Target ischemic stroke model creation method using photoacoustic microscopy with simultaneous vessel monitoring and dynamic photothrombosis induction

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\textbf{ABSTRACT}

The ischemic stroke animal model evaluates the efficacy of reperfusion and neuroprotective strategies for ischemic injuries. Various conventional methods have been reported to induce the ischemic models; however, controlling specific neurological deficits, mortality rates, and the extent of the infarction is difficult as the size of the affected region is not precisely controlled. In this paper, we report a single laser-based localized target ischemic stroke model development method by simultaneous vessel monitoring and photothrombosis induction using photoacoustic microscopy (PAM), which has minimized the infarct size at precise location with high reproducibility. The proposed method has significantly reduced the infarcted region by illuminating the precise localization. The reproducibility and validity of suggested method have been demonstrated through repeated experiments and histological analyses. These results demonstrate that our method can provide the ischemic stroke model closest to the clinical pathology for brain ischemia research from inducement, occurrence mechanisms to the recovery process.

1. Introduction

Strokes are a leading cause of death worldwide \cite{1} and are primarily classified into ischemic and hemorrhagic strokes \cite{2}. An ischemic stroke accounts for approximately 70–90\% of all strokes and is primarily caused by thrombotic or embolic occlusion of the brain-supplying blood vessels \cite{3}. Decreased local cerebral blood flow caused by occluded vessel(s) evokes neural dysfunction and impairment in the brain and eventually leads to long-term disability, persistent damage, and even death \cite{4,5}. The pathophysiology of ischemic stroke involves primary and secondary neuronal damage. There is a need to develop neuroprotective therapies for improving functional outcomes as suggested by the stroke treatment academic industry roundtable VII (STAIRVII) \cite{6}. This can alleviate secondary ischemic damages following persistent occlusion or ischemia-reperfusion injury with recanalization therapies. The standardized animal models that mimic the physiochemical processes can evaluate the pathophysiology of ischemic stroke and subsequently assess the efficacy of candidate neuroprotective strategies following ischemic stroke \cite{7}.

Two representative techniques, middle cerebral artery occlusion (MCAO) and photothrombotic stroke, have been widely used to induce the ischemic stroke model \cite{8}. Among these, the MCAO model does not require craniectomy \cite{8,9} and is used frequently; however, it requires a high degree of surgical skill due to the small anatomical structure of the...
carotid artery in the mouse [10,11]. Furthermore, the induced infarct size, which is primarily dependent on the anatomical structure of the MCA and collateral vessels, is different in each case because there are unpredictable anatomical variations [8,12]. Additionally, the large size of the induced infarct territory in MCAO models can induce severe neurological deficits and high mortality rates, which limits its application in the long-term research [13,14]. Photothrombosis is an intra-vascular photo-oxidation mechanism-based focal cerebral ischemia, which is frequently used in the animal models [7,13]. It is characterized by rapid progression of ischemic cell death, which is caused by photo-sensitive dye (e.g., Rose Bengal) with light illumination-based local cerebral artery occlusion [13,15]. This requires simple surgical procedures with minimal surgical intervention, invasiveness, and high reproducibility of ischemic lesions with low mortality [12,16-18]. Since it is hard to determine the exact location of the part to be irradiated as the target point, it is difficult to control the collateral damage and extent of the infarction.

Magnetic resonance imaging (MRI) [19,20] and computed tomography (CT) [21,22] were utilized for monitoring and evaluating the photothrombotic model. These neuroimaging techniques are widely used in clinical settings to diagnose ischemia, but the static nature in both modalities, low sensitivity of CT, and long acquisition time in MRI might affect continuous monitoring of animal ischemic stroke model [23-25]. To overcome these shortcomings, optical coherence tomography has been implemented as a non-invasive method for the dynamic imaging of photothrombotic models [26,27]. However, it only provides morphology-based structural details, which is insufficient to obtain blood vessel-based dynamic information. The laser speckle contrast imaging (LSCI) and confocal microscopy (CM) have also been demonstrated for simultaneously inducing and monitoring the photothrombotic model. However, in LSCI, the skull is opened to induce and image the photothrombotic model [28,29]. In addition, the scanning range and penetration depth of CM are limited, which makes it hard to capture the whole brain [30]. Moreover, the measurement time is comparably long for inducing the photothrombotic model with simultaneous monitoring in a short duration.

Photoacoustic microscopy (PAM) is a non-invasive and label-free functional imaging technique, which provides high absorption contrast of light and high spatial resolution [31-34]. PAM utilizes the nanosecond pulse laser to generate the photoacoustic (PA) wave through the thermoelastic expansion of the tissue [35-37]. It has been widely applied to in vivo studies of morphological, functional metabolism, anatomical, and molecular imaging for animals and humans [38-41]. PAM enables functional imaging of cerebral metabolism, vascular morphology, and even ischemic stroke with long period of continuous monitoring [42-45]. However, as in the previously mentioned various neuroimaging methods, since PAM was employed to monitor the progress of the disease, the limitations of conventional methods for inducing stroke mouse model with simultaneous monitoring remained as well.

