Communication

Optogenetic control of body movements via flexible vertical light-emitting diodes on brain surface

Seung Hyun Lee, Jeongjin Kim, Jung Ho Shin, Han Eol Lee, Il-Suk Kang, Kiuk Gwak, Dae-Shik Kim, Daesoo Kim, Keon Jae Lee

A R T I C L E   I N F O

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A B S T R A C T

The microstimulation of specific neural populations of the brain is one of the facile and reliable methods used in neuroscience for deduction of functional movement, complex behavior and even long-range connectivity. Recent advanced biomedical tools now employ flexible optoelectronic devices combined with optogenetic mouse models to induce high spatiotemporal modulation of specific brain activity. However, most current applications are limited to activation of small functional regions using blue-light driven channelrhodopsin. In this report, we introduce flexible AlGaInP vertical light-emitting diodes (VLEDs) for perturbation of specific functional areas of mouse cortex. Micro-scaled LEDs effectively compress the conductive balls dispersed in anisotropic conductive film (ACF) resulting red light emissions with high optical power density, capable of stimulating motor neurons deep below layer III from the brain surface. Selective operation of pulsed red light from f-VLEDs induces mouse body movements and synchronized electromyogram (EMG) signals. The expression of chrimson, red-shifted channelrhodopsin, enables red-light excitation of targeted functional area of motor cortex. This demonstration opens new opportunities for entire cortical mapping, to explore the connectivity between undefined motor areas in the mouse brain.

1. Introduction

Cortical functional mapping using micro and macro stimulation is an essential tool in both clinical diagnostics and for obtaining a fundamental understanding of brain functions [1,2]. Among the different cortical regions, the motor cortex is the region involved in controlling body movements and was thus the first area to be mapped in relation to overt physical functions [3]. Advanced cortical mapping techniques such as direct cortical motor-evoked potentials (dc-MEP) and intracortical microstimulation (ICMS) have facilitated the ability to apply stimulation with high spatial resolution while reducing side effects (i.e., incidence of seizures during tumor lesion) [2,4]. However, these techniques are not suitable for investigating freely moving animals because of the inevitable large craniotomy, or the loss of cortical integrity. Transcranial magnetic stimulation (TMS) is a non-invasive method, but with limited resolution and focality [5]. To obtain further understanding of the motor cortex in its conventional role (generating movements) as well as new aspects (learning motor skills) [6] for the behavioral study of freely moving animals, it is imperative to have high spatiotemporal manipulation of the motor cortex, and biocompatible neural devices.

The optogenetics has pioneered new opportunities for studying neuronal properties, behaviors and even new therapeutic strategies for various neurological diseases, including Depression, Parkinson’s disease and Compulsive disorder [7-9]. Compared to electric/magnetic neuromodulation, photostimulation of opsin-expressed neurons enables high resolution mapping of the motor cortex by cell type and pathway specific activation. Although transcranial light-based mapping has successfully generated motor maps of limb movement without damaging brain tissue [10], optogenetics-based two-dimensional cortical stimulation is necessary for the analysis of complex movements. For that purpose, multiple arrays of inorganic light-emitting diodes (ILEDs) have been utilized as a promising optogenetic modulation tool,
providing advantages in scalability, and multisite operation [11,12]. Nevertheless, ILEDs present intrinsic problems for cortical stimulation mapping of behaving animals, including limited flexibility, and the lack of an insertion method [13–15].

A number of recently introduced flexible technologies, such as transfer printing [16,17], microscale molding of stretchable materials [18,19], inorganic laser lift-off [20,21], light-induced nanomaterial modification [22,23], and soft interconnects [24,25], have facilitated the realization of high performance flexible optoelectronic devices for biomedical applications. Kim et al. developed a deep brain optogenetic stimulation tool by penetrating a flexible LED probe into brain tissue [26]. Our group also demonstrated flexible ILEDs designed with vertical interconnection packaging that could offer a solution to increase optical performance [25,27]. However, these flexible ILEDs were still unable to reach the level of power, of at least several mW/mm² or more, that is performance [25,27]. However, these

Park et al. reported an effective insertion procedure using a flexible optogenetic system with electrocorticography (ECOG), called iWEBS (insertable wrapping electrode array beneath the skull), which accomplishes intracranial implantation through a small cranial slit without brain damage [30].

