROI of a certain neuron are averaged to generate a single value F_g or F_r representing the fluorescence intensity of this neuron in each channel. The fluorescence intensity of DA9 motor neuron is normalized by the formula: $[Ca^{2+}] = (F - F_0) / F_0$, where $F = F_g / F_r$ is the ratio of GCaMP fluorescence to RFP fluorescence and F_0 is the neuron-specific baseline being the average of the lowest 100 values. When the worms want to make a backward movement, the motor neuron DA9 will generate calcium oscillation. As is shown in Fig. 6, there are four spikes in the ablated group but no spike in control group. Because the worms in the ablated group could only move backward, the DA9 calcium oscillation of the animals in the ablated group is stronger than control group.

Miniature PSCs in body wall muscle

In accordance with the above experiments, we nearly assure that the LED light can work well and the INs and B-MNs of the *C. elegans* *hpls603*, *hpls459*, *hpls603*; *hpls459* are ablated by blue light. We know that worms make forward or reverse movements by dorsal or ventral muscles, and INs and B-MNs innervate with muscles. Whether the muscle activities change or not when these INs and B-MNs are killed remains unclear. The whole cell patch clamp has been performed on *hpls603* strains. We find that the frequency and amplitude of miniature PSCs in body wall muscle decrease when the INs and B-MNs are ablated (Fig. 7).

Conclusions

Photosensitizer MiniSOG is used in neurobiology more and more frequently, because it can selectively kill groups of neurons in a precise manner. However, there are no special lamps on the market to generate high-power blue light for relevant experiments. In the past experiments, researchers usually used fluorescence microscope to generate light, which was expensive and made other experiments impossible. In this paper, we develop a homemade LED light for photo-inducible cell ablation by photosensitizers. The LED light contains six groups of lamps, and it could illuminate a plurality of culture dish drawers simultaneously with different light intensities. Some commonly used biological experiments are used to verify the effectiveness of the lamp. The *C. elegans* are exposed under blue light for about 40 minutes, almost all premotor IN/B-MN have been ablated. The worms can only move backward, and the calcium oscillation increases in reverse movement motor neurons. The whole frequency and amplitude of miniature PSCs in body wall muscle decrease. Therefore, the LED light is verified useful and convenient through these experiments. Because of its low cost, the lamp can be used in high-throughput cells ablation experiments.

Declarations

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Availability of data and material

The data used to support the findings of this study are included within the article.

Competing interests

The authors have declared that no competing interests exist.

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Tables

Table 1
A list of transgenic arrays that were used in this research

| Purpose | Plasmid | Description | Host Strain | Transgene | Strain |
|---|---------------------|---|----------------------|-------------------------|--------|
| Neuron Ablation & Behavior Analyses & Electrophysiology | pJH3449/ | <i>Plgc-55B/Pnmr-1/Pacr-5::</i> | <i>lin-15/Lin-15</i> | <i>hpls603</i> | ZM9176 |
| | pJH3453/ | tomm20-miniSOG-SL2::BFP | | | |
| | pJH3456 | (all premotor IN/B-MN/others) | | | |
| Ca ²⁺ imaging | pJH3137 | <i>Punc-4::GCaMP6::RFP</i> (A-MNs/others) | <i>lin-15/Lin-15</i> | <i>hpls459</i> | ZM8428 |
| Neuron Ablation & Ca ²⁺ imaging | pJH3449/ | <i>hpls603; hpls459</i> (all premotor IN/B-MN ablation with A-MN Ca ²⁺ + imaging) | <i>lin-15/Lin-15</i> | <i>hpls603; hpls459</i> | ZM9228 |
| | pJH3453/ | | | | |
| | pJH3456/ pJH3137 | | | | |