In this study, we introduce the single-source-based localized target ischemic stroke model development method of by simultaneous dynamic vessel monitoring and photothrombosis induction under PAM guidance. By applying PAM-based target region localization for in vivo experiments, we break the conventional limitations by integrating the procedure of inducing and monitoring the photothrombotic model at an accurate location of the target region using a single source and path. In addition, we performed multiple inductions of occlusion at the target region to validate the reproducibility and effectiveness of the proposed method. We also compared conventional and proposed PAM-based methods for infarcted area and depth. Moreover, histological analysis was done using including triphenyl tetrazolium chloride (TTC), glial fibrillary acidic protein (GFAP), Iba1 polyclonal antibody (Iba1), and hematoxylin-eosin (H&E) staining for visualization.

2. Methods

2.1. Simultaneous inducing and monitoring method of photothrombotic stroke model

To compare the conventional [46-48] and proposed PAM-based photothrombosis induction and monitoring, the overall process of both methods was demonstrated in Fig. 1(a). Both methods were used to select the target region for inducing photothrombosis. Since the proposed PAM-based method can focus the region, the illuminated area was comparably smaller (up to a single vessel) than the conventional method (~ 2 mm diameter). Next, we injected the Rose Bengal into the tail vein and illuminated the target region after 5 min. For conventional method, two different systems, which were sequentially operated, were required each for inducing and monitoring the photothrombosis results. Therefore, it was not possible to image the photothrombosis inducing mechanism unless these two systems were integrated to have one common path. In contrast, the proposed method used a single source for simultaneously inducing and monitoring the photothrombosis, it was possible image the dynamic inducing mechanism.

The laser energy fluence at the brain surface during the experiment of PAM method was ~ 200 mJ and the calculated energy power density was 16 mJ/cm² (beam spot size at the sample surface: 40 µm), which adhered to the American Standard Institute safety (ANSI) safety limit (20 mJ/cm²). We gradually increased the energy density from a low level (10 mJ/cm²) to a value at which photothrombosis was formed with the minimized infarcted lesion (16 mJ/cm²), according to the PAM-based real-time monitoring. Moreover, the light illumination time was also chosen following the average blockage time of the target blood vessel after irradiation with selected pulse energy. Supplementary movie 3 shows that the photothrombosis model was not reproducible when the appropriate pulse energy and time were not set. In the case of conventional method, the illuminated power of the cold light source (FOK-150 W, Fiber Optic Korea, South Korea) was set to 150 W [46]. The illuminated time of the PAM-based method was 5 min, which was comparably shorter than the conventional method (15–20 min) (Table 1).

A whole-brain PAM MAP image was acquired to select the localized region of interest (ROI) with the actually driven signal of the galvanometer scanner Fig. 1(b). Then, we injected the Rose Bengal and illuminated the position feedback-based localized ROI using a single PAM laser source with a compensated operating voltage of the galvanometer scanner after 5 min of injection. The photothrombosis mechanism was simultaneously induced and monitored at the localized target region for 5 min. Finally, the photothrombosis-induced whole-brain PAM MAP image acquired to verify the formation of occlusion every 10 min after the induction until 1 h. In addition, we acquired the PAM MAP images after 24, 48, and 72 h to confirm the consistency of induction (Supplementary Fig. S2). All PAM MAP images were acquired 30 min after the induction.

2.2. Optical configuration of photoacoustic microscopy for simultaneous vessel monitoring and photothrombosis induction

The optical configuration of the presented PAM is shown in Fig. 2. A 532 nm Q-switched diode-pumped laser (SPOT-10-200-532, Elforlight, UK) with a repetition rate of 1–50 kHz was used to simultaneously monitor and induce the target ischemic stroke model. To expand the beam diameter with collimation, L1 (AC254-030-A, Thorlabs, USA) and L2 (AC254-075-A, Thorlabs, USA) were utilized, and a pinhole (PH, P50C, Thorlabs, USA) was employed to decrease the noise light of the source. The collimated beam was reflected by the protected silver mirror (PF10-03-P01, Thorlabs, USA) and focused by an objective lens (AC254-100-AB, Thorlabs, USA). In the developed PAM system, we implemented the two-axis fully waterproof galvanometer scanner to stably enhance the scanning speed [49,50]. The scanner part, which is indicated as
red-dashed box, was magnified, and the beam was passed by the customized opto-acoustic beam combiner, which consisted of an uncoated BK7 prism (PS910, Thorlabs, USA), a dielectric-coated prism (MRA10-E02, Thorlabs, USA), a correction lens (67-147, Edmund Optics, USA), and an acoustic lens (45-384, Edmund Optics, USA) (Fig. 2b). The optical beam transmitted through the opto-acoustic beam combiner was reflected by a customized fluid-resistant designed two-axis galvanometer scanner (GVS102, Thorlabs, USA). To illuminate the sample with the focused beam, a window with a polyethylene membrane was used to transmit the optical beam and PA signal.

