LEBRA FREE-ELECTRON LASER ELICITS ELECTRICAL SPIKES FROM THE RETINA AND OPTIC NERVE OF THE SLUG LIMAX VALENTIANUS

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Abstract

Since 2001, the Laboratory for Electron Beam Research and Application (LEBRA) has been providing tunable free-electron lasers (FELs) encompassing the near-infrared (IR) region and part of the mid-IR region (0.9–6.5 μm), and generating visible wavelengths up to 400 nm by means of nonlinear optical crystals. We used LEBRA-FEL to irradiate the retina of slugs (*Limax valentianus*), and determined which FEL wavelengths generate electrical spikes from a retina-optic nerve preparation. In the dark-adapted state, blue FEL light (peak wavelength: 470 nm) efficiently elicited electrical spikes from the retina. The results are consistent with a previous study where a xenon arc lamp with interference filters was used to produce monochromatic visible light. The retina produced detectable electrical spikes when repeatedly irradiated with pulsed FEL below 5 Hz. We extended the wavelengths to the near-IR regions (0.8–2.5 μm); however, we detected no electrical response.

INTRODUCTION

Free electron lasers (FELs), such as the one developed by the Laboratory for Electron Beam Research and Application (LEBRA), produce high-energy, tunable pulsed radiation (wavelength range: 0.4–6.5 μm), which is ideal as a radiation source for investigating photochemical reactions in living organisms. Previously, we verified that visible FELs can control the germination of lettuce seeds, a well-known photochemical reaction in plants that is promoted by red light (660 nm) and inhibited by far-red light (740 nm) [1].

In this work, we investigated the efficiency of FEL for photic stimulation in an electrophysiological study. The eye (or retina) and optic nerve of the slug *Limax valentianus* is particularly useful for this purpose because the retina and optic nerve can be readily dissected free from the amputated eyestalk of the adult animal. The dissected retina-optic nerve preparation can be used for more than 12 h in a plastic chamber filled with snail Ringer solution [2]. Furthermore, the retina is big enough to be illuminated easily by the LEBRA-FEL micro-irradiation system, which contains a quartz fiber, and the optic nerve is large enough for signals to be recorded using a conventional capillary suction electrode. In the dark-adapted state, FEL irradiation experiments show that the peak wavelength of the spectral sensitivity curve is 470 nm.

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MATERIALS AND METHODS

Animals

L. valentianus slugs, which are terrestrial and nocturnal, were collected locally and maintained for at least seven generations. These animals were kept under dark, wet conditions in plastic boxes placed in an incubator (SLC25A, Mitsubishi-Engineering Co., Japan) at 19.0 °C.

Dissection

Adult specimens (2.1–2.4 g) were used. Each animal was anesthetized with an injection of 500 μL of snail Ringer's solution [2] containing 50 mM $MgCl_2$. The snail Ringer's solution was a modification of Ramsey's Ringer solution [3]. The retina (about 0.25 mm diameter) and optic nerve (about 40 μm diameter) were isolated by micro-dissection in snail Ringer's solution. The optic nerve was removed free from surrounding tissue, and cut apart from the base of the retina. The retina-optic nerve preparations of both eyes were fixed with small tungsten wire pins (3 mm long, 0.1 mm diameter) on a sloping transparent sheet of silicon in fresh Ringer's solution in a small plastic chamber. The preparations in Ringer's solution were used in experiments for 1 day.

FEL Stimulation

In an earlier experiment [2], a xenon arc lamp (500 W) with a series of interference filters was used for producing monochromatic light. Here, we used the LEBRA-FEL as a radiation source, the beam specifications of which are detailed elsewhere [4,5]. LEBRA-FEL can generate sharp peak emissions of high-energy, high-coherency, tunable pulsed radiation from 0.4–6.5 μm, and narrow spectral bandwidths. LEBRA can generate 4 or 5 wavelengths for the irradiation experiments over a day.

The setup of the LEBRA-FEL micro-irradiation systems has been described in our previous study [1]. A quartz fiber (0.6 mm diameter; Edmund Optics, Tokyo, Japan) was installed on a holder (H-7; Narishige Group, Tokyo, Japan) of a micro-manipulator (MMO-220A, Narishige Group), and delivered visible light wavelengths and near-infrared (IR) wavelengths up to 3 μm. A dissection microscope and recording apparatus were placed in a Faraday cage, as shown in Fig. 1.