Herein, we introduce an optogenetic tool for controlling body movements using high performance flexible AlGaInP vertical LEDs on the brain surface. The vertical interconnection of flexible LEDs using anisotropic conductive film (ACF) reduced the length of the current path and increased the optical power density to more than 25 mW/mm², enough to stimulate the motor neurons below layer III from the brain surface. For red light optogenetic excitation, red-shifted channel rhodopsin (chrimson) was selectively injected into the primary and secondary motor cortices of mouse brains. After viral expression, the f-VLEDs were slid through a cranial slit and settled on the meningeal brain surface. For red light optogenetic excitation, red-shifted channel rhodopsin (chrimson) was selectively injected into the primary and secondary motor cortices of mouse brains. After viral expression, the f-VLEDs were slid through a cranial slit and settled on the meningeal space between the skull and brain surface. By illuminating the motor cortex with the pulsed red light of the f-VLEDs, overt movements of whiskers and forelimbs were elicited that were directly proportional to the optical stimulation, as confirmed by a video monitoring system and a histological analysis of the photoactivated region. In addition, synchronized electromyogram (EMG) signals were simultaneously measured during the optogenetic f-VLED stimulation.

2. Experimental section

2.1. Animals

Animal care and handling were conducted according to the guidelines of the Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology (KAIST, Korea). C-fos EYFP transgenic mice (Jackson Laboratory, USA) were generated by mating heterozygous transgenic mice (C57BL/6 J background). The mice were crossbred by a video monitoring system and a histological analysis of the photoactivated region. In addition, synchronized electromyogram (EMG) signals were simultaneously measured during the optogenetic f-VLED stimulation.

The I-V characteristics of the f-VLEDs were measured using a semiconductor parameter analyzer (4200-SCS, Keithley Instruments, Inc.). Probe tips were contacted directly on the p-side and the n-side of the top metal lines. The flexible optoelectronic devices were detached and connected to PCB using a similar ACF bonding process. The flexible VLEDs and the PCB were aligned on the same plane, and thermo-compressed by pre-heated bonding tool (ANT Corp.) for 20 s, to minimize plastic damage, as shown in Fig. S2b in the Supporting information.

2.5. Electrical and optical measurement of the flexible VLEDs

The I-V characteristics of the f-VLEDs were measured using a semiconductor parameter analyzer (4200-SCS, Keithley Instruments, Inc.). Probe tips were contacted directly on the p-side and the n-side of the top metal lines. The optical power of different sized f-VLEDs (50 × 50 μm² ~ 300 × 300 μm²) were analyzed using a digital optical power meter (PM160, Thorlabs, Inc.), by changing the DC voltage sweep mode with the compliance current level. For each sweep mode, the compliance current level was maintained for ~ 5 s. To measure exact
optical power densities, the detector part was fixed to the center of the f-VLEDs. Pulsed currents were induced by connecting an ultra-fast I-V module (4225-PMU). In electrical pulse generation through PMU, compliance current cannot be fixed to exact level, and the resulting current was 10% overdriven during the pulsed operation of the f-VLEDs. Therefore, the peak current of the pulses showed 110 µA for the compliance current of 100 µA. All devices and systems were placed in a darkroom for accurate measurement.

2.6. Optogenetics stimulation of the motor cortex

Cranial windows ($2.5 \times 4 \text{ mm}^2$) on each hemisphere starting at 1.0 mm posterior from the bregma were made after 3 weeks of viral injection. The optogenetic f-VLED was implanted beneath the skull by sliding the device through the cranial window to contact the cortical surface [30]. Two tungsten electrodes (#796000; A-M Systems) were implanted in the neck muscles to record the EMG. To monitor the limb and whisker movements, video recording was acquired when the motor cortical areas were being stimulated by the f-VLEDs. To measure the movements of the whiskers and limbs the video recordings were analyzed using EthoVision XT 8.5 (Noldus Information Technology). EMG signals were recorded with a Digital Lynx acquisition system (Neuralynx). Data were digitized at 32 kHz and band-pass filtered at 30–5 kHz for the EMGs. Muscle activation was calculated using a Neuroexplorer (Neuralynx Inc.).