An ultrasound transducer (V214-BB-RM, Olympus NDT, USA), whose center frequency was 50 MHz and with a $-6$ dB bandwidth of 82 %, was used to convert the acoustic signal to an analog electrical signal. The measured PA signal was amplified (52 dB gain) by two amplifiers (ZFL-500LN+, Mini-Circuits, USA) connected in series and was converted to a digital signal by a digitizer (ATS9350, Alazar Technologies, Canada). A data acquisition board (DAQ, NI PCIe-6323, National Instrument Corporation, USA) was used to accurately synchronize the scanning timing and data acquisition.

The lateral and axial resolutions were 4.5 $\mu$m and 32.7 $\mu$m, respectively (Supplementary Fig. S1 and Supplementary Text). The obtained axial resolution was matched well with the theoretical value of 33 $\mu$m.

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**Fig. 1.** Comparison of photothrombosis induction and monitoring processes between conventional and our proposed method, and specific description of PAM-based method. (a) Comparison between the two methods. (b) Detailed sequence of PAM-based method.

**Table 1**

Comparison of experimental parameters between conventional and proposed PAM-based method.

|                  | Irradiation area | Laser power | Irradiation time (min) | Vessel targeting | Induction + monitoring |
|------------------|------------------|-------------|------------------------|------------------|------------------------|
| Conventional     | $\sim 2$ mm      | 150 W       | 15–20                  | X                | X                      |
| Our proposed     | $\sim 500$ $\mu$m| 5 mW        | 5                      | O                | O                      |

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which was determined by the ultrasound transducer (50 MHz of center frequency and 40.63 MHz of $-6$ dB bandwidth). In addition, the calculated signal to noise ratio using carbon fiber was 37.8 dB, which was sufficient to image a single blood cell, consistent with the previous studies [50,51]. The maximum scanning range was 14.5 mm × 9 mm (fast axis × slow axis), which was sufficient to cover the whole range of the mouse brain. We adjusted the pixel size of the PA image for whole-brain imaging (3 s, 400 × 300 pixels) and localized it to induce a targeted ischemic stroke model (0.5 s, 200 × 100 pixels).

### 2.3. Position-feedback-based water pressure compensation of PAM scanner for precise localization

Since the mirror part of both scanners was submerged in the water as shown in Fig. 2, the dynamic pressure, caused by the water resistance was projected on the perpendicular direction of motion [52]. Therefore, the applied fluid resistance on the mirror surface of both fast and slow axes hindered the proper movement, which caused the scanning position difference between the input and the actual case. To precisely localize the target blood vessel for photothrombosis induction, it was required to compensate for the position error that occurred by water resistance at high scanning speed. We feedbacked the actual scanning voltage of the scanner by the diagnostics connector of the galvanometer scanner driver (GVS series driver, Thorlabs, USA), which did not require the additional hardware components. The feedback of actual scanning voltage of both axes at representative frequencies (5, 10, 25, 50, 75, and 100 Hz) and the control data without water resistance (i.e., in the air) are shown in Fig. 3(a) and 3(b), respectively. Since the higher scanning frequency needed to endure the higher water resistance, the differences between control and actual scanning position were increased according to the enhanced frequency in both fast and slow axes. In addition, actual angle differences of the scanner movement according to various scanning speeds were compared with the ideal case (Supplementary Fig. S3 and Supplementary Text), which showed the necessity of the position-feedback-based water pressure compensation for precise localization.

The overall process of water pressure compensation is described in Fig. 4. In this case, we used the leaf skeleton target as a representative sample and set the scanning range to 7 V, which was also utilized for simultaneously inducing and monitoring the photothrombosis. Compared with the ideal case in Fig. 4(a), the actual scanning voltage was distorted, and the degree of differences was increased according to the enhanced scanning frequency. The position differences in each case are clearly observed in Fig. 4(c), which is the magnified image of the red-dashed box in Fig. 4(b). Since the diameter of the target region for photothrombosis induction is approximately 500 $\mu$m, even the slightest difference in scanning voltage (even in a decimal place) affects the illuminating region of PAM. Although the manually shifting of the field of view for localization is precisely conducted in the air condition, in the water, real scanning voltage feedback must be required to accurately localize the target region and compensate for the signal distortion between the input and the output.

