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Abstract. As the most abundant cell type in the central nervous system, astrocyte has been one of main research topics in neuroscience. Although various tools have been developed, at present, there is no tool that allows noninvasive activation of astrocyte *in vivo* without genetic or pharmacological perturbation. Here we report a noninvasive label-free optical method for physiological astrocyte activation *in vivo* using a femtosecond pulsed laser. We showed the laser stimulation robustly induced astrocytic calcium activation *in vivo* and further verified physiological relevance of the calcium increase by demonstrating astrocyte mediated vasodilation in the brain. This novel optical method will facilitate noninvasive physiological study on astrocyte function.
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Keywords: femtosecond laser; astrocyte; *in vivo*; label-free; noninvasive.

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1 Introduction

Astrocyte is the most abundant cell type in the central nervous system, comprising approximately half of the volume of the adult mammalian brain.[1] It extends numerous fine processes that physically interact with all other cellular elements in the brain, including neurons, glial cells, and blood vessels. Especially, more than 99% of the cerebrovasular surface is ensheathed by the astrocyte processes.

Because astrocyte is electrically nonexcitable, it had long been considered as mere structural support for neurons. However, this view began to change several decades ago by the finding that astrocyte expresses various membrane receptors and stimulation of these receptors evokes variety of cellular responses. The most well-known second messenger of cell signaling in astrocyte is Ca^{2+}. In response to elevation of intracellular Ca^{2+}, astrocyte can release neuro-modulatory signaling molecules, or modulate vasomotion of the artery enwrapped by the endfeet processes of the astrocyte.[2,3] Especially, as the fundamental mechanism of functional brain imaging and the promising therapeutic target in neurodegenerative diseases, the vasomotion mediated by astrocyte is currently under active research.[4]

To study the physiological role of astrocyte in a living animal, various tools for altering astrocyte Ca^{2+} have been developed, such as mechanical stimulation with glass pipette, membrane potential depolarization, and activation of metabotropic glutamate receptors; however, these tools require invasive pipette insertion to access a target astrocyte.[5,6] To avoid the pipette insertion, optical uncaging methods, such as caged Ca^{2+} or IP_3, have been developed but still, invasive skull opening and the dura damage are unavoidable to load the caged compounds.[7] Recently, an optogenetic approach which modifies a gene to express the light responding protein has been reported.[8] Although this technique ensures noninvasiveness with high spatiotemporal specificity, the need of laborious genetic modification limits the wide application and potential clinical translation.

In the biological tissue, unamplified femtosecond pulsed laser can evoke localized low-density plasma formation, leading to a photochemical effect without involving significant thermal damage.[9] This phenomenon allowed noninvasive modulation of biological functions such as gene transfection and intracellular calcium wave generation.[10–12] Photoactivation of intracellular Ca^{2+} in cultured astrocytes has also been demonstrated.[13] This suggested a tool for noninvasive and highly specific modulation of astrocyte function without an exogenous probe, but feasibility of this technique *in vivo* remains unexplored. Here, we report that a femtosecond pulsed laser irradiation can induce physiological astrocytic Ca^{2+} activation *in vivo*.

2 Materials and Methods

2.1 *Cell Culture*

Primary cultures of rat astrocytes were prepared as described previously.[14] Briefly, the cerebral cortices were isolated from neonatal rat brains, and then meninges and capillary vessels were removed completely. The tissues were minced and enzymatically dissociated with trypsin and DNase. After centrifugation, the cells were plated into a culture flask and grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% nonessential amino acids. After incubating for 7 days, primary astrocytes were separated by shaking for 24 h at 110 rpm at 37°C. The immunofluorescence staining for glial fibrillary acidic protein (GFAP) revealed that most of the cultured cells (>90%) were GFAP-positive astrocytes.