![Fig. 1. Flexible vertical light-emitting diodes (f-VLEDs) for the optogenetic stimulation of mouse cortex. (a) Schematic illustration of the experimental concept. i) Layered components of the f-VLEDs. ii) Insertion of the f-VLEDs through a small cranial slit for position-fixed and multipoint optogenetic stimulation, directly on the motor cortex. (b) Photograph of the $4 \times 3$ passive matrix f-VLED array connected to the printed circuit board and wired connector, which enables the individual operation of each device. (c) Cross section view of the soft-ACF bonded f-VLED. (d) Magnified image of the ball capture area exposed by focused ion beam milling of the square dotted area in (c). (e) Comparison of the stiffness of various flexible optogenetic devices.](image-url)
3. Results

3.1. f-VLEDs for optogenetic stimulation

Fig. 1a illustrates the f-VLEDs and its insertion through the narrow cranial slit for multipoint optogenetic stimulation of the mouse motor cortex. To construct the high performance flexible optoelectronic device, vertically structured LED (VLED) chips were used. VLEDs have advantages in both light extraction and heat dissipation due to improved current spreading [31]. In this work, AlGaInP VLEDs with the small size of 50 × 50 µm² were vertically connected to the bottom flexible electrodes by ACF bonding process. In order to prevent contamination of the f-VLEDs, the components were passivated by an SU-8 epoxy in the same way of our previous work [30], followed by contact hole patterning and top electrode deposition. The total device thickness of ~ 35 µm enabled the flexible inorganic VLEDs (4 × 3 array with 500 µm pitch) to be smoothly inserted through the cranial slit for multi-spot photostimulation of the motor cortices. To modulate the mouse motor cortex, the f-VLEDs were implanted through a small cranial window and positioned between the skull and the cortical surface. The intact skull firmly pressed the devices in the targeted location, facilitating long-term and stable illumination of the cortices of the freely moving mice. Fig. 1b shows photographs of the f-VLEDs connected to the printed circuit board and wired connector. The optoelectronic devices were stably illuminated using accurate current and voltage control from a power generator. The optic modules were composed of three p-
metal lines and four n-metal lines to individually operate each pixel, enabling sophisticated analysis of the brain network through optogenetics. Stable ACF interconnection between the VLEDs and the bottom electrodes was required to achieve reliable f-VLED packaging. Fig. 1c and Fig. S3 in the Supporting information show scanning electron microscopy (SEM) images of the cross-section of the AlGaInP VLED chip connected to the bottom metal line. The conductive film was squeezed homogeneously along the metal line, adhering the chip to the bottom electrode. Under the same compression, the thickness of the conductive film under the chip was lower than that of the peripheral film, which indicates that the pressure was concentrated on the LED chips. Fig. 1d is a magnified SEM image of the thermo-compressed ACF, composed of the conductive ball and the polymer matrix. The Au/Ni-coated ball in the deformed tape was squeezed to construct effective current paths between the AlGaInP LED and the bottom metal line on the polyimide substrate [24].

Insertion under the mouse skull required the optogenetic tools to have an optimum thickness and bending stiffness. Fig. 1e shows the thickness and bending stiffness of the flexible VLEDs and optogenetic tools based on this work, as compared with previous reports (see Supporting information for details). Considering the meningeal space of the commercial transgenic mice, a device that was thicker than 100 µm could induce severe damage to the cortex of the mice during the insertion through the cranial slit. The thickness of the f-VLEDs was far smaller than the maximum thickness needed for the implantation under the skull of the mouse without creating cortical injury. In addition, the bending stiffness of our optogenetic tools (2.6 × 10^{-8} N m) was close to that of iWEBS (1.5 × 10^{-9} N m) for optimized insertion through the space between the skull and the cortex of the mice [30]. These data demonstrate that f-VLEDs are appropriate for making contact with curved cortical surfaces, yet rigid enough to slide into the subcranial space. In order to stimulate rounded motor cortex, f-VLEDs are required to have the mechanical robustness along with high flexibility. Fig. 54 in the Supporting information exhibits the I-V characteristics and the optical power density changes under varied bending radius down to 2.5 mm. Both electrical properties and the optical output of the f-VLEDs in the bent states were almost identical to the f-VLEDs under flat state. As shown in Fig. S5 in the Supporting information, the optical power and the voltage changed within 5% and 8% respectively during 1000 bending and releasing cycles with a bending radius of 5 mm, which guarantees the robust operation of the device on the curved cortical areas after the insertion through the cranial slit.

