Laser system refinements to reduce variability in infarct size in the rat photothrombotic stroke model

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Abstract

Background—The rat photothrombotic stroke model can induce brain infarcts with reasonable biological variability. Nevertheless, we observed unexplained high inter-individual variability despite using a rigorous protocol. Of the three major determinants of infarct volume, photosensitive dye concentration and illumination period were strictly controlled, whereas undetected fluctuation in laser power output was suspected to account for the variability.

New method—The frequently utilized Diode Pumped Solid State (DPSS) lasers emitting 532 nm (green) light can exhibit fluctuations in output power due to temperature and input power alterations. The polarization properties of the Nd:YAG and Nd:YVO₄ crystals commonly used in these lasers are another potential source of fluctuation, since one means of controlling output power uses a polarizer with a variable transmission axis. Thus, the properties of DPSS lasers and the relationship between power output and infarct size were explored.

Results—DPSS laser beam intensity showed considerable variation. Either a polarizer or a variable neutral density filter allowed adjustment of a polarized laser beam to the desired intensity. When the beam was unpolarized, the experimenter was restricted to using a variable neutral density filter.

Comparison with existing method(s)—Our refined approach includes continuous monitoring of DPSS laser intensity via beam sampling using a *pellicle beamsplitter* and *photodiode sensor*. This guarantees the desired beam intensity at the targeted brain area during stroke induction, with the intensity controlled either through a polarizer or variable neutral density filter.

Conclusions—Continuous monitoring and control of laser beam intensity is critical for ensuring consistent infarct size.

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Keywords

Continuous monitoring of laser intensity; DPSS laser stability; Intensity adjustments; Laser beam polarization; Photothrombotic stroke; Variability

1. Introduction

The photothrombotic stroke model is one of the commonly utilized pre-clinical rodent models of focal ischemia. In this model, vascular thrombosis is induced by transcranial illumination with a light source in combination with intravenous injection of a photosensitive dye. Intravascular thrombotic material, red blood cell stasis, and platelet aggregates adhering to luminal surfaces inside blood vessels result in thrombosis; this in turn is responsible for induction of ischemia leading to infarction in the cortex (Watson et al., 1985). As with all preclinical models of stroke, the photothrombotic model has both advantages and disadvantages. Whereas the photothrombotic stroke model has limitations (e.g. the presence of vasogenic edema) for mimicking all aspects of human stroke (Carmichael, 2005), it has the advantage of providing excellent control over infarct location and size. While the infarct location defines what function will be affected after stroke (e.g. forelimb vs. hindlimb), the infarct size highly contributes to the subsequent pathophysiological response to stroke, infarct evolution (or maturation), and the degree of functional abnormalities and functional improvement after stroke. Therefore, it is critical to precisely control the initial infarct size in order to understand the cascade of post-stroke pathophysiological processes and the potential for neuroplasticity and angiogenesis in the remaining brain tissue and to develop treatment strategies that promote brain repair and functional recovery.

The ability to consistently induce a predetermined infarct size and location in the brain is often stated as a major advantage of the photothrombotic stroke model (Krakauer et al., 2012; Murphy and Corbett, 2009; Watson et al., 1985). However, inter-individual variability has also been reported (Alaverdashvili et al., 2008; Brown et al., 2007; Metz et al., 2005). The source of variability in the degree of functional deficit and recovery after stroke is mostly biological and likely associated with: (i) the variability in topography of motor, sensory and sensory-motor maps (Donoghue and Wise, 1982; Neafsey et al., 1986) and/or (ii) topography of the cortical and sub-cortical vascular tree (Fox et al., 1993). Nevertheless, we observed an unexplained source of high variability in a group of rats exposed to photothrombotic stroke in the motor cortex despite following a rigorous protocol that controlled the major determinants of infarct volume in this model.

The photothrombotic stroke model employs a light source in combination with a photosensitive dye to induce vascular thrombosis (Watson et al., 1985). The key variables, the dose of the photosensitive dye Rose Bengal, the duration of transcranial illumination with the 532 nm wavelength green Diode Pumped Solid State (DPSS) laser, and the demography of experimental animals (age, strain, gender, health status) had been carefully controlled for all rats subjected to stroke in our laboratory. Although the power output generated by the laser had been verified by several measurements, undetected fluctuations in

power output both within and between surgeries used to induce stroke were suspected to account for high inter-individual variability in infarct size. The aim of the present study, therefore, was to measure the properties of two commercially available laser light sources widely used in pre-clinical rodent stroke studies. Most importantly, the study addresses critical refinements and methodological improvements in induction of photothrombotic focal cortical ischemia in rats to prevent an important non-biological source of inter-individual variability in initial infarct size.

