Journal of Visualized Experiments

Modeling stroke in mice: directed brain injury using photothrombotic model

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62536R1
Full Title:	Modeling stroke in mice: directed brain injury using photothrombotic model
Corresponding Author:	Gemma Llovera Ludwig-Maximilians-Universitat Munchen Munich, Bayern GERMANY
Corresponding Author's Institution:	Ludwig-Maximilians-Universitat Munchen
Corresponding Author E-Mail:	Gemma.Llovera-Garcia@med.uni-muenchen.de
Order of Authors:	Gemma Llovera
	Kelsey Pinkham
	Arthur Liesz
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Neuroscience
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Munich, Bayer, Germany
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Modeling Stroke in Mice: Directed Brain Injury using Photothrombotic Model

AUTHORS AND AFFILIATIONS:

Gemma Llovera[1], Kelsey Pinkham[1], Arthur Liesz[1,2]

[1]Institute for Stroke and Dementia Research, LMU Munich, Feodor-Lynen-Strasse 17, Munich, Germany

[2]Munich Cluster for Systems Neurology (SyNergy), Munich, Germany

Email addresses of co-authors:

Gemma Llovera (gemma.llovera-garcia@med.uni-muenchen.de)

Kelsey Pinkham (kelsey.pinkham@med.uni-muenchen.de)

Arthur Liesz (arthur.liesz@med.uni-muenchen.de)

Corresponding authors:

Gemma Llovera (gemma.llovera-garcia@med.uni-muenchen.de)

KEYWORDS:

stroke, brain ischemia, animal model, photothrombotic, permanent, Rose Bengal, laser illumination

SUMMARY:

Described here is the photothrombotic stroke model, where a stroke is produced through the intact skull by inducing permanent microvascular occlusion using laser illumination after administration of a photosensitive dye.

ABSTRACT:

Stroke is a leading cause of death and acquired adult disability in developed countries. Despite extensive investigation for novel therapeutic strategies, there remain limited therapeutic options for stroke patients. Therefore, more research is needed for pathophysiological pathways such as post-stroke inflammation, angiogenesis, neuronal plasticity, and regeneration. Given the inability of *in vitro* models to reproduce the complexity of the brain, experimental stroke models are essential for the analysis and subsequent evaluation of novel drug targets for these mechanisms. To overcome the so-called replication crisis, detailed standardized models for all procedures are urgently needed. As an effort within the ImmunoStroke research consortium, a standardized photothrombotic mouse model using an intraperitoneal injection of Rose Bengal and the illumination of the intact skull with a 561 nm laser is described. This model allows the performance of stroke in mice with allocation to any cortical region of the brain without invasive surgery; thus, enabling the study of stroke in various areas of the brain. In this video, the surgical methods of stroke induction in the photothrombotic model as well as histological analysis are demonstrated.

INTRODUCTION:

Ischemic stroke remains a principal cause of death and acquired adult disability in developed countries in the 21st century accounting for approximately 2.7 million deaths in 2017 worldwide[1]. Even with the immense efforts of the scientific community, few treatments are available. Furthermore, with such high exclusion criteria, these already limited options are not accessible to many patients. Therefore, there is an urgent need for novel treatments to improve functional recovery after stroke.

Considering the incapability of *in vitro* models to replicate the complex interactions of the brain, animal models are essential for preclinical stroke research. Mice are the most frequently used animal model in the stroke research field. The majority of these mouse models aim to induce infarctions by blocking the blood flow within the middle cerebral artery (MCA) since the majority of human stroke lesions are located in the MCA territory[2]. Although these models very well reproduce human stroke lesions, they involve complex surgeries and high infarct volume variability.

Since Rosenblum and El-Sabban's proposal of the photothrombotic model in 1977[3], and later the application of this model to rats Watson et al.[4], it has become widely used in ischemic stroke research[5,6]. The photothrombotic stroke model induces a local and defined cortical infarct as a result of the photoactivation of a light-sensitive dye previously injected into the blood flow. This causes local thrombosis of the vessels in the areas exposed to light. Briefly, upon exposure to light from the injected photosensitive dye, localized oxidative injury of the endothelial cell membrane is induced, leading to platelet aggregation and thrombus formation, followed by local disruption of cerebral blood flow[7].

The principal advantage of this technique resides in its simplicity of execution and the possibility to direct the lesion to the desired region. Unlike other experimental stroke models, minor surgical expertise is needed to perform the photothrombotic stroke model as the lesion is induced through simple illumination of the intact skull. Moreover, the well-delimited borders (**Figure 2A** and **Figure 5B**) and the flexibility to induce the lesion to a specific brain region can facilitate the study of cellular responses within the ischemic or intact cortical area[8]. For these reasons, this approach can be suitable for the study of cellular and molecular mechanisms of cortical plasticity.

Over the past few decades, the growing concern regarding the lack of reproducibility between research groups has been coined the so-called replication crisis[9]. After the coordination of the first preclinical randomized controlled multicenter trial study in 2015[10], a proposed tool to improve preclinical research[11-13], it was confirmed that one cause for failing reproducibility between preclinical studies from independent laboratories was the lack of sufficient standardization of experimental stroke models and outcome parameters[14]. Accordingly, when the ImmunoStroke consortium was established (https://immunostroke.de/), which aims to understand brain-immune interactions underlying the mechanistic principles of stroke recovery, the standardization of all the experimental stroke models among each research group was essential.

Described here is the standardized procedure for the induction of the photothrombotic model as

used in the above-mentioned research consortium. Briefly, an animal underwent anesthetics, received a Rose Bengal injection (10 µL/g) intraperitonally, and then the intact skull, 3 mm left from bregma, was immediately illuminated by a 561 nm laser for 20 min (**Figure 1**). Additionally, a related histological and behavioral method to analyze the stroke outcome in this model is reported. All methods are based on standard operating procedures developed and used in the laboratory.

PROTOCOL:

The experiments reported in this video were conducted according to the national guidelines for the use of experimental animals, and the protocols were approved by the German governmental committees (Regierung von Oberbayern, Munich, Germany). Male C57Bl/6J mice, 10–12 weeks old, dispatched by Charles River Germany were used in this study. The animals were housed under controlled temperatures (22 °C ± 2 °C), with a 12 h light-dark cycle period and access to pelleted food and water *ad libitum.*

1. Preparation of the material and instruments

1.1. Dissolve Rose Bengal in 0.9% saline solution to reach a final concentration of 10 mg/mL. Connect the heat blanket to keep the operation area warm and maintain the mouse body temperature during anesthesia at 37 °C.

1.2. Prepare scissors, forceps, pieces of cotton, dexpanthenol eye ointment, and suture material. Prepare a syringe with saline solution (without needle) to maintain the operation area hydrated. Prepare the anesthesia gas (100% O_2 + isoflurane).

2. Preparation of the animal

2.1. Inject analgesia 30 min before surgery (4 mg/kg Carprofen and 0.1 mg/kg Buprenorphine).

2.2. Record the mouse body weight to adjust the dose of Rose Bengal to be injected (10 µL/g i.e., 100 µg/g).

2.3. Place the mouse in the induction chamber with an isoflurane flow rate of 4% to anesthetize it until the spontaneous movement of the body and vibrissae stops.

2.4. Transfer the mouse into the stereotactic frame and place it in a prone position with its nose into the anesthesia mask. Fix the animal and maintain the isoflurane concentration at 4% for another minute. Then reduce and maintain the isoflurane concentration at 2%.