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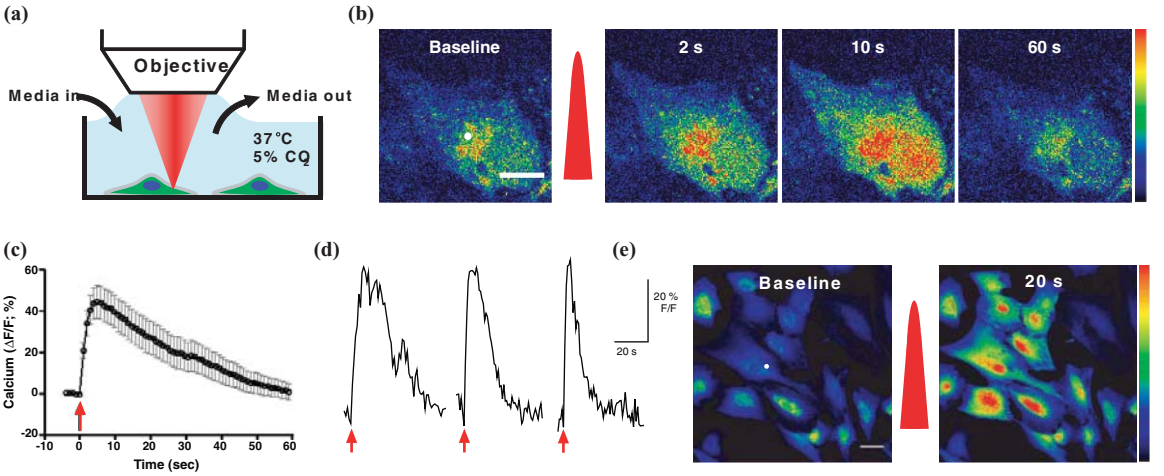

Fig. 1 Laser-induced calcium activation of astrocyte *in vitro*. (a) A schematic of the *in vitro* experimental setup. (b) Representative temporal dynamics of the laser-induced calcium wave in a cultured astrocyte. The white dot indicates the irradiated region. Scale bar, 10 μm. (c) Quantification of laser-induced calcium dynamics in the irradiated cells ($n = 18$). The arrow indicates the time of laser irradiation. (d) Repeatability of the laser-induced calcium activation. The arrows indicate the time of laser irradiation. (e) Propagation of calcium wave to the neighboring astrocytes. The white dot indicates the irradiated region. Scale bar, 50 μm.

2.2 *Animal Models*

All experiments used 7- to 9-week-old male ICR mice (Charles River Japan, Yokohama, Japan). For anesthesia, intraperitoneal injection of ketamine-xylazine was used. The procedures for the open skull window preparation were performed as described previously.[15] After anesthesia, dexamethasone (Sigma, 0.2 mg/Kg) was subcutaneously administered, the scalp was removed, and the skull was glued to a custom-designed metal plate (Namil Optical Components, Inchon, Korea) using dental cement. Using a high-speed microdrill, a circular area of skull, 3 to 4 mm in diameter, over the region of interest was removed under a dissection microscope. After bleeding had stopped spontaneously, the dura layer was removed for bath loading of dyes. After the staining procedure, 1.5% agarose gel with cover slip was added to reduce movement artifacts. The animal care and experimental procedures were performed under the approval of the Animal Care Committee of KAIST (Daejeon, Republic of Korea).

2.3 *Dye Loading and Two-Photon Fluorescence Imaging*

We used an upright two-photon laser scanning microscopy (LSM510; Carl Zeiss, Oberkochen, Germany) with a femtosecond pulsed laser (Chameleon, Coherent, Santa Clara, California) that provides 140 fs duration at 800 nm wavelength with 80 MHz repetition rate, and a water immersion objective lens