Table 2
The light intensity of the six LED lamps

| No. | Middle(<i>mW/cm²</i>) | Top left(<i>mW/cm²</i>) | Top right(<i>mW/cm²</i>) | Bottom left(<i>mW/cm²</i>) | Bottom right(<i>mW/cm²</i>) | Average(<i>mW/cm²</i>) |
|-----|------------------------------------|--------------------------------------|---------------------------------------|---|--|-------------------------------------|
| 1 | 4.2 | 3.8 | 3.4 | 3.6 | 3.8 | 3.8 ± 0.3 |
| 2 | 5.1 | 4.7 | 4.2 | 4.6 | 4.3 | 4.6 ± 0.4 |
| 3 | 4.2 | 3.9 | 3.9 | 4.0 | 4.1 | 4.0 ± 0.1 |
| 4 | 4.4 | 4.3 | 3.6 | 3.8 | 3.7 | 4.0 ± 0.4 |
| 5 | 5.1 | 4.9 | 4.3 | 4.1 | 4.3 | 4.5 ± 0.4 |
| 6 | 4.5 | 4.8 | 4.3 | 3.4 | 3.7 | 4.1 ± 0.6 |

Table 3
The light intensity of each floor

| No. | First floor(<i>mW/cm²</i>) | Second floor(<i>mW/cm²</i>) | Third floor(<i>mW/cm²</i>) | Fourth floor(<i>mW/cm²</i>) | Fifth floor (<i>mW/cm²</i>) | Sixth floor (<i>mW/cm²</i>) |
|-----|---|--|---|--|--|--|
| 1 | 7.5 ± 0.3 | 6.7 ± 0.1 | 5.8 ± 0.4 | 4.9 ± 0.3 | 4.3 ± 0.2 | 3.8 ± 0.3 |
| 2 | 7.1 ± 0.4 | 6.6 ± 0.3 | 6.3 ± 0.2 | 5.7 ± 0.2 | 5.2 ± 0.3 | 4.6 ± 0.4 |
| 3 | 7.3 ± 0.2 | 6.5 ± 0.4 | 5.7 ± 0.2 | 5.2 ± 0.3 | 4.7 ± 0.2 | 4.0 ± 0.1 |
| 4 | 7.4 ± 0.1 | 6.6 ± 0.2 | 5.8 ± 0.2 | 5.1 ± 0.2 | 4.4 ± 0.2 | 4.0 ± 0.4 |
| 5 | 8.0 ± 0.4 | 6.5 ± 0.2 | 6.0 ± 0.1 | 5.3 ± 0.3 | 4.9 ± 0.2 | 4.5 ± 0.4 |
| 6 | 7.2 ± 0.5 | 6.1 ± 0.2 | 5.4 ± 0.3 | 4.8 ± 0.3 | 4.6 ± 0.1 | 4.1 ± 0.6 |