3.2. Electrical and optical characteristics of different sized f-VLEDs

For the optogenetic modulation of specific brain areas, the size of the f-VLEDs need to be carefully determined based on their electrical/optical properties. Fig. 2a shows an optical microscope image of f-VLEDs with 6 different sized chips (From the upper left, the LED size is 50 × 50 µm², 100 × 100 µm², 150 × 150 µm², 200 × 200 µm², 250 × 250 µm² and 300 × 300 µm², respectively). The optogenetic stimulators are required to activate one of the light-sensitive actuators, including channelrhodopsin, halorhodopsin and archaerhodopsin, used for neural tissue stimulation. As shown in Fig. 2b, the f-VLEDs exhibited a peak wavelength of 638 nm, which was suitable to activate a red-shifted channelrhodopsin (chrismom, CnChR1) [32]. Furthermore,
compared with conventional LED and the optic fiber for optogenetics, f-VLEDs exhibited the smallest radiation angle, which facilitates the high resolution stimulation of the cortical areas as shown in Fig. S6 in the Supporting information. Fig. 2c is the luminescence-current-voltage (L-I-V) characteristic curve of the 50 × 50 µm² sized f-VLED. The forward bias and the optical power density of the smallest device were measured to be about 4.4 V and 16 mW/mm² at 1 mA current. The 50 × 50 µm² f-VLED required the electrical power of 2.4 mW for the optical power density of 10 mW/mm², comparable to the performance of the conventional LED for wireless optogenetics, as shown in Fig. S7 in the Supporting information. Fig. 2c illustrates the optical power densities of the different sized f-VLEDs as a function of current density. The smallest LED exhibited the highest optical power density of 25 mW/mm², which far exceeds the minimum optical power density required for the optogenetic stimulation of chrimson (~ 1 mW/mm²) [32,37]. At constant optical power density, the smallest LED exhibited the lowest current density compared to the rest of f-VLEDs. Considering the chip size of each f-VLED, this comparison indicates that the current of the smallest optic module was lower than that of other devices with an identical light output. Based on the electrical/optical characteristics of the different sized f-VLEDs, the amount of heat released from the optoelectronic devices during the same operating time can be expressed as follow [38]:

\[ Q = I^2 \times R \times t \]

where Q is the amount of heat, I is the electric current flowing through the device, R is the amount of electric resistance present in the device, and the t is the device operation time. At constant operation time and the same optical power density level, the 50 × 50 µm² f-VLED showed the lowest total resistance and current compared to the other f-VLEDs. Therefore, the smallest chip generated the lowest amount of heat, resulting in the least cell damage while stimulating the nerve systems. In addition, Fig. S10 in the Supporting information displays that the

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**Fig. 4.** Enhanced muscle activities and body movements induced by f-VLED operation (638 nm, 10 Hz, 10 ms). The peak wavelength of the light was 638 nm. The frequency, and the width of the light pulses were 10 Hz, and 10 ms respectively. (a) EMG activation of the neck muscle with f-VLED stimulation. (b) Comparison of EMG activities between pre-trials and on-trials. All bars indicate means ± SEM (*p < 0.05). (c) Distance of whisker movement with f-VLED stimulation. Yellow triangles, the whiskers. (d) Confocal fluorescent images. Top, the expression of chrimson in frontal motor cortical areas. Bottom, the co-expression of chrimson and c-fos protein in cortical neurons.
smallest device raised temperature only about 0.5 °C during 300 s of illumination, which confirms that the 50 × 50 µm² f-VLED is the most optimized device for optogenetics among the different sized f-VLEDs to prevent nerve tissue damage along with its low resistance, and high output efficiency.