2.5. Gently insert the rectal probe to monitor the temperature throughout the surgical procedures. Set the associated feedback-controlled heating pad to maintain the mouse body temperature at 37 °C.

2.6. Apply dexpanthenol eye ointment to both eyes and clean the skin and the surrounding fur with a disinfectant agent.

3. Photothrombosis model

3.1 Make a 2.0–2.5 cm longitudinal incision and retract to expose the skull. Perform the skull exposure with a single cut to avoid wound complications.

3.2 Remove the periosteum gently with cotton and identify the coronal sutures.

3.3 Put on the protective glasses, switch on the 561 nm laser and mark the bregma +3 mm left.

3.4 Switch off the laser, hook a sticker with a 4 mm diameter hole placed at the marked coordinates mentioned above.

3.5 Inject the mouse with Bengal Rose (10 µL/g), intraperitoneally. Place the laser beam at 4–5 cm from the skull, switch on the 561 nm laser and illuminate the skull for 20 min.

3.6 Apply two drops of 0.9% saline to the skull to rehydrate, suture the wound, and place the animal in a recovery chamber at 37 °C to recover from anesthesia. After 1 h, return the mice to their cages in a temperature-controlled room.

3.7 Inject analgesia every 12 h for 3 days after surgery (4 mg/kg Carprofen and 0.1 mg/kg Buprenorphine).

4. Sham operation

4.1. Carry out two different procedures of Sham operations as described in steps 4.1.1 and 4.1.2.

4.1.1. Perform all the procedures identically to the operation described above. Inject Rose Bengal without switching on the laser. After 20 min under anesthesia, allow the animals to stay in the recovery chamber for 1 h to recover, before being returned to their cages.

4.1.2. Perform all the procedures identically to the operation described above, switching on the laser. Do not inject Rose Bengal. After 20 min of laser illumination, allow the animals to stay in the recovery chamber for 1 h to recover from anesthesia, before being returned to their cages.

5. Laser speckle

5.1. Connect the heat blanket to maintain the operation area warm and maintain the mouse body temperature during anesthesia at 37 °C.

5.2. Place the mouse into the induction chamber with an isoflurane flow rate of 4% to anesthetize it until the spontaneous movement of the body and vibrissae stops and then transfer the mouse into the stereotactic frame.

5.3. Place the mouse in a prone position with its nose into the anesthesia mask. Fix the animal and maintain the isoflurane concentration at 4% for another minute. T hen reduce and maintain it at 2%.

5.4. Gently insert the rectal probe to monitor the temperature throughout the surgical procedures. Set the associated feedback-controlled heating pad to maintain the mouse body temperature at 37 °C and apply dexpanthenol eye ointment to both eyes. Clean the skin and the surrounding fur with a disinfectant agent.

5.5. Make a 2.0–2.5 cm longitudinal incision and retract to expose the skull. Perform the skull exposure with a single cut to avoid wound complications.

5.6. Place the sterotactic frame under the laser speckle and adjust the height to obtain a sharp image. Focus the laser speckle perfusion imaging (LSI) camera on the cranial window. Configure the high resolution laser speckle imaging (LSI) camera system as previously described[15].

5.7. Acquire data from a 1 cm x 1 cm field of view using a 785 nm wavelength and 80 mW lasers with a frame rate of 21 images/s at a working distance of 1 cm for 1 min.

5.8. After imaging, apply two drops of 0.9% saline to the skull to rehydrate, suture the wound and place the animal in a recovery chamber at 37 °C to recover from anesthesia for 1 h. After 1 h, return the mice to their cages in a temperature-controlled room.

6. Neuroscore

NOTE: For the neurological deficit analysis, a modified neurological scale published by Eckenstein et al. in 1997 is used[15].

6.1. Score the animals for general (**Table 1**) and focal deficits (**Table 2**). This composite scale ranges from 0 (no deficits) to 46 (severe impairments).

6.2. Perform the neuroscore always at the same time each day and use surgical clothes to keep a neutral smell.

6.3. Habituate the mice for 30 min in the room with an open cage before the test and allow them to observe each item for 30 s.

7. Perfusion

7.1. Prepare a 20 mL syringe containing PBS-heparin (2 U/mL) and place it 1 m above the bench to facilitate/ensure gravity-driven perfusion.

7.2. Inject intraperitoneally 100 µL of ketamine and xylazine (120/16 mg/kg body weight, respectively). Wait for 5 min and corroborate the cessation of spontaneous body movement and vibrissae.

7.3. Fix the animal in a supine position and disinfect the abdominal body surface with 100% ethanol. Make a 3 cm long incision in the abdomen; cut the diaphragm and the ribs to completely visualize the heart.

7.4. Make a small incision in the right atrium and insert the perfusion cannula into the left ventricle and perfuse with 20 mL PBS-heparin.

7.5. After perfusion, decapitate the animal and remove the brain, freeze it using dry ice and store them at -80 °C until further use.

8. Infarct volumetry

8.1 Cryosectioning: Cut the brain serially on a cryostat to 20 µm thick sections every 120 µm on slides. Store the slides at -80 °C until use.

8.2 Cresyl violet (CV) staining

8.2.1 To prepare the staining solution, mix 0.5 g of CV acetate in 500 mL of H_2O. Stir and heat (60 °C) until the crystals are dissolved. Allow the solution to cool and store it in a dark bottle. Reheat to 60 °C and filter (paper filter) before every use.

8.2.2 Dry the slides at room temperature for 30 min. Place them in 95% ethanol for 15 min, followed by 70% ethanol for 1 min, and afterwards in 50% ethanol for 1 min.

8.2.3 Place the slides in distilled water for 2 min, refresh the distilled water, and place the slides in water again for 1 min. Then, place the slides in the pre-heated staining solution for 10 min at 60 °C. Wash the slides twice in distilled water for 1 min.

8.2.4 Place the slides in 95% ethanol for 2 min. Then place them into 100% ethanol for 5 min, refresh the 100% ethanol and place the slides in 100% ethanol again for 2 min. Afterwards, cover the slides with a mounting medium.

8.2.5 Analysis: Scan the slides and analyze the indirect infarct volume by the Swanson method[16] to correct for edema: Ischemic area = (ischemic region)-((ipsilateral hemisphere) - (contralateral hemisphere)).

9. Tunel staining (*in situ* apoptosis detection kit)

9.1. Dry the slides, post-fix in 4% paraformaldehyde in PBS (ph 7.4) for 10–20 min at RT. Wash in PBS, post-fix in precooled ethanol: acetic acid 2:1 for 5 min at -20 °C.

9.2. Wash in PBS and apply equilibration buffer (10 s to a maximum of 60 min at RT) and apply working strength TdT enzyme (1 h at 37 °C in humidified chamber)

9.3. Apply working strength stop/wash enzyme (10 min at RT), wash in PBS and apply warmed (RT) working strength anti-digoxigenin conjugate (30 min at RT in dark)

9.4. Wash in PBS, incubate with DAPI for 5 min at RT and mount the slides with fluoromount media.

REPRESENTATIVE RESULTS:

The model described here is a photothrombotic stroke model by Rose Bengal injection and intact skull illumination for 20 min, at a constant 561 nm wavelength and 25 mW output power at the fiber. Although the complete photothrombotic surgery lasts 30 min, the animal is kept under low anesthesia and the brain damage is moderate. Approximately 10 min after transfer to their cages, all the animals were awake, freely moving in the cage, and interacting with littermates.