Figures

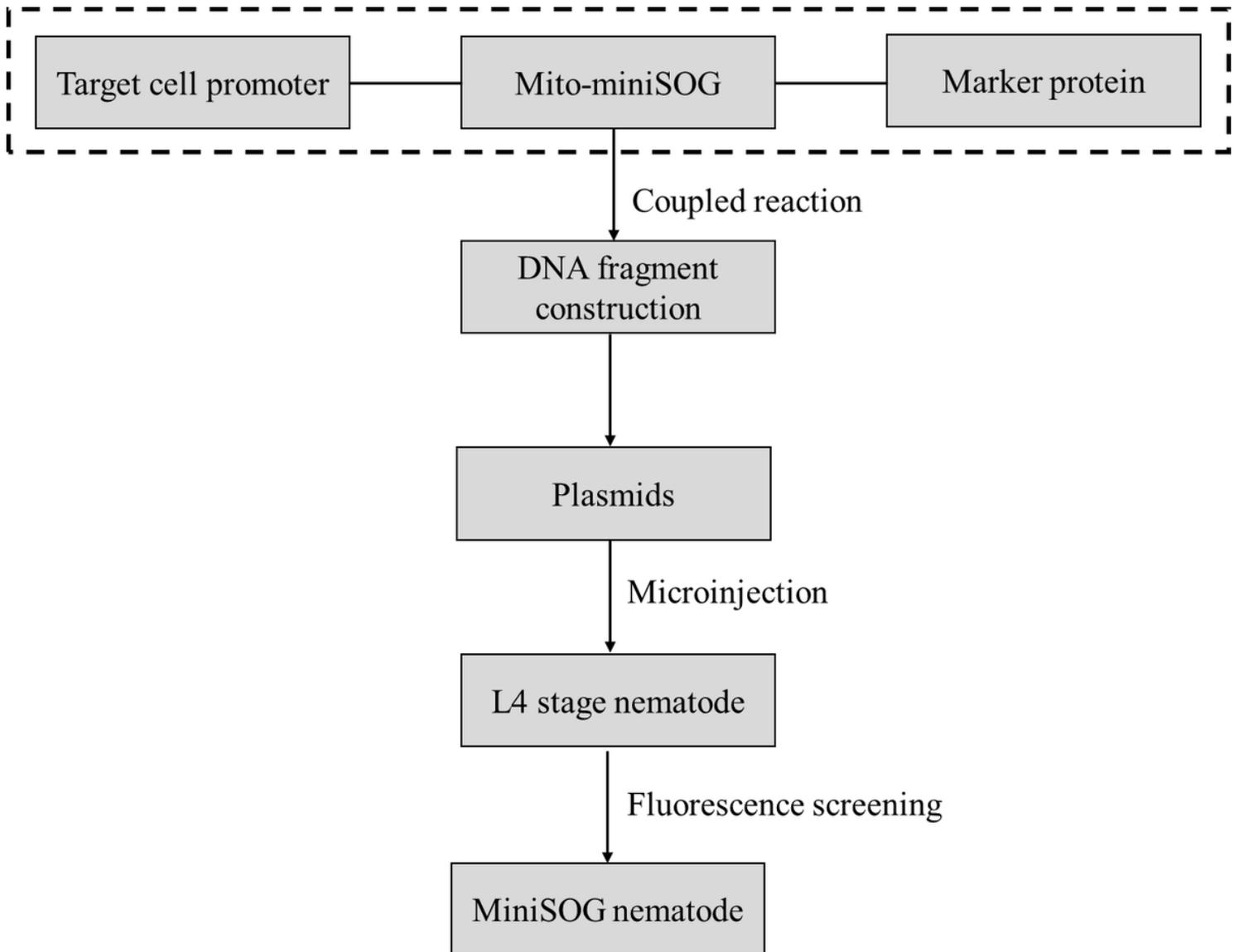


Figure 1

Construction of miniSOG nematode strain.