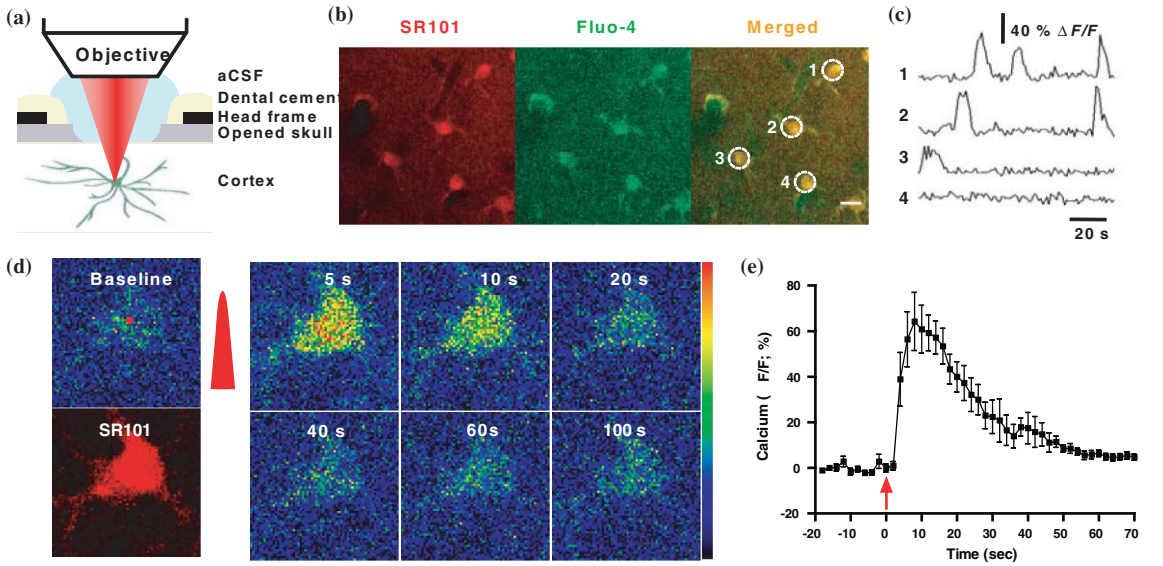

Fig. 2 Laser-induced calcium activation of astrocyte *in vivo*. (a) A schematic of the *in vivo* experimental setup. (b) Brain cortex stained with an astrocyte marker (SR101) and a calcium indicator (Fluo-4). Scale bar, 10 μm. (c) Spontaneous calcium oscillation in astrocytes. (d) Representative temporal dynamics of laser-induced calcium wave in an astrocyte. Scale bar, 10 μm. (e) Temporal dynamics of astrocyte calcium after the laser stimulation ($n = 12$). The arrow indicates the time of laser irradiation.

(20×, 1.0 numerical aperture). For *in vitro* experiments, cultured cells were loaded with 10 μm of Fluo-4AM (Molecular Probes, California) for 40 min in DMEM, washed with phosphate-buffered saline, placed in a perfusion chamber [Harvard Apparatus, Massachusetts, Fig. 1(a)], and imaged. For *in vivo* experiments, the dye mixture (1 mm for Fluo-4AM and Rhod-2, 100 μm for SR101) was bath loaded for 1 h and washed for 15 min. 100 μL of 5% 2MDa FITC-dextran was intravenously administered for visualizing the lumen of the blood vessel. The exposed cortex was sealed with agarose gel and cover glass, and imaged at 0.5 Hz [Fig. 2(a)]. The average laser output power used for imaging was less than 90 mW to avoid unwanted stimulation of astrocytes.

2.4 *Laser Stimulation*

The optical path and laser source for optical intervention were the same as those used in the two-photon fluorescence imaging. The laser stimulation was pinpoint irradiation with the duration of 100 μs and the average laser output power of 300 to 3000 mW. We started with low laser power and increased it until a calcium wave was observed. For the *in vitro* study, we selected a target region in the cytosol of a cultured astrocyte using baseline calcium fluorescence. For the animal experiments, we selected target astrocytes that had subsurface depths of 50 to 150 μm, in an open skull window model. Vascular type was confirmed by measuring the direction of blood flow in the branching vessels as previously described.[16]

2.5 *Data Analysis*

We used IMAGEJ or MATLAB for image processing and data quantification, respectively. Data are expressed as the means ± SEM. Statistical analyses were performed using Graph Pad Prism software. We expressed data as mean ± SEM.

3 Results

To characterize laser-induced calcium in astrocyte, we used cultured rat primary astrocytes. After loading with a calcium indicator, Fluo-4AM, astrocytes exhibited baseline fluorescence. The spatial profile of the baseline fluorescence was used to target the cytosolic region for laser stimulation. In our culture condition, astrocytes showed no noticeable spontaneous calcium oscillation. After the pinpoint irradiation of a femtosecond pulsed laser in the cytosol, the astrocyte showed rapid calcium increase and subsequently recovered the baseline calcium level [Figs. 1(a)–1(c)]. The slow temporal dynamics of the laser induced calcium elevation is consistent with physiological calcium activity in astrocytes.[17] This laser induced calcium can be induced repeatedly, suggesting no irreversible damage to the cell [Fig. 1(d)]. In physiology, astrocyte calcium elevation is accompanied by intercellular calcium propagation to neighboring astrocytes.[17] The laser induced calcium also showed propagation to neighboring astrocytes [Fig. 1(e)]. Collectively, these data implied that a femtosecond laser irradiation can induce physiological calcium elevation in a cultured astrocyte.

We next addressed whether the laser induced astrocyte activation is feasible *in vivo*. To load a calcium indicator and an astrocyte specific dye, sulforhodamine 101 (SR101), we used an open skull cranial window model [Fig. 2(a)]. With the bath loading protocol, astrocytes specifically showed a high level of baseline calcium,[18] therefore we could utilize the calcium staining for identifying astrocytes in the subsequent experiments [Fig. 2(b)]. For *in vivo* experiments, we used astrocytes with subcortical depth of 50 to 150 μm. In the calcium stained astrocytes, we could observe spontaneous calcium oscillation, reflecting cell viability [Fig. 2(c)]. In agreement with the previous reports, this baseline calcium activity is slow and infrequent.[19] For the laser stimulation experiments, we checked flat baseline activity for 20 s and irradiated in the cell body. After the laser irradiation, astrocytes showed rapid calcium increase within several seconds and recovered the baseline calcium level [Figs. 2(d) and 2(e)]. The *in vivo* calcium dynamics was similar to the *in vitro* response. We then questioned the feasibility of this technique for astrocytic endfeet activation. To aid the endfeet targeting, we stained the blood plasma with 2M-Da dextran conjugated fluorescein isothiocyanate (FITC) and the astrocytes with a calcium indicator, Rhod-2 [Fig. 3(a)]. Regardless of the vascular types, artery and vein, the laser irradiation evoked calcium activation in the astrocytic endfeet [Figs. 3(b) and 3(c)].