Infarct volumetry was performed using cresyl violet stained serial coronal brain sections 24 h after stroke induction (**Figure 2A**). The mean infarct volume was 29.3 mm^3, representing 23% of one brain hemisphere. Moreover, the variability of this stroke model is exceptionally low with a standard deviation of approximately 3.5% (**Figure 2B**). The lesion area encompasses the motor cortex without the affection of subcortical structures.

Phototrombosis lesion caused a moderate, long-term sensorimotor impairment, indicated by the composite Neuroscore[17] (**Figure 3**); general and focal deficits were measured 24 h, 3 days and 7 days after surgery. The general Neuroscore has five items, including the evaluation of the fur, ears, eyes, posture, and spontaneous activity, with a maximum score of 18 (**Table 1**). The focal Neuroscore comprises seven items, including the evaluation of body symmetry, gait, climbing, circling behavior, forelimb symmetry, compulsory cycling, and whiskers response, with a maximum score of 28 (**Table 2**). Stroke animals had a significant change in the composite neuroscore 24 h after surgery compared to Sham-operated animals. These differences persisted, although stroke mice improved over time (**Figure 3**).

Mortality during the observation time rarely occurs in 1–2% of the animals. In this report, none of the 10 animals studied had to be excluded and all of them survived the 7-day observation period. The body weight and temperature changes in the mice were monitored at 24 h, 3 days, and 7 days after surgery (**Figure 4A**,**B**). Data showed that body weight and temperature were decreased 24 h after surgery only in the Rose Bengal + illumination group, but recovered to the level of the Sham-operated animals in 3 days after surgery.

To confirm an induction of ischemic changes, 24 h after surgery, the animals underwent a laser

imaging test. A laser speckle contrast imaging measured blood perfusion of the cortex for a duration of 1 min and an averaged color-coded picture was obtained for each animal, showing that Rose Bengal or laser illumination alone do not produce a lesion, while simultaneous application of Rose Bengal and laser illumination generate a round hypoperfused area of 4 mm diameter surrounded by a narrow oligemic zone (**Figure 5A**). In addition, a cresyl violet and Tunel staining for assessment of the infarct volume 24 h after surgery revealed no tissue damage either in Rose Bengal or laser illumination surgeries. On the other hand, Rose Bengal + laser illumination generated a well-demarcated lesion (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Table 1: General Neuroscore. For each of the five general deficits measured, animals can receive between 0 and 4 points depending on the severity. The scores on the five areas are then summed to provide a total general score ranging from 0–18.

Table 2: Focal Neuroscore. For each of the seven general deficits measured, animals can receive between 0 and 4 points depending on the severity. The scores on the five areas are then summed to provide a total general score ranging from 0–28.

Figure 1: Photothrombosis (PT). Diagram depicting the photothrombotic area, 3 mm from Bregma. The green dot indicates the position of the laser.

Figure 2: Volumetric infarct analysis and infarct outcome 24 h after PT. (**A**) Representative cresyl violet stained coronal brain, sections every 120 µm at 24 h after PT. Dashed lines demarcate the lesion area. (**B**) Infarct volume analysis of 10 brains (each dot representing one individual brain) 24 h after PT. The horizontal red line represents the mean (29.32 mm^3), error bars indicate standard deviation (3.45 mm^3).

Figure 3: Neuroscore for functional deficits after PT. Composite Neuroscore before, 24 h, 3 days, and 7 days after PT. BL = before PT, RB = Rose Bengal. n = 5 per group. *$p < 0.05$.

Figure 4: Body weight and temperature analysis after PT. (**A**) Body weight and (**B**) temperature was slightly reduced in PT animals compared to Sham-operated groups at 24 h and it recovered 3 days after PT. BL = before PT, RB = Rose Bengal. n = 5 per group.*$p < 0.05$.

Figure 5: Lesion confirmation after PT. (**A**) Laser Speckle imaging (**B**) Cresyl violet (upper panels) and Tunel staining (lower panels) confirmed the lesion only after administration of Rose Bengal and subsequent laser illumination. RB = Rose Bengal. Scale bar = 1,000 µm in upper panel B, scale bar = 20 µm in lower panel B.

DISCUSSION:

The presented protocol describes the experimental stroke model of photothrombosis by illuminating the intact skull with a 561 nm laser, with a previous intraperitoneal injection of Rose Bengal. Until recently, the use of this model has been low but is steadily increasing.

Mortality during stroke induction in this model is absent. The overall mortality of less than 5% arises during operation due to anesthesiological complications or sacrifice after meeting the exclusion criteria. To warrant the low variability of this model and its reproducibility, the following exclusion criteria are suggested: 1) operation time longer than 30 min; 2) infection of the suture; 3) bite wound; and 4) no infarct or no fore asymmetry at 24 h after PT.

A widely used experimental stroke models is the transient occlusion of the MCA, by using a suture filament, which is introduced in the internal carotid artery until the silicon-coated tip occludes the origin of the MCA. This model allows the reperfusion by removing the filament and mimics the human clinical scenario, in which there is a restoration of the cerebral blood flow after spontaneous or therapeutic (rtPA) lysis of an embolic clot[18,19]. However, it involves a complex surgery with high variability of the final infarct and high mortality rate[10]. In contrast, the permanent occlusion of the MCA distal of the lenticulostriatal arteries can be achieved by coagulation of the artery[20,21], which induces locally defined lesions in the neocortex[22]. Although this model has a lower mortality rate, it requires invasive surgery to the animal by trepanation of the skull over the MCA to later coagulate it[23]. Consequently, high surgical skills are required for a successful and unbiased *in vivo* stroke study.

Compared to other brain ischemia models, the photothrombotic model as carried out in this video has the advantage of no craniotomy or major surgery on the animal unlike other models that involve complex surgeries or brain craniotomy. Moreover, the simple execution of the model makes the surgery accessible to many with low time-consuming training. Low mortality, moderate infarct volume, and flexibility to induce the lesion to a specific brain region, emphasize the advantage of this experimental paradigm for brain regeneration and stroke studies[24–27].

Despite the obvious advantages, a few limitations of this stroke model need to be taken into consideration. The long exposure of anesthetics to the animal might be a critical factor to take into account, as the impact of anesthetics on neuroprotection and stroke outcome is already well-known[28]. Although the duration of this surgical procedure takes approximately 30 min, the animal can be under low anesthetic concentrations due to the minimal manipulation of the animal during the 20 min of laser illumination. Because this model induces moderate brain injuries, only minor behavioral deficits are detectable. Thus, more advanced test systems with higher sensitivity and qualitative test parameters, such as the skilled reaching test[29] and Neuroscore[17], as described in here, maybe more suitable for detecting long-term functional outcomes in this model. Finally, due to the permanent aggregation of the platelets into the illuminated blood vessels, no reperfusion can be obtained, which is a feature observed in a substantial percentage of stroke patients due to spontaneous clot lysis or therapy[30].