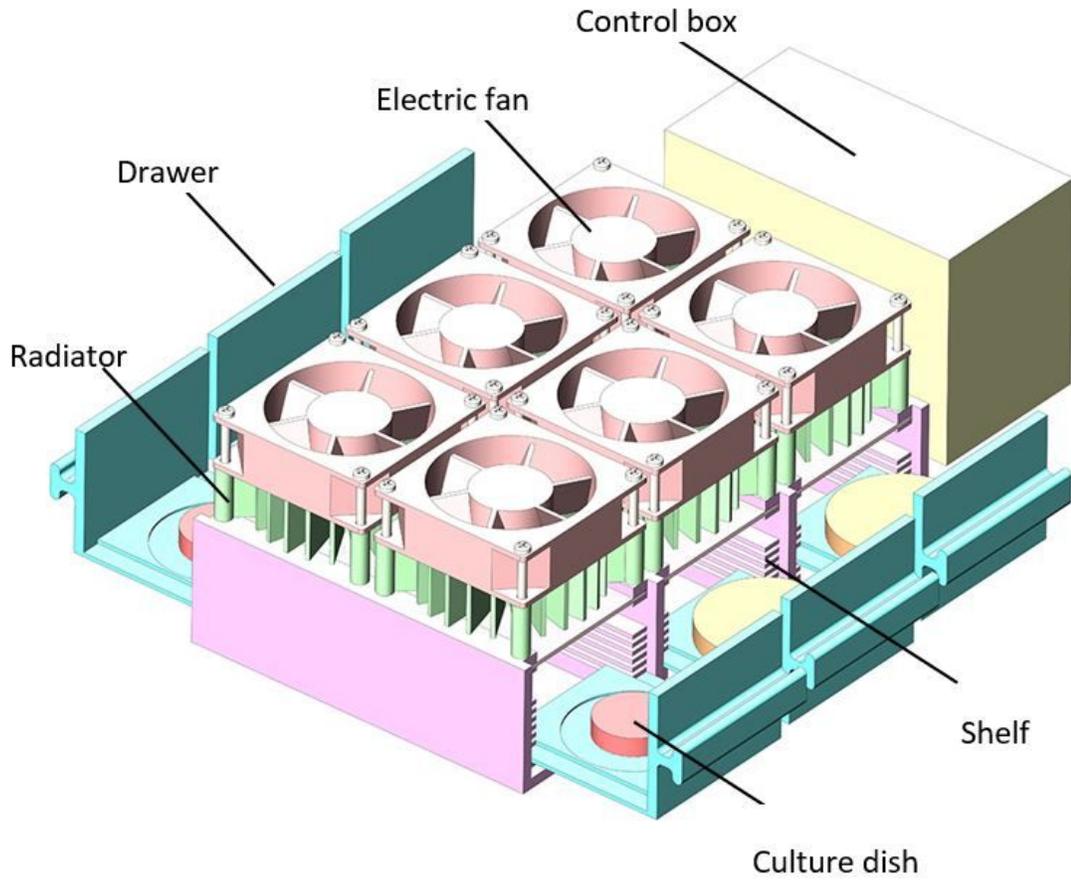


Figure 2

The diagram of the LED light, consist of six groups of lamps, and each group of lamps is composed of six 1W lamp beads.