2. Methods

Male, young adult (90–100 days old) Sprague Dawley rats ($n = 27$) were obtained from Charles River (QC, Canada). This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use, that is in agreement with in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC). Rats were housed in Plexi-glas cages (39.5 cm long, 34.6 cm wide, and 22.7 cm deep) with absorbent bedding (hardwood and softwood shavings), in groups of two or three in a colony room maintained on a 12 h light/12 h dark cycle (07:00–19:00 h) with controlled temperature and humidity.

The study also followed the recommendations of Workplace Safety and Environmental Protection (WSEP) of the University of Saskatchewan. The WSEP recommendations follow conventional ANSI Z136 and IEC 60825 standards.

2.1. Photothrombotic stroke

2.1.1. Photothrombotic reaction—Vascular thrombosis in the caudal region of motor cortex of the rat was induced using a method modified from that described by Watson et al. (1985). Focal ischemia was induced by transcranial illumination with a 532 nm laser source in combination with injection of 10 mg/kg of the photosensitive dye, Rose Bengal (Sigma-Aldrich, <http://www.sigmaaldrich.com>, Cat. no. R3877-25G) dissolved in 0.9% saline (see theoretical details in Supplementary material).

Photothrombosis of actively conducting blood vessels is a threshold effect that is driven by the rates of endothelial damage and the platelet response to it. Thus, the rate ($R_{\text{photochem}}$) of a photochemical reaction is proportional to the light beam intensity (I) as well as the concentration of the photochemical absorbing dye (C_{dye}), as follows:

$$R_{\text{photochem}} \propto I \times C_{\text{dye}} \quad (1)$$

Empirically, the desired intensity of a laser beam for inducing cortical focal infarct in the caudal region of forelimb motor cortex under our conditions has been found to be 285 ± 5 mW/cm². The intensity I of a laser beam (sometimes also referred to as the irradiance) is the power density expressed as power per unit area of the beam and is thus given by:

$$I = \frac{P}{\pi/4} \times D^2 \quad (2)$$

where P is the total beam power and D is the beam diameter. For the beam diameter $D \approx 4$ mm used throughout this work, a laser power $P \approx 36 \pm 0.6$ mW was required to reach the target beam intensity of 285 ± 5 mW/cm².

2.1.2. Photothrombotic stroke surgery—The rats were anesthetized with 1.75–3.00% isoflurane and 40% O₂/60% N₂O. The rat head was secured in a stereotaxic frame, and a midline incision was made through the scalp. The skin was displaced laterally to expose the skull surface. The skull was thinned with a high speed bone drill across a rectangle with the following coordinates: 2 mm anterior and 1 mm posterior to bregma and 2–5 mm lateral to midline (Paxinos and Watson, 2004). To avoid variability in the occlusion process due to differences in skull thickness, the skull was thinned under a microscope until the experimenter observed vasculature under the thin sheet of the skull. In order to have precise control of the size and shape of the infarct, the adjacent area was protected by a mask fabricated from aluminum sheet shim stock (~0.1 mm thick), in which a 3 × 3 mm square hole was punched to allow passage of the laser beam. The Rose Bengal (10 mg/kg) was injected via a catheter (BD Isyte™, i.v. catheter 24 GA × 0.75 in.) into the tail vein over a 2 min period, and the catheter was flushed with sterile saline. Immediately, the center of the laser beam (4 mm diameter) was directed at the predetermined thinned rectangle on the surface of the skull for 10 min. The targeted area corresponded to the caudal area of the forelimb region of the motor cortex as defined by behavioral (Whishaw, 2000), anatomical (Donoghue and Wise, 1982) and electrophysiological (Neafsey et al., 1986) studies.

During the surgery, respiration, heart rate, and oxygen saturation were carefully monitored using the MouseOx Plus (STARR Life Sciences Corp.) and maintained at 40 – 48 breathes/min, 250–320 beat/min and 98–99% of oxygen, respectively. The rectal temperature was monitored and kept constant at 37 ± 1 °C using a homeothermic blanket system (www.harvardapparatus.com). Following photostimulation, the catheter was removed from the tail vein, and the incision was closed. At the end of the surgery, the rat received 2 mg/kg of bupivacaine hydrochloride (Marcaine™, Hospira, Inc.) subcutaneously. Dehydration associated with surgery was prevented via subcutaneous administration of 10 ml/kg/h of sterile saline.