A similar phototrombotic stroke model was published in 2013 by Labat-gest and Tomasi, describing a PT protocol using a cold light lamp instead of a 561 nm green laser[8]. Both laser and cold light sources can be used to induce Rose Bengal excitation. An advantage of laser-based light sources over cold light lamps is that lasers can be used to target individual surface arterioles for *in vivo* vessel-specific clotting[31]. Although we were not targeting specific arterioles, we used a 561 nm green laser for brain illuminationn and phototrombosis induction, because of the Rose

Bengal absortion peak at 562 nm. To ensure a proper laser intensity during the illumination, the Cobolt Monitor Software-6.1.0.0 was used to calibrate the laser. Moreover, in the present study a Rose Bengal dosage of 10 µL/g (100 µg/g) was sufficient to induce phototrombosis, while the previous protocol reported a higher dose (150 µg/g)[8]. In addition, the protocol provides a behavioral method to analyze the stroke outcome (Neuroscore) and an additional sham-control group (laser illumination) in order to prove that the laser itself does not produce any tissue damage, so only the combination of Rose Bengal + laser illumination induce a brain lesion.

Taken together, this non-invasive straightforward surgical procedure enables high reproducibility and directionality of the stroke lesion to the brain alongside the possibility of long-term observation due to minimal mortality. This photothrombotic stroke model is distinguished as a valuable experimental paradigm for basic and translational stroke research.

ACKNOWLEDGMENTS:

We thank all our collaboration partners of the Immunostroke Consortia (FOR 2879, From immune cells to stroke recovery) for suggestions and discussions. This work was funded by the Deutsche Forschungsgemeinschaft (DFG,German Research Foundation) under Germany's Excellence Strategy within the framework of the Munich Cluster for Systems Neurology (EXC 2145 SyNergy – ID 390857198) and under the grants LI-2534/6-1, LI-2534/7-1 and LL-112/1-1.

DISCLOSURES:

The authors have no competing interests to disclose.

REFERENCES:

1. GBD 2016 Causes of Death Collaborators. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet*. **390** (10100), 1151–1210 (2017).
2. Carmichael, S. T. Rodent models of focal stroke: size, mechanism, and purpose. *NeuroRx: The Journal of the American Society for Experimental Neuro Therapeutics*. **2** (3), 396–409 (2005).
3. Rosenblum, W. I., El-Sabban, F. Platelet aggregation in the cerebral microcirculation: effect of aspirin and other agents. *Circulation Research*. **40** (3), 320–328 (1977).
4. Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S., Ginsberg, M. D. Induction of reproducible brain infarction by photochemically initiated thrombosis. *Annals of Neurology*. **17** (5), 497–504 (1985).
5. Bergeron, M. Inducing photochemical cortical lesions in rat brain. *Current Protocols in Neuroscience*. **Chapter 9** Unit 9.16 (2003).
6. Lee, J. K. et al. Photochemically induced cerebral ischemia in a mouse model. *Surgical Neurology*. **67** (6), 620–625 (2007).
7. Dietrich, W. D., Watson, B. D., Busto, R., Ginsberg, M. D., Bethea, J. R. Photochemically induced cerebral infarction. I. Early microvascular alterations. *Acta Neuropathologica*. **72** (4), 315–325 (1987).
8. Labat-gest, V., Tomasi, S. Photothrombotic ischemia: a minimally invasive and reproducible photochemical cortical lesion model for mouse stroke studies. *Journal of Visualized Experiments: JoVE*. **76**, 50370 (2013).

9. McNutt, M. Journals unite for reproducibility. *Science*. **346** (6210), 679 (2014).
10. Llovera, G. et al. Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia. *Science Translational Medicine*. **7** (299), 299ra121 (2015).
11. Dirnagl, U. et al. A concerted appeal for international cooperation in preclinical stroke research. *Stroke*. **44** (6), 1754–1760 (2013).
12. Bath, P. M., Macleod, M. R., Green, A. R. Emulating multicentre clinical stroke trials: a new paradigm for studying novel interventions in experimental models of stroke. *International Journal of Stroke: Official Journal of the INternational Stroke Society*. **4** (6), 471–479 (2009).
13. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., Altman, D. G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *Journal of Pharmacology & Pharmacotherapeutics*. **1** (2), 94–99 (2010).
14. Llovera, G., Liesz, A. The next step in translational research: lessons learned from the first preclinical randomized controlled trial. *Journal of Neurochemistry*. **139 Suppl 2**, 271–279 (2016).
15. Gnyawali, S. C. et al. Retooling laser speckle contrast analysis algorithm to enhance non-invasive high resolution laser speckle functional imaging of cutaneous microcirculation. *Scientific Reports*. **7**, 41048 (2017).
16. Swanson, G. M., Satariano, E. R., Satariano, W. A., Threatt, B. A. Racial differences in the early detection of breast cancer in metropolitan Detroit, 1978 to 1987. *Cancer*. **66** (6), 1297–1301 (1990).
17. Clark, W. M., Lessov, N. S., Dixon, M. P., Eckenstein, F. Monofilament intraluminal middle cerebral artery occlusion in the mouse. *Neurological Research*. **19** (6), 641–648 (1997).
18. Longa, E. Z., Weinstein, P. R., Carlson, S., Cummins, R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. **20** (1), 84–91 (1989).
19. Engel, O., Kolodziej, S., Dirnagl, U., Prinz, V. Modeling stroke in mice - middle cerebral artery occlusion with the filament model. *Journal of Visualized Experiments: JoVE*. **47**, 2423 (2011).
20. Tamura, A., Graham, D. I., McCulloch, J., Teasdale, G. M. Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism*. **1** (1), 53–60 (1981).
21. Chen, S. T., Hsu, C. Y., Hogan, E. L., Maricq, H., Balentine, J. D. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. *Stroke*. **17** (4), 738–743 (1986).
22. Tureyen, K., Vemuganti, R., Sailor, K. A., Dempsey, R. J. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. *Journal of Neuroscience Methods*. **139** (2), 203–207 (2004).
23. Llovera, G., Roth, S., Plesnila, N., Veltkamp, R., Liesz, A. Modeling stroke in mice: permanent coagulation of the distal middle cerebral artery. *Journal of Visualized Experiments: JoVE*. **89**, e51729 (2014).
24. Cramer, J. V. et al. In vivo widefield calcium imaging of the mouse cortex for analysis of network connectivity in health and brain disease. *Neuroimage*. **199**, 570–584 (2019).
25. Heindl, S. et al. Automated morphological analysis of microglia after stroke. *Frontiers in Cellular Neuroscience*. **12**, 106 (2018).
26. Nih, L. R., Gojgini, S., Carmichael, S. T., Segura, T. Dual-function injectable angiogenic

biomaterial for the repair of brain tissue following stroke. *Nature Materials*. **17** (7), 642–651 (2018).
27. Rust, R. et al. Nogo-A targeted therapy promotes vascular repair and functional recovery following stroke. *Proceedings of the National Academy of Sciences of the United States of America*. **116** (28), 14270–14279 (2019).
28. Kitano, H., Kirsch, J. R., Hurn, P. D., Murphy, S. J. Inhalational anesthetics as neuroprotectants or chemical preconditioning agents in ischemic brain. *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism*. **27** (6), 1108–1128 (2007).
29. Farr, T. D., Whishaw, I. Q. Quantitative and qualitative impairments in skilled reaching in the mouse (Mus musculus) after a focal motor cortex stroke. *Stroke*. **33** (7), 1869–1875 (2002).
30. Kassem-Moussa, H., Graffagnino, C. Nonocclusion and spontaneous recanalization rates in acute ischemic stroke: a review of cerebral angiography studies. *Archives of Neurology*. **59** (12), 1870–1873 (2002).
31. Sigler, A., Goroshkov, A., Murphy, T. H. Hardware and methodology for targeting single brain arterioles for photothrombotic stroke on an upright microscope. *Journal of Neuroscience Methods*. **170** (1), 35–44 (2008).