We used a light-emitting diode (LED) at a constant wavelength of 460 nm (20 $\mu W\ s^{-1}$ at 4.7 V) as a test light that was placed in the FEL delivery path and removed when the FEL was switched on. The intensities of the

light sources, FEL and LED, were measured at the tip of the quartz fiber by power meters (FieldMax-II and OP-2VIS, respectively, Coherent, Inc., Portland, OR) at the beginning and end of experiments. The FEL radiation energies could be reduced by a series of combined neutral density (ND) glass filters (Kenko Tokina Co. Ltd., Tokyo, Japan). In contrast to the LED power, the optical power of the FELs varied between experiments, and the average power was 8.70 μJ/pulse for visible wavelengths (420–710 nm) and 35.10 μJ/pulse for near-IR wavelengths (0.8–2.5 μm).

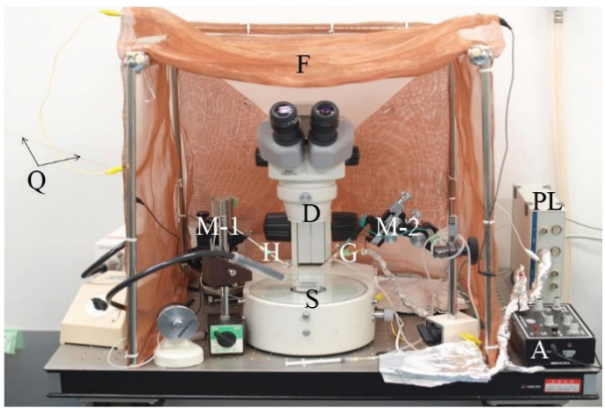

Figure 1: FEL micro-irradiation system. A, amplifier; D, dissection microscope; F, Faraday cage; G, glass capillary suction electrode and reference electrode; H, holder and quartz fiber; M-1, manipulator 1 for irradiation; M-2, manipulator 2 for recording electrodes; PL, data acquisition device (PowerLab 2/26); Q, quartz fiber; S, sample.

Recording and Analysis

Prior to irradiation experiments the retina-optic nerve preparations were fixed on a sloping silicon plate in a small plastic chamber (30 × 60 mm, 8 mm deep). The whole optic nerve bundle was suctioned by the tip of a glass capillary suction electrode at one of the three suction sites (Fig. 2). We used a silver chloride reference electrode consisting of an Ag/AgCl wire (0.25 mm diameter) coiled around the glass capillary suction electrode and immersed in snail Ringer's solution. Using manipulators, a FEL wavelength delivered through the quartz fiber could precisely irradiate the surface of the lens by bringing the tip of the fiber into contact. The retina-optic nerve preparations were irradiated for 1 s at 2 to 5 min intervals in most experiments, as this interval allowed the signal to return to the background discharge as a control.

Electrical signals were fed into a high-input impedance amplifier (DAM80 Differential Amplifier, World Precision Instruments, Inc., Sarasota, FL), which was connected to a data acquisition device (PowerLab 2/26, ADInstruments, Inc., Dunedin, New Zealand; settings: low filter, 1 Hz; high filter, 10 kHz; and gain, 1000). The oscilloscope trace was recorded continuously on a personal computer. Recordings and all irradiation experiments were performed at room temperature (22 °C) in a darkened room. Data analyses were performed with LabChart 7 software (ADInstuments, Inc.).

RESULTS AND DISCUSSION

Recording Site on the Optic Nerve

The recording sites on the optic nerve are shown in Fig. 2 (top left), and are designated *a*, *b*, and *c* according to their proximity to the retina. The results from site *a* gave large, steady electrical spikes when stimulated by the LED test light and the FEL. The suction electrode was fixed firmly to site *a* without losing electrical signals, and was used for the study. Suzuki et al. [2] used a site similar to site *a*, allowing us to compare our results with their previous results.