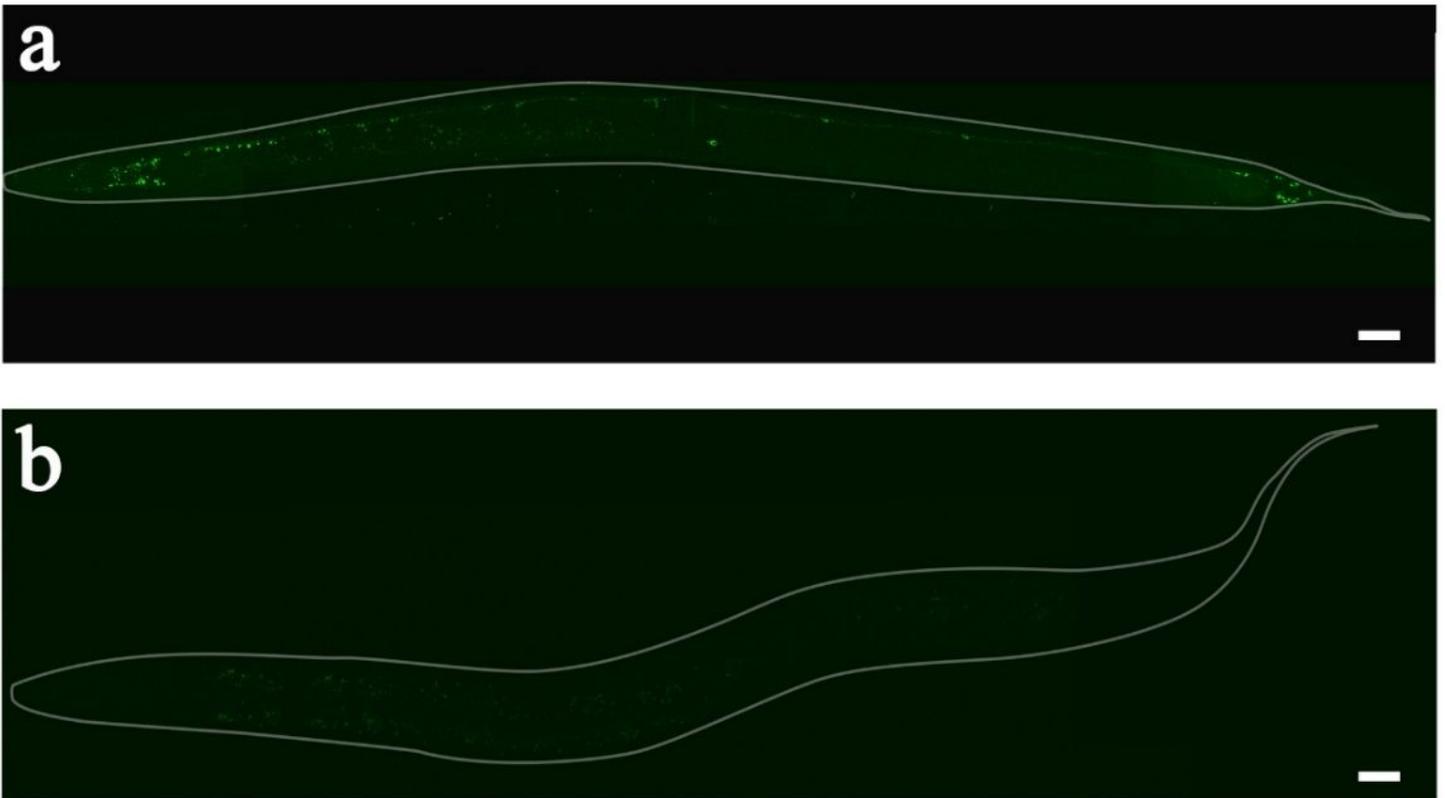


Figure 3

Confocal micrographs showing the dorsal nerve cord of *hpls603* mutant animals without (a) or with (b) the ablation of neurons. Scale bar: 50 μ m

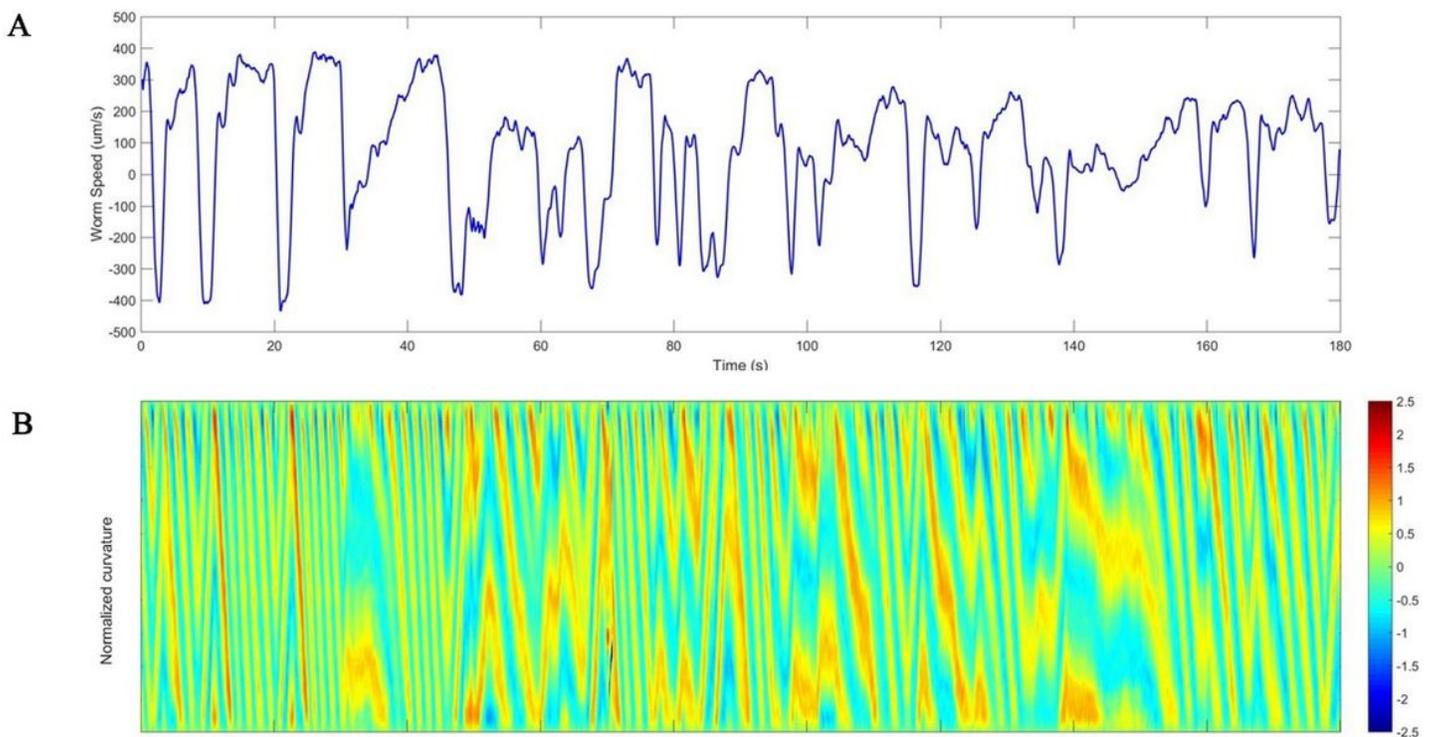


Figure 4

The locomotor phenotypes of animals without the ablation of neurons. An example velocity profile of an animal(A) and representative body curvature kymographs from head to tail(B).