Figure 1

Click here to

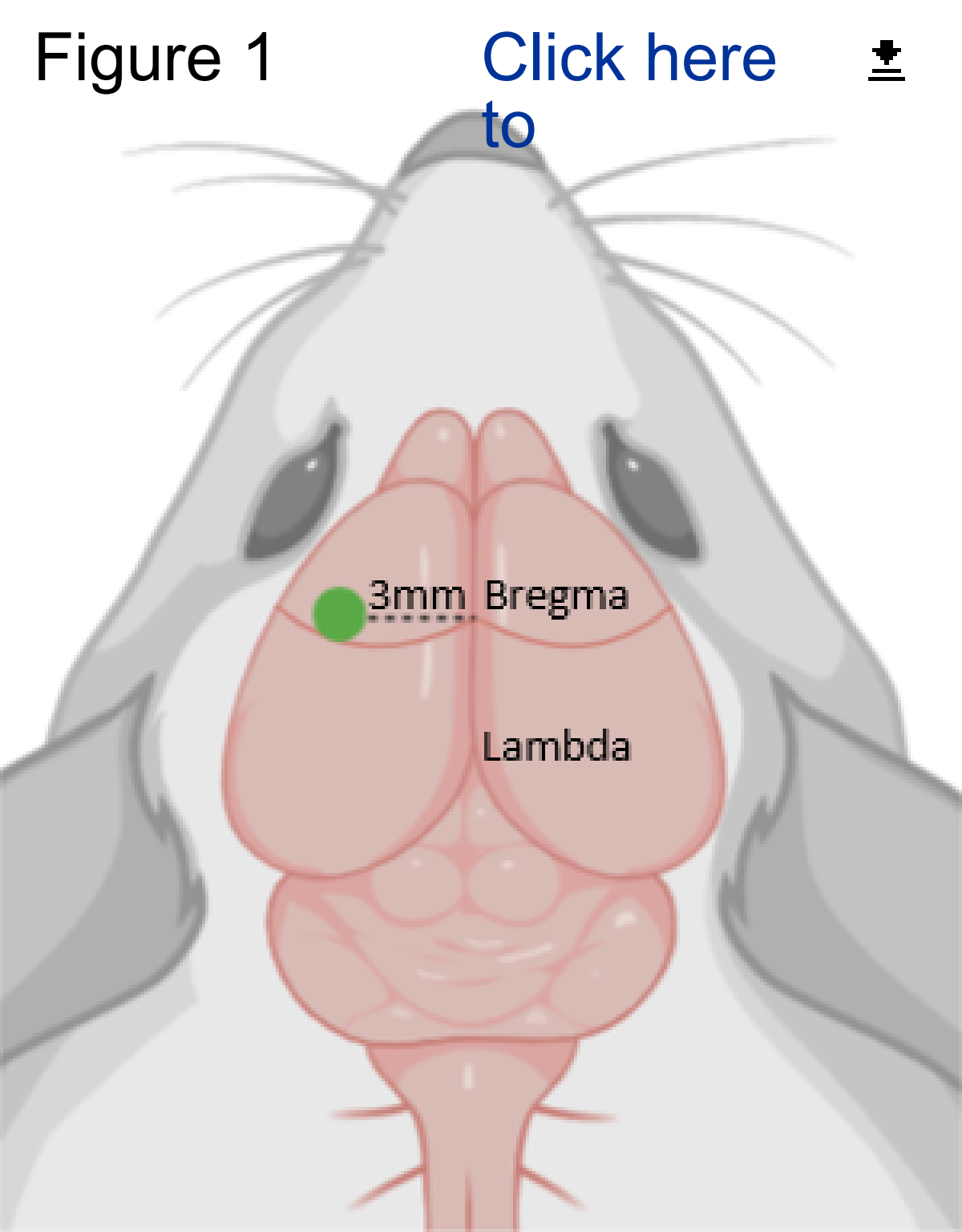
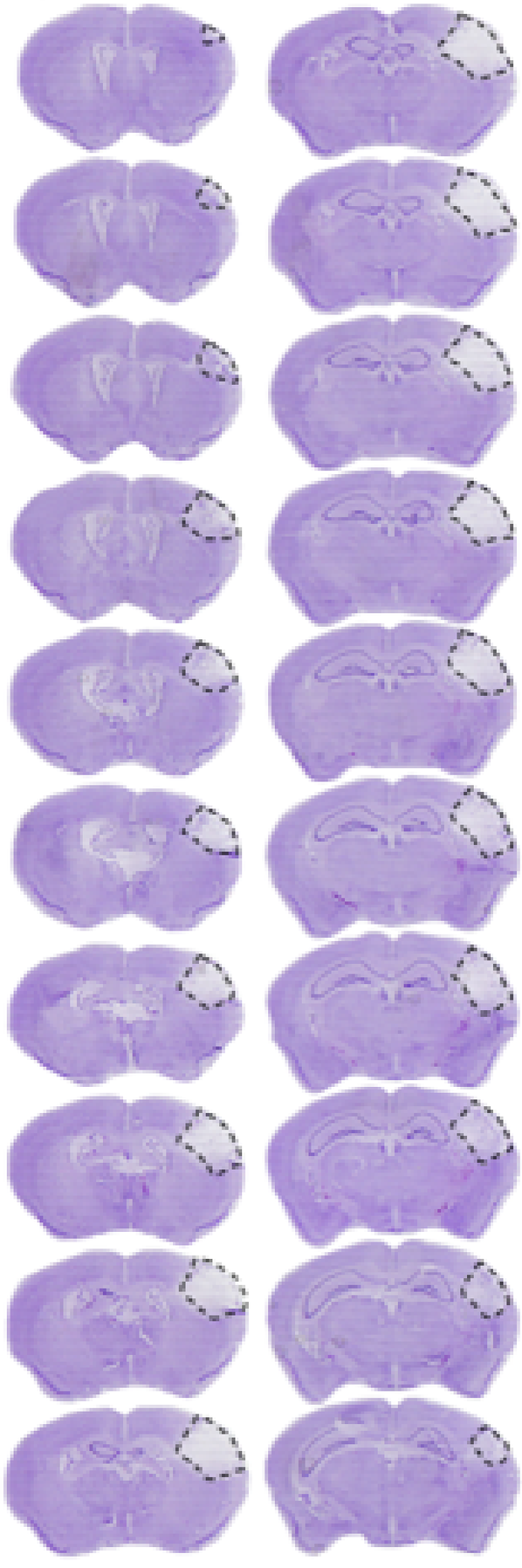