Having confirmed the laser induced astrocyte activation technique, we investigated whether this technique can trigger physiological vasomotion mediated by astrocyte activation. Arterial dilation by astrocytic endfeet activation has previously been reported using a caged calcium compound. We selected a penetrating artery based on the direction of perfusion in the branching point. The arterial type was confirmed by the thick layer of unlabeled smooth muscle cell between the lumen and the encasing astrocytic endfeet. Upon laser irradiation, the astrocytic endfeet showed calcium increase followed by vasodilation [Figs. 4(a) and 4(b)]. The time delay between the endfeet calcium activation and vasodilation was less than several seconds. The peak lumen diameter increase was 11.1 ± 1.5% (n = 10) which was a 23.6 ± 3.4% increase in the luminal area [Fig. 4(c)]. There was positive correlation between the peak endfeet calcium

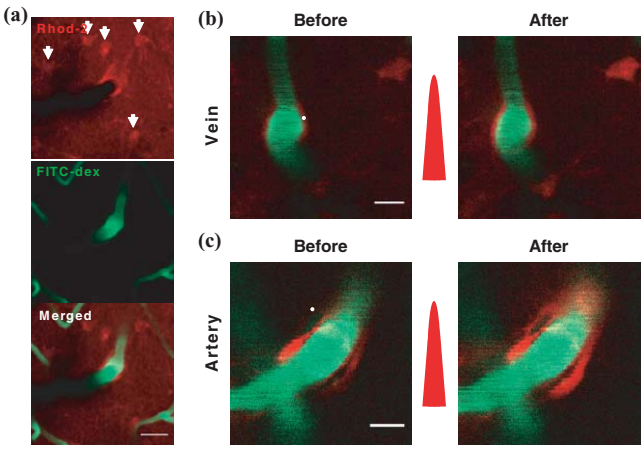

Fig. 3 Targeted calcium activation of astrocytic endfeet *in vivo*. (a) After the bath loading of a calcium indicator (Rhod-2), 2M-Da dextran conjugated fluorescein (FITC-dex) is intravenously injected to visualize blood vasculature. The arrows indicate the stained astrocytes. Scale bar, 10 μm. [(b) and (c)] Calcium activation in the astrocytic endfeet by the laser stimulation. The white dot indicates the irradiated region. Scale bar, 10 μm.