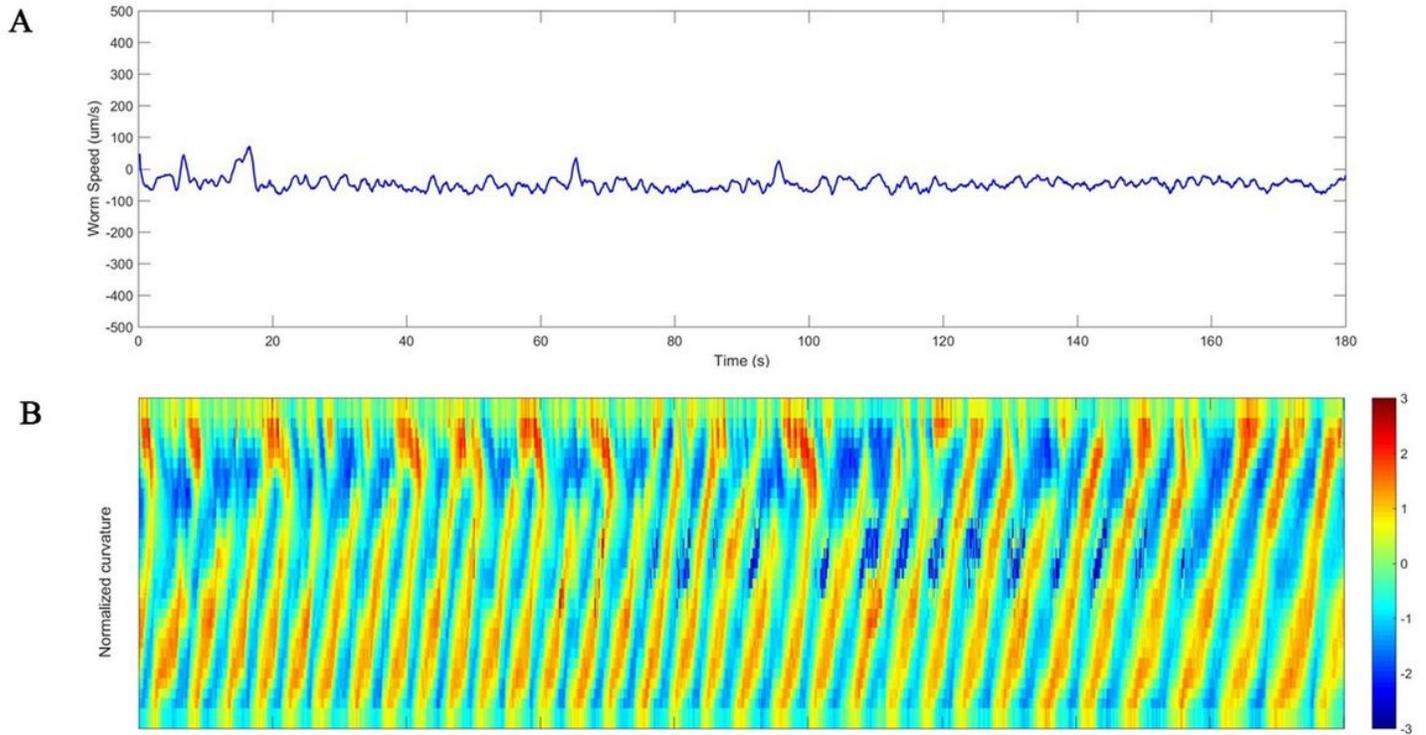


Figure 5

The locomotor phenotypes of animals upon the ablation of neurons. An example velocity profile of an animal(A) and representative body curvature kymographs from head to tail(B).