Figure 2



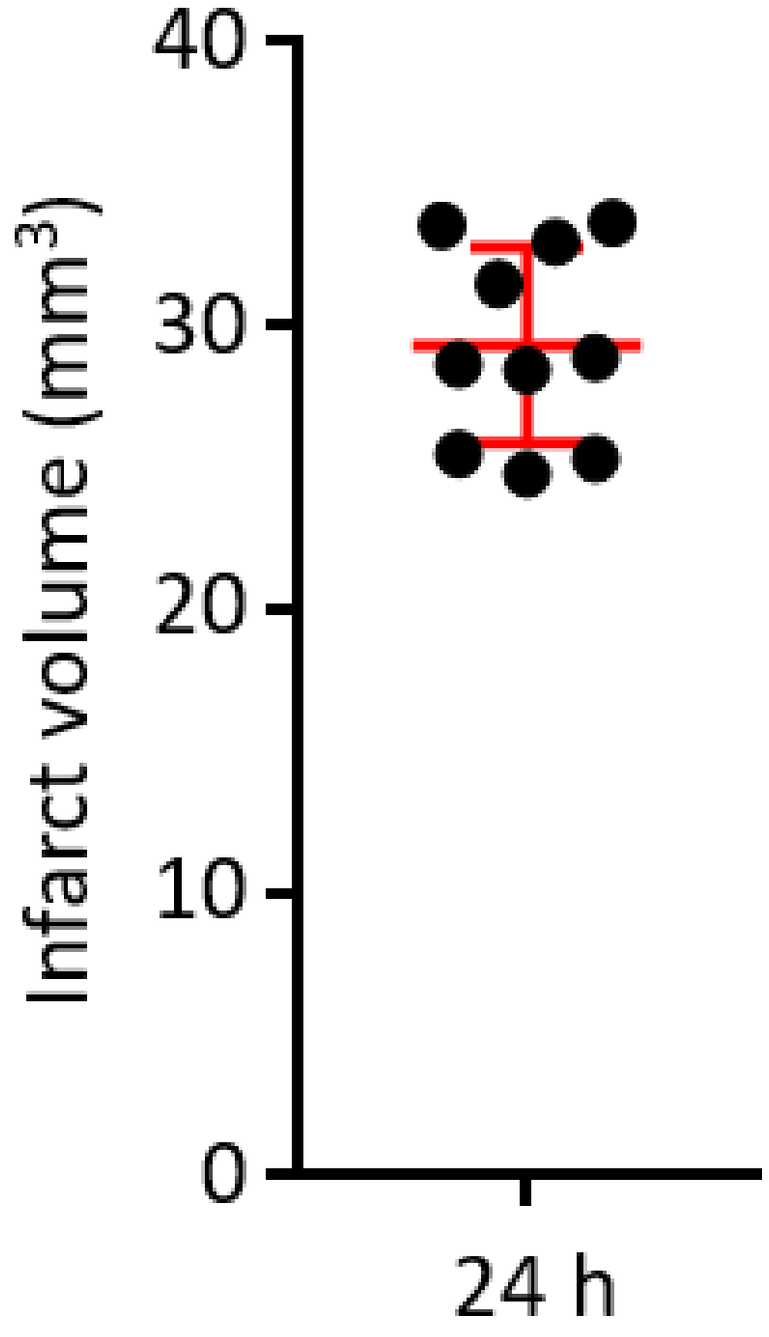


Figure 3

Click here to access/download;Figure;Figure 3-62536R1.pdf

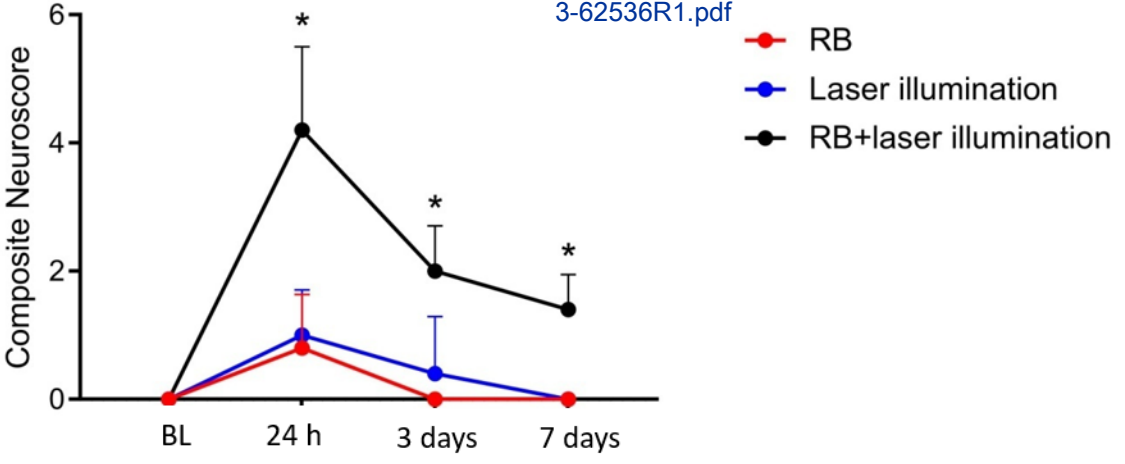


Figure 4

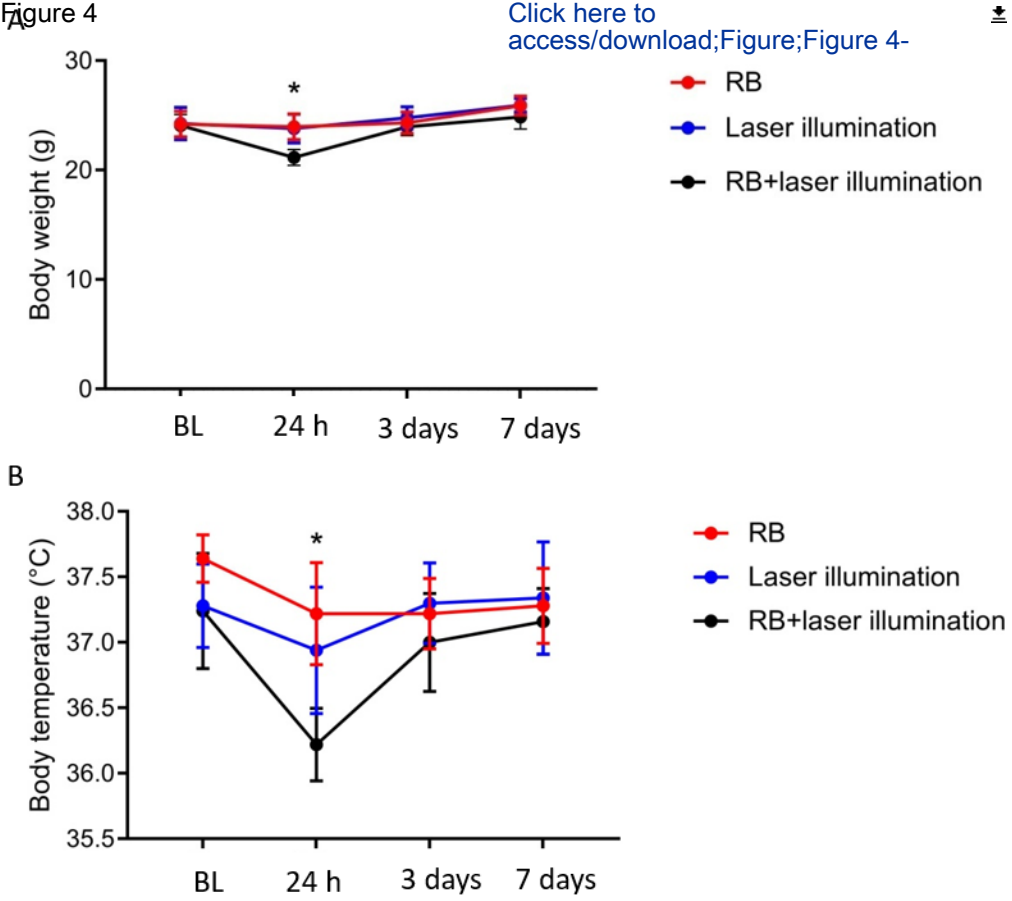


Figure 5

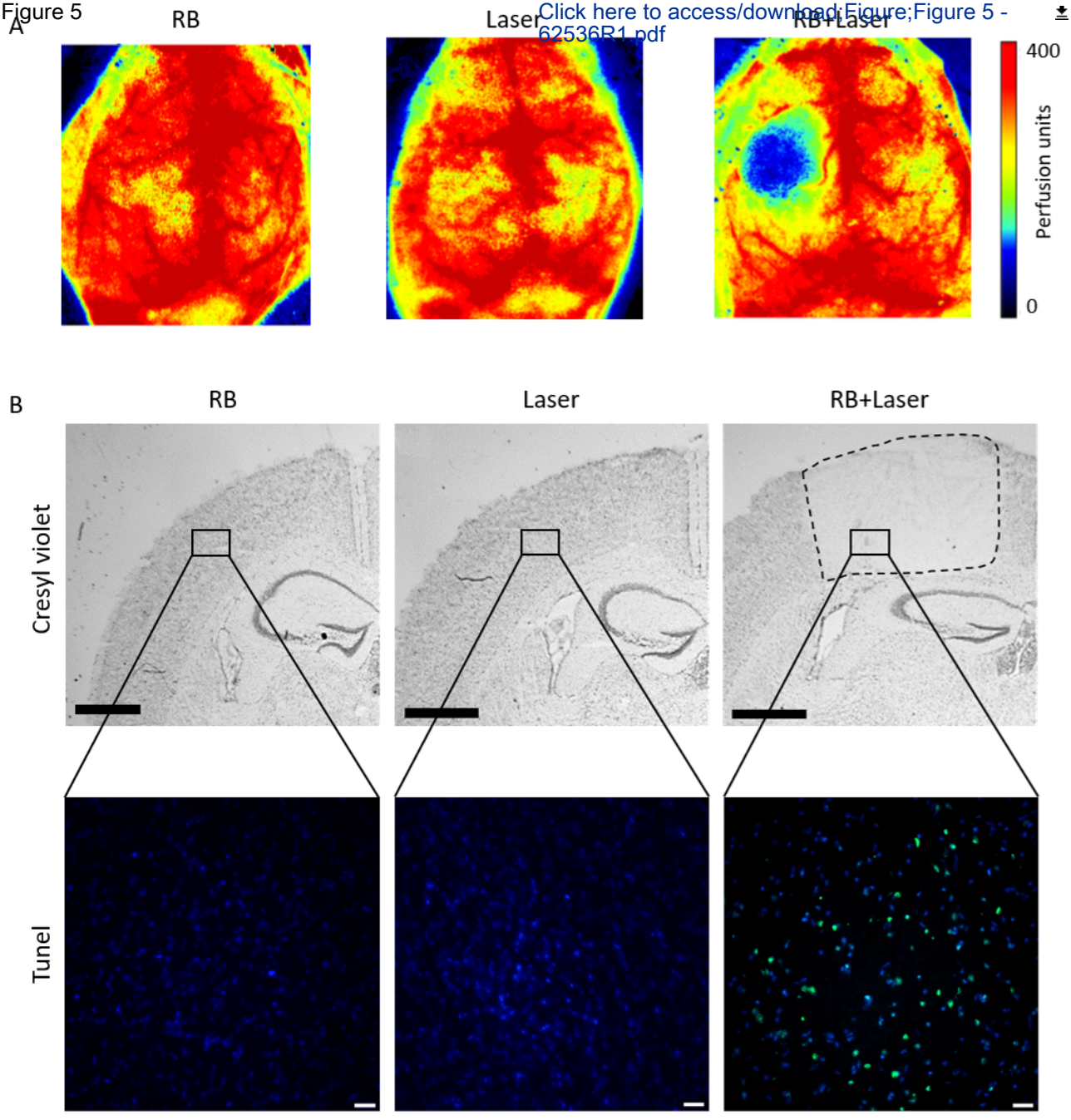


Table 1

Table1: General Neuroscore

		Time-point of scoring	score
General Neuroscore	**Hair**	0. Hair neat and clean	
		1. Localized piloerection and dirty hair in 2 body parts (nose and eyes)	
		2. Piloerection and dirty hair in >2body parts	
	Ears (mouse on an open bench top)	0. Normal (ears are stretched laterally and behind, they react by straightening up following noise)	
		1. Stretched laterally but not behind (one or both), they react to noise	
		2. Same as 1. NO Reaction to noise.	
	Eyes (mouse on OBT)	0. Open, clean and quickly follow the surrounding environment	
		1. Open and characterized by aqueous mucus. Slowly follow the surrounding environment	
		2. Open and characterized by dark mucus	
		3. Ellipsoidal shaped and characterized by dark mucus	
		4. Closed	
	Posture (place the mouse on the palm and swing gently)	0. The mouse stands in the upright position with the back parallel to the palm. During swing, it stands rapidly.	
		1. The mouse stands humpbacked. During the swing, it flattens the body to gain stability.	
		2. The head or part of the trunk lies on the palm	
		3. The mouse lies on one side, barely able to recover the upright position.	
		4. The mouse lies in a prone position, not able to recover the upright position.	
	Spontaneos activity (mouse on OBT)	0.The mouse is alert and explores actively	
		1.The mouse seems alert, but it is calm and sluggish	
		2.The mouse explores intermittently and sluggishly	
		3.The mouse is somnolent and numb, few movements on-the-spot	
		4.No spontaneous movements	
	Total score for general scoring (normal=0 max=18)		

Table 2

Table2: Focal Neuroscore

		Time-point of scoring	score
Focal Neuroscore	**Body symmetry** (mouse on OBT, observe the nose-tail line)	0. Normal (Body: normal posture, trunk elevated from the bench, with fore and hindlimbs leaning beneath the body. Tail: straight)	
		1. Slight asymmetry (Body: leans on one side with fore and hindlimbs leaning beneath the body. Tail: slightly bent.)	
		2. Moderate asymmetry (Body: leans on one side with fore and hindlimbs stretched out. Tail: slightly bent).	
		3. Prominent asymmetry (Body: bent, on one side lies on the OBT. Tail: bent)	