3.3. Optogenetic multi-site modulation of the motor cortex via f-VLEDs

Fig. 3a presents the experimental procedures used for the optogenetic stimulation of mouse motor cortex by f-VLEDs. We transfected the AAV viral vector harboring the Chrimson (CnChR1)-mCherry to the frontal motor cortical areas, including the primary and secondary motor cortex, which project to the peripheral motor neurons. The flexible micro LEDs were densely arranged to precisely stimulate multi-sites, facilitating the functional mapping of the mouse motor cortex. As shown in Fig. 3b, these small pixels were placed on the surface of the motor cortex in regions related to the jaw, wrist, neck, and whisker movement. Based on previous studies, it is known that the mouse motor cortex has ambiguous functional boundaries, and requires high resolution analysis for detailed mapping [1,4]. Prior to the implantation under the skull, the f-VLEDs had soaking test in PBS solution to evaluate the robustness against biofluids, as presented in Fig. S11 in the Supporting information. The optical power of the f-VLEDs was stably maintained even after soaking in PBS solution for 100 h, indicating that the packaged LED chips were well isolated from the external environment. To stimulate the frontal motor areas, the small-scale f-VLEDs under the skull successfully wrapped the motor cortical areas in a less invasive manner, as shown in Fig. 3c and d. Concurrently, we implanted bipolar electrodes in the neck muscles and acquired EMG signals to observe the neck muscle activities produced by photostimulation of the motor cortex, as presented in Fig. 3c. Video-monitoring was performed to measure the body movements induced by muscle contraction. To trigger the photo-induced body movements, the f-VLEDs were connected to the power source, and produced 10 Hz red light with 10 ms pulse duration. The red light was successfully limited, to illuminate the primary and secondary motor cortex, as shown in Fig. 3d.

Fig. 4a indicates that EMG signals were vigorously modulated by irradiating the frontal motor regions with pulsed red light. 10 Hz frequency modulation of red light by f-VLEDs efficiently generated rhythmic muscle activation. Fig. 4b compares the quantified EMG signals between pre-state and ON-state under same level of chrimson expression, confirming that the optogenetic proteins were well controlled in the targeted regions during the stimulation of the mouse motor cortex with f-VLEDs. Fig. 4c and Fig. S12 in the Supporting information show that photostimulation of the motor cortical areas triggered the movements of whiskers and forelimbs, respectively (see Video S2 in the Supporting Information). Furthermore, the mice with the implantable devices in the different skull position exhibited no behavioral problems, indicating no severe side-effects of the f-VLED insertion (see Video S3 in the Supporting information). These results show that f-VLEDs can be used to define the boundary of motion maps hidden in the motor cortical areas. Postmortem histological analysis presents that chrimson (red signals) were well expressed in the frontal motor cortical regions, as shown in Fig. 4d. To determine whether the f-VLEDs efficiently triggered neuronal excitation, we detected EYFP signal, a reporter for c-fos, a neuronal excitation protein marker, after optical stimulation. The expression level of c-fos (green signals) was highly enhanced in the chrimson expressed neurons (red signals) of the motor cortex following optogenetic f-VLED activation. This observation indicates that the optical stimulation efficiently activated neuronal and muscular excitation, without triggering any unintended artifact which can modulate the mouse behavior. In addition, Fig. S13 and S14 in the Supporting information showed the identical brain morphology, confirming the minimal damage in the frontal motor cortex after f-VLED implantation through DAPI staining. These demonstrate that the f-VLEDs are powerful optogenetic tools for the microstimulation of animals to elucidate the functional macro-interactions between the cortical columns and the circuit mechanisms underlying motion generation.