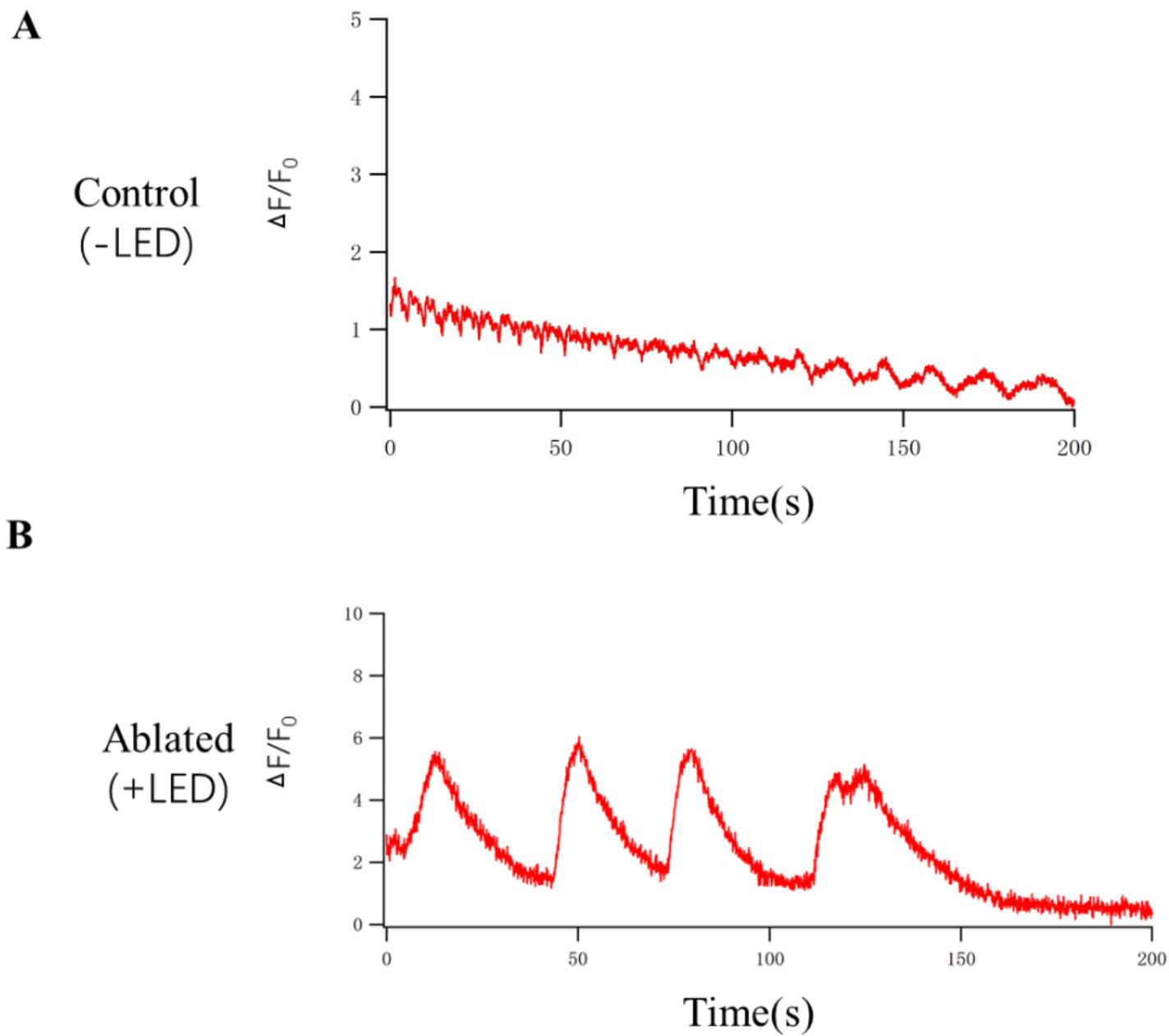


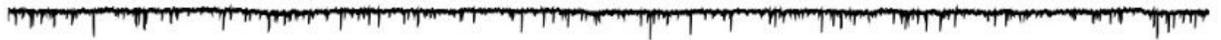
Figure 6

DA9 calcium oscillation of the animals without (Control, -LED) and with (Ablated, +LED) the ablation of premotor INs and B-MNs.

**Control
(-LED)**



**Ablated
(+LED)**



30 pA
1 S

Figure 7

Spontaneous mini-postsynaptic currents (mPSCs) recorded at -60 mV without (Control, -LED) and with (Ablated, +LED) the ablation of premotor INs and B-MNs.