Before conducting water pressure compensation, we measured PAM MAP images of the sample to verify the degree of scanning position error.
caused by external resistance, and the intended target and actual position were observed to be different, which affected the precise targeting (Fig. 4(e)). We initially obtained the actual scanning position using a digital acquisition board (DAQ, PCIe-6323, National Instrument Co., USA)-based on real-time feedback to compensate for the position error (Fig. 4(f)). The actual scanning voltage of the scanner was obtained by the feedback port (i.e., diagnostic connector) of the scanner driver. Second, a specific target region (from P1 to P2 in Fig. 4(g)) was selected and scanning angle of the scanner was compared for the correction of localization. The start and end scanning angle of the input signal were denoted by $\theta'_1$ and $\theta'_2$, respectively, and those of the output signal were denoted by $\theta_1$ and $\theta_2$ (Fig. 4(h)). Then, based on the obtained value, we calculated the angle difference of the start ($\Delta \theta_1$) and end positions ($\Delta \theta_2$) of the scanning range and corrected the applied input signal of the scanner, and we obtained the localized PA images (Fig. 4(i)). The detailed explanation using the representative specific case of the PAM-based localization process is described in Supplementary Fig. S4 and Supplementary Text. In our experiment, the maximum scanning frequency of the slow axis was 2 Hz (laser repetition rate: 40 kHz, total scanning points: 200 × 100), which was not distorted by the water pressure. Therefore, we compensated the scanning voltage difference for solely the fast axis. The performance of position-feedback-based localization results are demonstrated in Supplementary Fig. S5, which used leaf phantom and mouse brain as representative samples.

2.4. Animal preparation

All experimental procedures of this study were conducted in accordance with the Institutional Animal Care and Use Committee of Kyungpook National University (No. KNU-2021-0123). The illumination beam for simultaneously induction and monitoring the target ischemic stroke model adhered to the ANSI limit of 532 nm wavelength. A total of 13 normal healthy Balb/c mice (male, 7–9 weeks old, 20–22 g body weight) were used for this study (4 as controls and 9 as photothermotic models). We used a ketamine solution (100 mg/kg ketamine chloride and 10 mg/kg xylazine mixed solution) to anesthetize the mouse before conducting the experiment. The body temperature of mice was maintained during the experiment by an electronic heating pad combined with the imaging stage. The scalp was cut to expose the skull, and the skull was protected. A photosensitive dye, 0.1 ml Rose Bengal (20 mg/ml; Sigma-Aldrich, USA) with absorption spectrum ranging from 500 to 600 nm (Supplementary Fig. S6), was diluted in saline and filtered with a 0.2 µm pore size Syringe filter (Whatman, UK) before injecting into the tail vein. The preliminary photo-oxidation effect of the Rose Bengal and PAM laser was verified by simulation experiment using the Rose Bengal filled micro-tube with laser illumination (Supplementary Fig. S7 and Supplementary Text). After inducing the target ischemic stroke model, the injury was closed with a 3–0 nylon (Blue nylon, Ailee co., South Korea) suture, and the wound was disinfected with povidone-iodine.
2.5. Triphenyl tetrazolium chloride staining

After 24 h of inducing occlusion, ischemic stroke mice were sacrificed by an overdose of ketamine and xylazine mixture and perfused by phosphate-buffered saline (PBS) to remove blood. After perfusion, the brains were removed and sliced (2 mm thick) by the brain matrix of a mouse (AL-1175, Roboz Surgical Instrument Co., USA). Each section was stored in 1.5 % TTC solution (Sigma-Aldrich, USA) for 30 min at 37 °C.

2.6. Hematoxylin and eosin staining

The brain tissue was fixed and cut into 2 mm thick slices and were kept in 4 % paraformaldehyde for 24 h and embedded in Tissue-Tek O.C. T compound (Sakura Finetek Japan, Japan). Frozen-Section (5 μm) were cut in the coronal plane and thaw-mounted onto Superfrost plus slides (Thermo Fisher Scientific, USA). Sections were then stored at −20 °C until use. The slides were dried for 30 min at room temperature and the samples were fixed on a slide for 20 min with 4 % paraformaldehyde. H&E staining was performed and the slides were observed using Panoramic scan (3D Histech, Hungary) slide scanner.