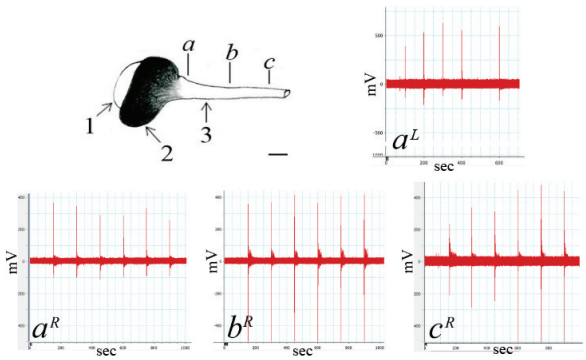

Figure 2: Effect of recording site on the optic nerve. Top left: schematic of the slug's right eye. Labels *a*, *b*, and *c* indicate recording sites. During recording, the eye was held at one of the sites with a glass capillary suction electrode. Signals were recorded at a^R, b^R, c^R, and a^L were obtained from the right eye (^{R}) and the left eye (^{L}), respectively, of the same individual. The stimulus lasted 1 s and was repeated 5 or 6 times at 100 or 200 s intervals. 1: Lens; 2: retina; 3: optic nerve. The scale bar indicates 50 μm.

Comparison of Spike Responses to Continuous Wavelength (LED) and Pulsed Wavelength (LEBRA-FEL)

The patterns of electrical spikes for continuous and pulsed wavelength light are compared in Fig. 3A and B. The patterns are similar, except for two prominent spikes detected in Fig. 3A in response to 2 Hz pulsed light. The first electrical spike was always large compared with the subsequent spikes. Figure 3B also shows a typical example; the two spikes generated by different light intensities demonstrated that the intensity of the electrical spikes was a function of the light source intensity. A 1–2 min interval between irradiation was sufficient for the signal to return to the static background discharge, even for full intensity signals (right side, Fig. 3B). However, when the irradiation continued, the intensity of the electrical spikes hardly decreased over 70 min (data not shown). The electrical responses from the retina-optic nerve of the slugs appeared to arise from complex reactions, as suggested by other studies [6,7].

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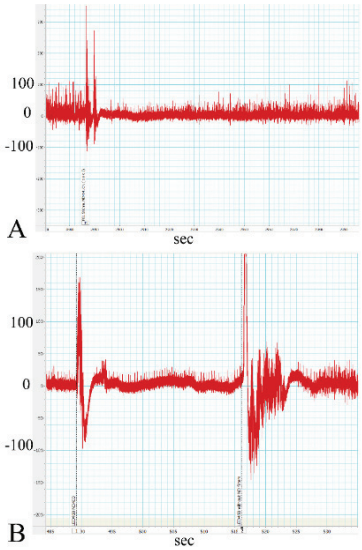

Figure 3: Comparison of electrical spikes between continuous (LED) and pulsed (LEBRA-FEL) irradiation. A: Data obtained from 560 nm LEBRA-FEL irradiation (1/16 intensity = 0.27 μJ/pulse), B; data obtained from LED 460 nm irradiation (left; 1/400 intensity = 0.05 μW s^{-1}, right; full intensity). Stimulus was 1 s in duration.

Spectral Sensitivity Curve

Figure 4 shows that the spectral sensitivity reached a maximum wavelength at about 470 nm, which is close to those of other gastropods, for example, 475 nm in *Helix pomatia* [8] and 480 nm in *L. flavus* [2]. At wavelengths from the near-IR region to 2.5 μm, any electrical spikes would have been too small to detect and would have been masked by the background discharge (Table 1). In contrast, for shorter wavelengths, the electrical spikes were visible up to 420 nm, which is the limit of LEBRA-FEL. Therefore, the slug's eyes responded well to blue light rather than to near-IR, suggesting the presence of only a single visual pigment having peak absorption at around 470 nm. Suzuki et al. [2] reported a shift in the maximum wavelength to 460 nm in response to the light-adapted state of the retina of *L. flavus*. However, we have not yet investigated the light-adapted state for our preparation. Kataoka [9] suggested that *L. flavus* slug eyes are sensitive to IR light, whereas our current data (Table 1) indicate that the eyes of *L. valentianus* are not sensitive to near-IR wavelengths (0.8–2.5 μm). We are currently performing further analysis of our data.