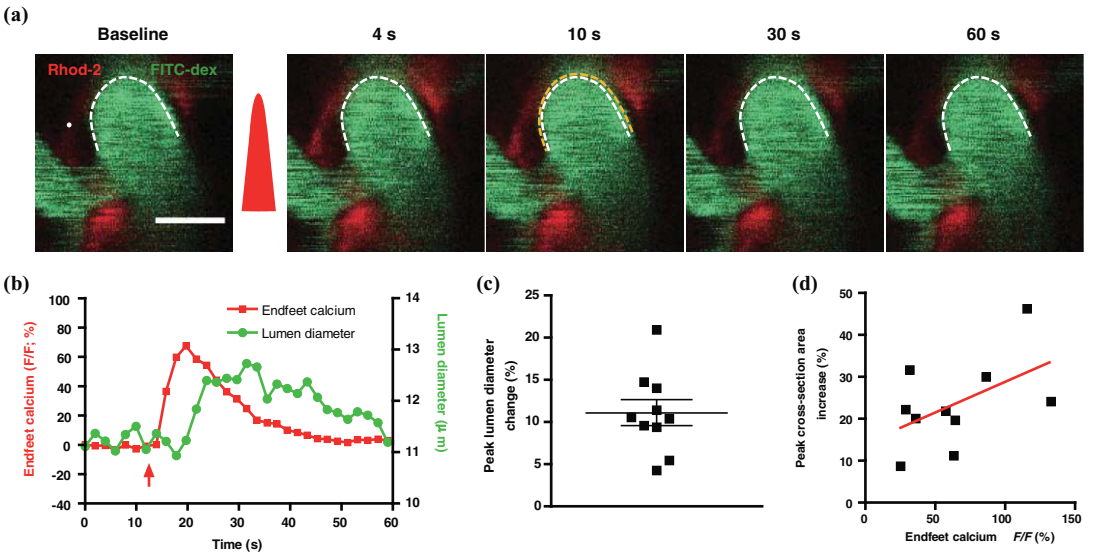

Fig. 4 Vasodilation by astrocytic endfeet activation. (a) Temporal dynamics of astrocyte mediated vasodilation. The dotted lines demarcate the arterial lumen at the baseline and the outer dotted line demarcate the arterial lumen at 30 s. The white dotted line indicates the irradiated region. Scale bar, 10 μm. (b) Time-course tracing of the endfeet calcium increase and arterial vasodilation in (a). (c) Summary of peak lumen diameter increase by laser-induced activation of astrocytic endfeet. $n = 10$, 4 mice. (d) Vasodilation as a function of the relative calcium increase in astrocytic endfeet. Correlation curve: $y = 0.065\ x + 6.89$, $R^2 = 0.26$. $n = 10$, 4 mice.

change and the lumen diameter change, albeit nonsignificantly [Fig. 4(d)]. Since these data were consistent with the previous report with a caged calcium compound,[18] we concluded that a femtosecond pulsed laser irradiation can induce physiological astrocytic Ca^{2+} activation *in vivo*.

4 Discussion

Here we reported a label-free optical method for astrocyte activation *in vivo* using an unamplified femtosecond pulsed laser. With the laser stimulation in the soma or in the endfeet, astrocyte Ca^{2+} could be robustly evoked. We verified physiological relevance of the laser induced astrocytic calcium response by demonstrating astrocyte mediated vasodilation in the mouse brain. This optical method will facilitate a noninvasive physiological study on astrocyte function.

Zhao et al. suggested that laser simulation formed transient holes which allowed influx of extracellular Ca^{2+} and triggered sequential release of intracellular Ca^{2+} stores.[13] On the contrary, we could also activate astrocytes optically by targeting cytosolic regions of the cell rather than membrane. We also confirmed that femtosecond pulsed laser irradiation can induce calcium wave generation even when extracellular calcium was depleted by calcium chelating agents (data not shown). Even though further investigation is needed to unveil detailed mechanism, we have demonstrated that exact targeting onto the plasma membrane is not necessary for optical activation of astrocytes. In live animal brains, visualizing cell membrane of astrocytes may not be easily achievable; therefore, current study provides a rationale for targeting the cell body for reliable optical activation of astrocytes *in vivo*. Another important difference is that Zhao's and our work used different optical parameters for laser stimulation. They exposed the femtosecond pulsed laser onto the cell for a duration of 1 to 4 ms and with an average power of 15 to 60 mW; whereas our group exposed the femtosecond pulsed laser onto the cytosolic region for a duration of 100 μs with an average power of 300 to 1500 mW. Both methods of laser stimulation delivered comparable energy onto the cell. We found that shorter duration of laser exposure is better for reliable generation of calcium wave *in vivo* because motion artifacts hinder consistent irradiation onto the exact location for a longer period.

Table 1 Comparison between conventional astrocyte stimulation methods, optogenetics, and laser stimulation method.

	Mechanical, electrical or chemical activation	Optogenetics	Label-free optical activation
Invasiveness	High	Low	Low
Time for preparation	Short	Long	Short to intermediate
Molecular mechanisms	Depolarization of membrane potential	Optical activation of specific ion channel	Unknown

Compared to the existing techniques, such as mechanical, electrical, pharmacological, and genetic methods, our method has advantages (Table 1). Most of all, it is noninvasive and label-free. Although we used an open skull cranial window model for molecular staining, no exogenous staining is needed for astrocyte activation. In combination with noninvasive labeling of astrocytes such as genetic engineering[20] or optical delivery of molecular probes,[21] the astrocyte activation can be performed without opening the skull which is known to cause deleterious effects, such as microglial activation and intracranial pressure change.[15] Due to the nonlinear nature of a femtosecond pulsed laser, subsurface targeting is possible without compromising the superficial layer. In addition, it has high spatiotemporal resolution, suitable for investigating dynamic glial network *in vivo*.

This method also has drawbacks. Unlike techniques with caged molecules or optogenetics, our method does not target a specific molecular event. Astrocytic calcium activation could be one aspect of multiple cellular responses by the laser stimulation and additional unknown molecular events can be associated. Detailed understanding on mechanism of the laser induced calcium will help to resolve this concern.

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