2.7. Immunofluorescence staining

Immunohistochemical staining to visualize GFAP and Iba1 expression was performed to delineate ischemic infarction. Slides stored at −20 °C were used, and before use, they were taken out at room temperature and dried sufficiently. The sections were blocked with 5 % goat serum in the PBS and were incubated with primary antibody at 4 °C overnight. GFAP (1:100, Abcam, UK) was used as an astrocyte marker and Iba1 (1:100, Abcam, UK) as a microglia marker. Sections were then incubated with secondary antibody Alexa Fluor 488 anti-rabbit (1:200,
Abcam, UK). The tissues were incubated for 5 min with DAPI (2 ml/100 ml, sigma, USA) to visualize cell nuclei. After washing with PBS, mounted with Prolong Gold mounting medium (Thermo Fisher Scientific, USA), fluorescence was observed in Panoramic scan (3D Histech, Hungary) slide scanner.

2.8. Measurement of ischemic lesion volume

Ischemic stroke mice were sacrificed by overdose of ketamine and xylazine mixture and perfused with 0.1 M PBS and 4% paraformaldehyde. After perfusion, brains were extracted and stored in 4% paraformaldehyde for 7 days, and four coronal sections corresponding to +1.28, +0.14, -0.86, and -1.93 were obtained based on bregma using the brain matrix of a mouse (AL-1175, Roboz Surgical Instrument Co., USA). It was dehydrated in 20% sucrose buffer for 24 h and embedded in Tissue-Tek O.C.T compound (Sakura Finetek Japan, Japan). Then, it was divided into frozen-sections with a thickness of 5 μm and mounted onto Superfrost plus slides (Thermo Fisher Scientific, USA). Slides were stained with H&E and observed using Panoramic scan (3D Histech, Hungary) slide scanner.

3. Results

3.1. Simultaneous photothrombosis induction and dynamic vessel monitoring for target ischemic stroke model creation using PAM in vivo

To induce the target ischemic stroke model with a specific infarcted region using PAM, the mice were injected with Rose Bengal dye (20 mg/kg) with an absorption spectrum ranging from 500 to 600 nm (Supplementary Fig. S6). Before induction, we conducted a preliminary experiment of laser illumination on a micro-tube, which was filled with Rose Bengal (equal concentration as used in the experiment) to verify the photo-oxidation effect of the proposed method (Supplementary Fig. S7 and Supplementary Text). Based on the result of phantom study, we compared the results of control (i.e., without Rose Bengal injection) and the proposed method (i.e., with Rose Bengal injection) (Fig. 5) to validate the performance of the PAM-based method. The entire process of inducing and monitoring the ischemic stroke model creation was conducted with the intact skull of the mouse. Before the illumination of the PA laser, vascular maps of the mouse brain were obtained to select the region (Fig. 5(a) and 5(k)). In the case of controls, the target blood vessel (presented by the white arrow) was undamaged after 5 min of irradiation (Fig. 5(b)), in contrast to the pre-acquired vascular map (Fig. 3(a)). However, in the target ischemia model, the selected blood vessel (white arrow in Fig. 3(k)) disappeared after photochemical thrombosis formation following the Rose Bengal injection and localized laser illumination (Fig. 3(l)). These results are demonstrated in the magnified view of ROI (white square of vascular maps) of each case (Fig. 3(c), (d), (m), and (n)). In addition, we continuously monitored the hemodynamics in the target micro-vessel for 5 min while simultaneously inducing a thrombus in a precisely localized area as indicated by white dotted box in Fig. 3(c) and Fig. 3(m) (Fig. 3(e)-(j) and Fig. 3(e)-(q)). Supplementary movies 1 and 2 illustrate the whole PA images of the vessel blockage process from the sequence of 60 frames for 5 min. In the target ischemic stroke model, broken target blood vessel was observed in real-time (Fig. 3(o)-(t) and Supplementary movie 2), unlike the control case (Fig. 3(e)-(q) and Supplementary movie 1), where the shape and intensity of the selected blood vessel were maintained. These results confirmed that the target ischemic stroke model using PAM was precisely induced and the entire formation process of infarction was clearly observed with continuous monitoring.

Supplementary material related to this article can be found online at doi:10.1016/j.pacs.2022.100376.