		4. Extreme asymmetry (Body: highly bent, on one side constantly lies on the OBT. Tail: highly bent)	
	Gait (mouse on OBT. Observed undisturbed)	0. Normal (gait is flexible, symmetric and quick)	
		1. Stiff, inflexible (humpbacked walk, slower than normal mouse)	
		2. Limping, with asymmetric movements	
		3. Trembling, drifting, falling	
		4. Does not walk spontaneously (when stimulated by gently pushing the mouse walks no longer than 3 steps)	
	Climbing (mouse on a 45° surface. Place the mouse in the center of the gripping surface)	0. Normal (mouse climbs quickly)	
		1. Climbs with strain, limb weakness present.	
		2. Holds onto slope, does not slip or climb	
		3. Slides down slope, unsuccessful effort to prevent fail	
		4. Slides immediately, no effort to prevent fail.	
	Circling behavior (mouse on OBT, free observation)	0. Absent circling behavior	
		1. Predominantly one-side turns.	
		2. Circles to one side, although not constantly.	
		3. Circles constantly to one side.	
		4. Pivoting, swaying, or no movement.	
	Forelimb symmetry (mouse suspended by tail)	0. Normal	
		1. Light asymmetry: mild flexion of contralateral forelimb.	
		2. Marked asymmetry: marked flexion of contralateral limb, the body slightly bends on the ipsilateral side.	
		3. Prominent asymmetry: contralateral forelimb adheres to the trunk.	
		4. Slight asymmetry, no body/limb movement.	
	Compulsory circling (forelimbs on bench, hindlimbs suspended by the tail: it reveals the presence of the contralateral limb palsy)	0. Absent. Normal extension of both forelimbs.	
		1. Tendency to turn to one side (the mouse extends both forelimbs, but starts to turn preferably to one side)	
		2. Circles to one side (the mouse turns towards one side with a slower movement compared to healthy mice)	
		3. Pivots to one side sluggishly (the mouse turns towards one side failing to perform a complete circle)	
		4. Does not advance (the front part of the trunk lies on the bench, slow and brief movements)	
	Whisker response (mouse on the OBT)	0. Normal	
		1. Light asymmetry (the mouse withdraws slowly when stimulated on the contralateral side)	
		2. Prominent asymmetry (no response when stimulated to the contralateral side)	
		3. Absent response contralaterally, slow response when stimulated ipsilaterally.	
		4. Absent response bilaterally	
Total score for focal deficits (normal=0 max=28)			