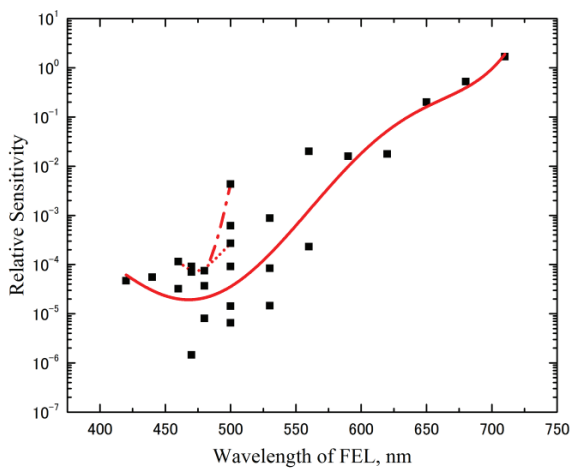

Figure 4: Spectral sensitivity curve of the dark-adapted *L. valentianus* retina-optic nerve as a function of the wavelength. Sensitivity is determined as the smallest amount of FEL energy at each wavelength required to generate electrical spikes slightly larger than background discharge. To examine the minimum radiation energy of each wavelength, a combination of ND glass filters was used. The dotted and dashed lines indicate results obtained from single preparations of the right and left eyes, respectively. The data was fitted with the straight line by OriginPro (OriginLab, Co., Northampton, MA).

Table 1: Slug Retina-Optic Nerve Responses to Near-IR Radiation Stimuli from LEBRA-FEL

Wavelength, μm	Energy, μJ/pulse	Electrical Spike [1]
0.80	5.86	ND [2]
0.90	13.37	ND
1.00	33.25	ND
1.10	12.12	ND
1.25	42.90	ND
1.50	33.54	ND
1.70	90.90	ND
1.90	121.60	ND
2.00	52.60	ND
2.50	33.80	ND

[1]: Results of 10–15 reciprocals.
[2]: Not detected.

Responses to Repetitive Stimulation

Figure 3A indicates that the retina-optic nerve system can elicit two electrical spikes during irradiation for 1 s by LEBRA-FEL (2 Hz). To measure the response of one eye to the frequency of light pulses, we used LEBRA-FEL and LED light sources. Figure 5 shows that the right and left eyes both responded to increasing rates of repetitive stimulation up to 5 Hz with difficulty (Fig. 5C). This rate was defined as the flicker fusion threshold of the slug's eye. To generate higher frequency stimulation, we used an arbitrary/function generator (AFG3052C, Tektronix, Inc., Portland, OR) connected to the 460 nm LED (20 μW s^{-1} at 4.7 V). The flicker fusion threshold was below 4 Hz (data not shown). The different frequencies for the light sources may arise from the different intensity of the peak emission. The flicker fusion threshold of *L. valentianus* was much lower than animals, insects (*Locusta migratoria*, *Glossina morsitans* and *Drosophila hydei*), and birds (*Sturnus vulgaris* and *Columba livia*) that stalk prey, which typically have flicker fusion thresholds of over 100 Hz [10,11]. *L.*

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valentianus can react to frequencies of up to 5 Hz at the higher peak emission energy for the LEBRA-FEL.

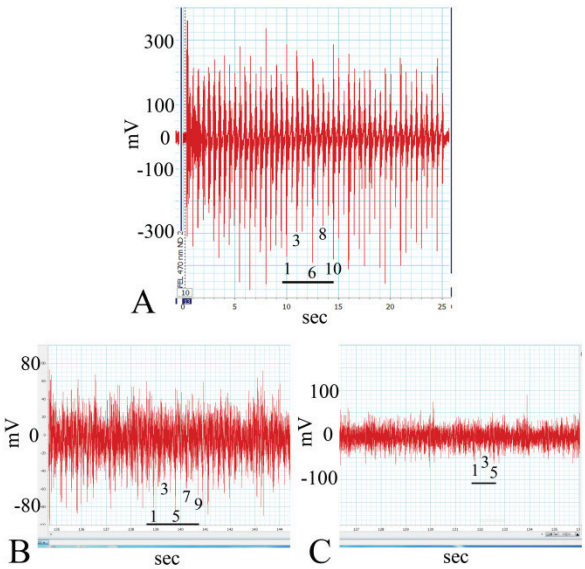

Figure 5: Flicker fusion rate (Hz) of *L. valentianus* eye preparation. The stimulus was 470 nm FEL (10 μJ/pulse) at A, 2.0 Hz; B, 4.55 Hz; and C, 5.0 Hz. Bars indicate irradiation times: A, 5 s; B, 2 s; and C, 1 s. The numbers on each graph indicate the frequency of LEBRA-FEL pulses.

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