3.2. Dynamic localization of infarcted site to induce the target ischemic stroke mouse models

To verify the reproducibility of the proposed PAM-based target ischemic stroke model creation method, we induced a total of nine photothrombosis induction and demonstrated three representative cases using our PAM-based method (Fig. 6). In mouse 1, we dynamically localized ROI presented by a blue and yellow square in before and after PA brain MAP images, respectively (Fig. 6(a), (b)). We magnified the target ROI of the mouse brain (Fig. 6(c), (d)) for the broken target blood vessel. The PA intensity profiling results (dashed lines in Fig. 6(c), (d)) before and after photothrombosis are displayed in Fig. 6(e) in blue and green color, respectively. The blocked target micro-vessel after inducing occlusion by water pressure compensated PAM with precise localization (Fig. 6(e)). To evaluate the performance of the presented method for photothrombosis induction, we conducted additional experiments on different mice, and brain PAM MAP images of the representative cases before induction are shown in Fig. 6(f) and (k). In accordance with the result shown in Fig. 6(a)-(e), intended infarcted regions were precisely localized, and target blood vessels were found to be broken in three different mouse models, which were qualitatively and quantitatively verified by whole-brain PAM MAP images after induction (Fig. 6(g) and (l)), magnified images of each ROI (Fig. 6(h), (i)-(n)), and line profiles of PA amplitude (Fig. 6(j) and (o)). Hence, the reproducibility and repeatability of the PAM-based target ischemic stroke model induction were verified, which is the closest clinical animal model possible. In addition, it was verified that photothrombosis was not formed when the PA laser was irradiated without PAM-based localization (Supplementary Fig. S8, and Supplementary Text). These results confirmed that combining precise localization using PAM and activated photosensitizer (e.g., Rose Bengal) following PA laser illumination is essentially required to induce the target ischemic stroke model.

To validate the effectiveness of inducing a target ischemic stroke model with a localized target region using PAM, we conducted quantitative analyses in terms of the PA amplitude (Fig. 7). After simultaneous monitoring and induction of occlusion for 5 min (red color, n = 9) compared to controls (black color, n = 4), the total average PA amplitude of the target region with normalization decreased with the passage of time (Fig. 7(a)). The target region for calculating the average intensity is the illuminating area of each mouse (e.g., Fig. 5(o)-(t) for mouse 1). Consequently, the average total PA amplitude of the target ischemic stroke model decreased by 77.7% compared to the beginning of the experiment (0.9305–0.2077). However, in controls, the average total PA amplitude increased by 3.1% (0.9123–0.9408), but this value was within the error range. In addition, to demonstrate the efficacy of precise localization of the target region, we compared average PA signal for representative parts of the mouse brain (target region, whole brain, and left hemisphere) before and after induction (Fig. 7(b)). Unlike the whole brain and left hemisphere, which had little difference before and after (0.0508–0.04994 and 0.05941–0.05562, respectively) induction, the average total PA signal of the target region decreased by 77.7%. The p-values showed that PAM precisely blocked the target vessel, and no other sites. These results verified the reproducibility of the proposed PAM-based simultaneous vessel monitoring and photothrombosis induction of target ischemic stroke model development. Moreover, the target area was precisely localized without affecting other regions of the mouse brain.

3.3. Comparison of infarcted lesion in conventional and PAM-based methods

We quantitatively compared conventional and PAM-based inducing methods in terms of both infarcted area and intracortical depth (Fig. 8). The accuracy of the correlation between PAM image and H&E staining results are shown in Supplementary Fig. S9 and Supplementary Text, by comparing results at the same position. To measure the contralesional
Fig. 5. Comparison results of simultaneous photothrombosis induction and vessel monitoring of target ischemic model between control and PAM. (a), (b) Mouse brain PAM MAP image of control before and after illumination. (c), (d) Magnified PAM MAP images of the ROI marked by the white square in (a) and (b), respectively. (e)-(j) PAM MAP images of the selected vessel (white dotted box in (c)) measured at 1 min intervals for 5 min. (k), (l) Mouse brain PAM MAP images of the target ischemic stroke model before and after illumination. (m), (n) Magnified PAM MAP images of the ROI marked by the white square in k and l, respectively. (o)-(t) PAM MAP images of the selected vessel (white dotted box in (m)) measured at 1 min intervals for 5 min. MAP, maximum amplitude projection.
and remaining ipsilesional cortical areas, four representative coronal sections were selected between approximately 1.28 mm anterior to 1.93 mm posterior to bregma (+1.28, +0.14, −0.86, and −1.93 mm). We stained each section with H&E as shown in Fig. 8 (a) and 8 (f). Based on the H&E stain results, we extracted the infarcted area and overlaid it on coronal section templates of each method (Fig. 8 (b)–(e), (g)–(j)), and differences in infarcted lesions were verified. There were several undamaged coronal section templates as shown in Fig. 8 (g) because of the PAM-based minimized infarcted region. In addition, we quantitatively evaluated the infarcted lesion in terms of depth and area (Fig. 8 (k), (l)). The infarction of all sections had more depth in the conventional method than in the proposed PAM method, and relative differences between the two methods were 94.0 %, 74.2 %, 86.9 %, and 83.1 %, respectively (Fig. 8 (l)). Based on this, the proposed PAM-based target ischemic stroke model-inducing method induced minimized infarction of both depth and volume by precisely targeting the local area compared with the conventional photothrombosis inducing method.