Name of Material/ Equipment	Company	Catalog Number
561 nm wavelenght laser	Solna	Cobolt HS-03
Acetic Acid	Sigma Life Science	695092
Anesthesia system for isoflurane	Drager	
ApopTag Peroxidase In Situ Apoptosis Detection Kit	Millipore	S7100
Bepanthen pomade	Bayer	1578681
C57Bl/6J mice	Charles River	000664
Collimeter	Thorlabs	F240APC-A
Cotons	NOBA Verbondmitel Danz	974116
Cresyl violet	Sigma Life Science	C5042-10G
Cryostat	Thermo Scientific CryoStarNX70	
Ethanol 70%	CLN Chemikalien Laborbedorf	521005
Ethanol 96%	CLN Chemikalien Laborbedorf	522078
Ethanol 99%	CLN Chemikalien Laborbedorf	ETO-5000-99-1
Filter paper	Macherey-Nagel	432018
Fine Scissors	FST	15000-00
Forceps	FST	11616-15
Heating blanket	FHC DC Temperature Controller	40-90-8D
Isoflurane	Abbot	B506
Isopentane	Fluka	59070
Ketamine	Inresa Arzneimittel GmbH	
Laser Speckle	Perimed	PeriCam PSI HR
Mayor Scissors	FST	1410-15
Phosphate Buffered Saline PH: 7.4	Apotheke Innestadt Uni Munchen	P32799
Protective glasses	Laser 2000	NIR-ZS2-38
Rose Bengal	Sigma Aldrich	198250-5G
Roti-Histokit mounting medium	Roth	6638.1
Saline solution	Braun	131321
Stereomikroskop	Zeiss	Stemi DV4
Stereotactic frame	Stoelting	51500U
Superfrost Plus Slides	Thermo Scientific	J1800AMNZ
Xylacine	Albrecht	

Comments/Description

Modeling stroke in mice: directed brain injury using photothrombotic model

We would like to thank the reviewers and editors for the very positive general evaluation of our manuscript and the constructive comments. Please find below a point-by-point reply to all individual comments:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: As suggested, we reviewed the manuscript thoroughly and confirmed that there are no spelling or grammatical problems.

2. Please revise the following lines to avoid previously published work: 74-80,86-88,243-256,274-277,305-310.
Response: As suggested, we have reviewed the different selected sections and added the appropriate references.

3. Line 120: Please specify the salinity and preparation procedure.
Response: As suggested, we have added the salinity and Rose Bengal solution preparation procedure (line: 117)

4. Line 194: How is this injected?
Response: As suggested, we add the injection method (intraperitoneally) in the protocol (line:157/222).

5. Line 222: Please specify the filter to be used.
Response: As suggested, we add the type of filter (filter paper) in the protocol as well as in the Material table (line: 245).

6. Use “mL, µL” instead of “ml, µl”. Use “g” instead of “gr”.
Response: As suggested, we change all the abbreviations for the proper ones.

7. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and a maximum of 4 sentences per step.
Response: As suggested, we combined some of the shorter steps.

8. Do not highlight steps involving anesthesia/euthanasia.
Response: As suggested, we have removed the anesthesia and euthanasia steps from the highlighted steps.

9. Please expand the Representative Results section in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.
Response: As suggested we expand the representative results section including the analysis of different outcomes (infarct volume, behavior analysis (Neuroscore) –Figure 3-, and body weight and temperature –Figure 4-) as well as Laser speckle imaging and Tunel staining for successful stroke (Figure 5); line 293-318.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. Do not use "&/and" in the author list for the references. Please include volume and issue numbers for all references.
Response: References are cited using ENDNOTE's JOVE style.

11. Please sort the Materials Table alphabetically by the name of the material.
Response: As suggested we sorted the Materials Table alphabetically by the name of the material.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe the photothrombotic stroke model, a well-known model of permanent ischemia in rodents. The description is accurate and will help other scientists to replicate the procedure. I have a few comments:

Major Concerns:

- How do the authors confirm the success of the stroke procedure? Ideally, you could add a Laser Doppler/Speckle Imaging technique to confirm a successful stroke before euthanasia.
Response: We agree with the reviewer and we added a laser speckle imaging 24h after PT to confirm a successful stroke. We add the different steps in the protocol (line 177), the materials in the excel table as well as the obtained results (line 310) (Figure 5).

- There is a quite similar publications on JoVE by Vivien Labat-gest and Simone Tomasi (2013) doi: 10.3791/50370. At least the authors should reference this publication since it is highly cited and might like to point out the main difference between the two publications
Response: We agree with the reviewer and we add the suggested citation and discussed the main differences between publications (line 392).

Minor Concerns:

- L120, please add how to dissolve Rose Bengal (usually you use 0.9% saline or ddH2O)
Response: As suggested, we have added the salinity and Rose Bengal solution preparation procedure (line: 117).

- L159, it is also possible to perform the illumination with a cold light source instead of a 561 nm laser. The authors should add the option or at least discuss the different light sources
Response: As suggested we discussed the different options for RB activation (line: 393).

- L187 ideally another control should be added for sham operation: only light but no rose Bengal

Response: We agree with the Reviewer and add an additional sham-control surgery using only laser illumination without Rose Bengal injection in the protocol (line 167) as well as in the result section (Figure 3-5).

- The authors may add some recent publications that have been performed using the photothrombotic stroke model e.g. PMID: 29784996, PMID: 31235580
Response: As suggested we add some recent publications that used the PT model (line 378).

Reviewer #2:

Manuscript Summary:

The article "Modeling stroke in mice: directed brain injury using photothrombotic model" describes a technique for performing photothrombotic stroke in mice. The model of photothrombosis of blood vessels in the rodent brain is well studied. There are various modifications of the photothrombotic stroke model, the next option is presented in the article.

Minor Concerns:

1. It is necessary to indicate the concentration of Rose Bengal in mg per kg of animal weight.
Response: As suggested, we added the concentration of Rose Bengal in mg per kg of animal weight (100µg/g) (line 129).

2. Correct operation of the laser is important for the photothrombosis model. It is necessary to clarify the intensity level (W/cm2) of laser radiation. How did the authors calibrate the laser (s)?
Response: We agree with the Reviewer and we add the output power of the laser (25mW) (line 283) and the calibration method (Cobolt Monitor Software) (line 399).