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4. Conclusions

In summary, we present high performance AlGaInP f-VLEDs, for the precise optogenetic control of a mouse’s body movements. These optogenetic tools, fabricated using an ACF bonding process, had a thickness of ~35 µm and a bending stiffness of 2.6 × 10⁻⁶ N m, which were the optimum features needed for insertion through a narrow cranial slit. Light with a peak wavelength of 638 nm and pulsed operation of ~10 Hz enabled the activation of red-shifted channelrhodopsin (chrimson) and the effective modulation of the mouse motor cortex. The successful activation of motor cortex by using red-light driven channelrhodopsin means that other blue-light driven technologies can be implemented together in the LED microarray for selective stimulation of different population of neurons or independent recording of neural activity from photostimulation. Among the various micro-sized LEDs, the 4 × 3 array of 50 × 50 µm² sized f-VLEDs exhibited the best performance for in vivo optogenetic application, with the lowest resistance, the highest output efficiency and the least heat release. The flexible LEDs were implanted into the meningeal space, and pressed by the intact skull, enabling stable stimulation of the primary and secondary motor cortical regions. When the f-VLEDs were illuminated, EMG signal modulation and distinct movement of the whiskers was observed, which was also verified by the co-expression of chrimson and c-fos in the targeted neural populations. These results demonstrate that f-VLEDs are powerful tools for defining the motion map hidden in motor cortical areas, and can be successfully employed to activate neuronal and muscular signals. Using our multi-site cortical stimulation by f-VLEDs, we are currently investigating the long range cortical interactions of cognition and perception, which leads to new therapeutic solutions for neurological diseases.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.nanoen.2017.12.011.

References

Seung Hyun Lee received his Ph.D. in Materials Science and Engineering (MSE) at KAIST. During his Ph.D. at KAIST, he pioneered flexible biomedical devices for optoelectronic applications, large-scale inorganic laser lift-off and selective laser treatment of functional materials. Under supervision of Prof. Keon Jae Lee, he has been an expert in flexible device process and packaging. His current research interest is new fabrication method of flexible electronic devices exceeding performance and yields of previous ones.

Jim Hoon Shin received his B.S in Material Science and Engineering (MSE) from Hanyang University in 2017. He is currently working toward his MS at KAIST under the supervision of Prof. Keon Jae Lee. His doctoral research topics were in the development of the inorganic-based flexible optoelectronics for biomedical applications.
Il-Suk Kang received his Ph.D in Materials Science and Engineering (MSE) from Seoul National University (SNU) in 2008. Since 2008, he has been with National Nanofab Center (NNFC) at Korea Advanced Institute of Science and Technology (KAIST). His main research interests are low-dimensional and nanostructured materials and devices for smart, soft and sustainable applications.

Prof. Dae-Shik Kim received his Ph.D at the Max-Planck-Institute for Brain Research in Frankfurt, Germany. Following postdoctoral works at The Massachusetts Institute of Technology (MIT), he served as Assistant (University of Minnesota), and Associate Professor and Director of the Center for Biomedical Imaging (Boston University School of Medicine) before joining KAIST as a tenured Full Professor of Electrical Engineering. His current research interest include fMRI, computational cognitive neuroscience, deep learning, artificial intelligence, and neuroengineering technologies.

Prof. Daesoo Kim received a Ph.D. degree in Genetics and Neuroscience from the Pohang University of Science & Technology (POSTECH) in 1998. He performed post-doctoral research at the State University of New York (SUNY) medical school and worked at Korea Institute of Science & Technology (KIST) as senior scientist. He is currently studying about the circuit-based mechanism of motor behavior at Korea Advanced Institute of Science & Technology (KAIST). He has presented as to ‘brain-inspired solutions for the crowded earth’ at the World Economic Forum at Davos and won the 3.1 prize from the Samil foundation.

Prof. Keon Jae Lee received his Ph.D. in Materials Science and Engineering (MSE) at University of Illinois, Urbana-Champaign (UIUC). During his Ph.D. at UIUC, he involved in the first co-invention of “Flexible Single-crystalline Inorganic Electronics”, using top-down semiconductors and soft lithographic transfer. Since 2009, he has been a professor in MSE at KAIST. His current research topics are self-powered flexible electronic systems including energy harvesting/storage devices, IoT sensor, LEDs, large scale integration (LSI), high density memory and laser material interaction for in-vivo biomedical and flexible application.