3.4. Histological validation of the induced model

To identify and evaluate the infarct region of the induced target ischemic stroke mouse model, three different histological analyses, including TTC staining (visualization of hypoxic brain tissue), immunostaining (confirmation of GFAP and Iba1; ischemic cerebral infarction biomarkers), and H&E staining (brain tissue and red blood cells confirm), were employed. TTC staining was applied to visualize hypoxic
Fig. 7. Quantitative PA amplitude analyses of target ischemic stroke model using PAM. (a) PA amplitude comparison between control and target ischemic stroke model for 5 min of simultaneous inducing and monitoring. (b) Comparison between PA amplitudes before and after photothrombotic inducing for three different regions of mouse brain (target region, whole brain, and left hemisphere); ns, \( p > 0.05 \). *** \( p \leq 0.001 \).

Fig. 8. Comparison of infarcted depth and area between conventional and PAM-based inducing methods. (a) H&E stained brain section using a conventional method. (b)–(e) Reconstructions of each infarcted site overlaid on templates of the coronal section using a conventional method. (f) H&E stained brain section using PAM. (g)–(j) Reconstructions of each infarcted site overlaid on templates of the coronal section using PAM. (k), (l) Graphs showing the comparison of the infarcted cortical depth and area, respectively, between the two methods.
brain tissue and defined the regions of cerebral infarction, intact tissues, and penumbra in a cerebral infarction model. In TTC staining, vital tissue was stained red and necrotic tissue, which had induced infarcts by thrombus, was not stained (white color) [4,53,54]. Fig. 9(a)–(e) demonstrate the whole brain of control, conventional method, and PAM-based target ischemic stroke models obtained by perfusing PBS after three days of photothermalysis induction. Compared with the control mouse (Fig. 9(a)), the experimental group mouse (i.e., conventional and PAM-based methods; Fig. 9(b)–(e)) showed local damage to the PAM irradiation site. Fig. 9(b), (g), (j) show that the infarcted region of the conventional method was significantly larger than that of the proposed method. In the case of the PAM-based method, retention of blood and dyes of the experimental group (Fig. 9(h)–(j)) was also confirmed in the cross-section of the irradiation site before TTC staining, in contrast to the results obtained for the control group (Fig. 9(i)). In addition, the infarcted site of each model was consistent with the infarcted area after TTC staining. The cortical lesions of the target infarcted area after TTC staining. The cortical lesions of the target ischemic stroke model were identified through immunostaining as the biomarkers of ischemic stroke. GFAP is found in astrocytes that are the main structural and functional support for neurons [55,56]. In addition, Iba1 was utilized to validate the photothermbotic model. It is a protein for detecting the activation of microglia in the ischemic brain. Rapid activation of microglia due to cerebral infarction leads to the release of inflammatory mediators that exacerbate ischemic injury or aid repair according to other molecular signals received by microglia receptors [57,58]. Unlike the control group, which showed a low concentration of GFAP as shown in Fig. 10(a)–(c), the PAM induced target ischemic stroke model showed an increased amount of GFAP because of necrosis of the infarcted region (Fig. 10(d)–(f)). Iba1 in the normal brain was merely detected (Fig. 10(g)–(i)), whereas in the PAM-based model, the increased number of activated microglia increased Iba1 in the peri-infarct area because of cerebral infarction (Fig. 10(j)–(l)). In addition to the TTC and immunostaining (GFAP and Iba1) analyses, we conducted H&E staining to identify the histological lesions (stained in purple color) and red blood cells (stained in red color) of the infarcted region. Compared to normal brain tissue (Fig. 10(m)–(o)), an infarcted lesion was clear in the target ischemic stroke model using PAM (Fig. 10(p)–(r)). The yellow arrows in Fig. 10(r) indicate the blood retention due to local tissue lesions and infarction in the PAM-based model, which further confirms that localization of the target vessel was well demonstrated using the proposed method.

4. Discussion

In this paper, we reported the novel creation method of the target ischemic stroke model by single-source-based simultaneous vessel monitoring and dynamic photothermalysis inducing method at precise localization using PAM. The position error of the scanner caused by generated water pressure on the surface was compensated using the obtained feedback from the actual scanning voltage. Consequently, we induced and monitored the target ischemic stroke model for the precisely tracked blood vessel with an intact skull. The reproducibility and efficacy of our method were proved by repeated experiments, and the results of inducing target ischemic stroke model were evaluated by quantitative statistical analyses comparing PA amplitude variance, infarcted sizes, and histological analyses (TTC, GFAP, Iba1, and H&E).

We developed a precise feedback-based position compensation method on a micrometer scale due to the small diameter of the blood vessel to form clots. The position difference error, which was caused by the fast scanning speed to induce occlusion, was delicately controlled by comparing the actual position and set value (Fig. 4 and Supplementary Fig. S4). The performance of the position-feedback method was validated by vascular tracking of the mouse brain and leaf phantom.
Fig. 10. Comparing staining results using GFAP, iba1, and H&E staining between control and PAM-based photothrombotic models. (a)–(c) GFAP stained brain section of control. (d)–(f) GFAP stained brain section of the target ischemic stroke model. (g)–(i) Iba1 stained brain section of control. (j)–(l) Iba1 stained brain section of the target ischemic stroke model. (m)–(o) H&E stained brain section of control. (p)–(r) H&E stained brain section of the target ischemic stroke model.
In our experiment, the average scanning range of the fast-axis scanner for single vessel localization was 0.33 v (470 µm). However, the average position difference was 0.43 v (612 µm), which scanned completely different positions from the set value. This difference demonstrates that the localization of the intended infarct site would not have been possible without the position feedback-based localization method. Our water pressure compensation method for localization is agent-free and does not require additional hardware components to image the target region. The position-feedback-based localization method is not only beneficial to our system, but can be potentially utilized in universal systems in which part of the scanner (e.g., galvanometer scanner and microelectromechanical system) is implemented where the resistance of the medium exists (e.g., water for PA imaging). Since the medium resistance increases according to the increased scanning speed, which raises the degree of position difference, our position-feedback-based localization method has the potential of high versatility.

The conventional in vivo photothermobilic models for ischemic stroke were found to be non-specific and have extensive brain damage and low reproducibility due to inaccurate targeting. In contrast, the PAM-based model demonstrates reproducible occlusion with specific targeting without unnecessary brain damage (Fig. 8). The differences between PAM-based method and the conventional method were not clearly shown in the PAM MAP image (Supplementary Fig. S10); however, the degree of the infarcted lesion was significantly different in histological analyses (Fig. 8 and Fig. 9). Hence, the target ischemic stroke model using PAM is the most practical and feasible model for studying cerebral ischemia. Although the PA signal at out of ROI after photothermosis induction decreased in a few cases, brain damage or infarcted lesions were not observed (Supplementary Fig. S8), which proves that the disappeared blood vessels out of ROI are instantaneous reactions, and not permanent damage. To avoid the signal decrease in PAM images, there is a need to block the spread of unnecessary light beams from the skull surface.

In addition, the presented PAM-based simultaneous vessel monitoring and photothermosis induction method can be improved further. First, the total measurement time can be reduced by using lasers with a high repetition rate. By implementing the high-speed source instead of the one utilized in this experiment, the time gap between each image can be reduced, which can result in more short-term variances during the inducing and monitoring process including hemodynamics and neuron activities. Since the position-feedback-based localization method was applied, targeting the exact position at a higher scanning speed was possible. Second, the imaging depth of the system can be enhanced to image the selected blood vessel, which is in a deep depth. Although our PAM system provides the image of the distinctive blood vascular features of the mouse brain (e.g., sagittal sinus, coronal suture, and middle cerebral artery), if the penetration depth of PAM can be adjusted according to the depth of the target vessel, a new approach for vessel-based stroke can be developed. The penetration depth of PAM can be controlled by adjusting the optical components (e.g., objective lens, acoustic lens, controlled beam diameter by lens pair, and center frequency of transducer). Third, we selected the specific target vessel, which is a branch of the anterior cerebral artery according to anatomical information in this experiment. To produce a closer human resembled photothermobilic mouse model, it should differentiate between the artery and the vein. Therefore, additional PA sources are required to obtain flow speed, oxygen saturation, and hemoglobin concentration, which provide functional information of vessel to be classified.

5. Conclusion

In this study, the dynamic inducing and monitoring method of the target ischemic stroke model using PAM was demonstrated. By developing this proposed position error compensation method with PAM, we have provided a promising localization method for universal systems, which uses a scanner that withstands the resistance of the medium. Using this system, we have precisely localized the target region for dynamic induction and monitoring of photothermobilic model in vivo. We presented local ischemic damage on an exact target using developed PAM for exact localization with high reproducibility and minimized infarcted lesions. Because of these capabilities of the PAM-based method, it is possible not only to set up various models depending on the size and extent of the lesion and anatomical variations, but also to observe the hemodynamics of infarcted lesion formation. Furthermore, it can be used in vessel-targeted stroke research with continuous monitoring from formation to recovery based on the functional information including neovascularization and vessel density variations.

CRediT authorship contribution statement

D.S., S.L., and S.H. designed and performed all experiments. D.S. and S.L. wrote the original manuscript. D.S., S.L., and S.H. edited the manuscript. D.S. and S.H. developed the PAM system. S.L. conducted histological analyses. D.S., S.H., J.L., and S.P. performed statistical analyses and conducted a photoacoustic signal-based quantitative evaluation. Y. H. and J.K. supervised the experiment and edited the paper. H. K. and M.J. supervised the study, provided input for all experiments and the study concept, and edited the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pacs.2022.100376.

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