2.1.3. Infarct location and extent—The infarct was visualized at 24 h after stroke induction, and the location and depth were evaluated by using the 2,3,5-Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, <http://www.sigmaaldrich.com>, Cat no. T8877-25G) staining method (Bederson et al., 1986).

Descriptive statistics for the main features (minimum, maximum, mean, standard deviation) of the infarct area (mm²) on the surface of the brain and maximal infarct depth (mm) were performed by SPSS 22. The coefficient of variation (CV) was calculated for the end-point measures as standard deviation/mean × 100%.

2.2. Instrumentation

The basic design of our laser system is based on systems described by Sigler et al. (2008) and Watson and Prado (2009). However, our laser system utilizes new elements that allow continuous monitoring of the laser power.

2.2.1. General instrument design—Fig. 1A shows a schematic view of our assembly, with relevant dimensions. We used the Thorlabs, Inc. cage mounts for 30 mm cage systems for mounting all components. A compact DPSS 532 nm laser with a nominal maximum output power of 100 mW was mounted to emit a horizontally propagating beam of circular cross section. The beam is initially defocused by diverging lens $L1$ ($f = -100$ mm) before passing through a second converging lens $L2$ ($f = +25$ mm) followed by center-adjustable lens $L3$ ($f = +75$ mm) to aid in alignment. Emerging from this three-lens setup ($L1$, $L2$ and $L3$) is a ~ 70 mW converging beam of circular cross section. The reduction in power is due to the $\sim 4\%$ reflection loss at each lens surface (2 surfaces per lens, no anti-reflection coating).

Because the DPSS lasers used in this study utilized two types of crystals, isotropic and anisotropic, we experimented with two different schemes for controlling the intensity of the beam. Each of these methods (polarizer or variable neutral density filter) is discussed in detail below. The “Intensity Adjustment” (IA) component was mounted after $L3$ (Fig. 1A). Following the IA element, the beam is reflected downward toward the target by a dielectric mirror (Thorlabs cat. no. CM1-EO2) mounted at 45° in a Thorlabs 30 mm cage cube block. The beam passes through an adjustable iris (fully open for the experiments in this paper) and a final converging lens $L4$ ($f = +75$ mm). The set of optics was adjusted to give a beam diameter $D = 4$ mm at the rat brain target plane. A photograph of an assembled system is shown in Fig. 1B.

2.2.2. Novelty in general instrument design: Laser power stability via power monitoring scheme—Based on pilot studies, the desired cortical infarction occurs for laser intensity $I = 285$ mW/cm² (see Supplementary material). The stable optical beam path of our system provided good control of beam diameter D , and thus fluctuations in I were entirely due to fluctuations in the total beam power P . Therefore, achieving total beam power that is close to the pre-determined threshold ($P \approx 36 \pm 0.6$ mW) that remains stable once set is important, since P lower than this will result in a smaller infarction in the caudal region of the motor cortex. Alternatively, P higher than this will induce the infarct to extend to the corpus callosum, striatum or other cortical regions. Thus, instability in P results in inconsistency in infarction size.

Although the scheme of our laser system is comparable to the systems by Sigler et al. (2008) and Watson and Prado (2009), our system is unique in that it adds the capacity for continuous monitoring of the laser power delivered to the target. A *pellicle beamsplitter* (Thorlabs CM1-BP108, nominal beamsplitting ratio 8:92; <http://www.thorlabs.com>) was mounted on the cage rails above the target (Fig. 1A). This *pellicle beamsplitter* is a thin ($2\ \mu\text{m}$ thick) transparent nitrocellulose membrane mounted at 45° to the main vertical beam that splits off a fraction of the beam for monitoring using a *photodiode sensor* or *meter*

(Standard Photodiode Power Sensor)(Thorlabs S120C) mounted on the cage rails in front of the *pellicle beamsplitter* (Fig. 1A and B).

During testing, the total power P_2 in the sampled beam fraction reflected by the pellicle was monitored by a photodiode sensor (Thorlabs S120C) and read out by a meter (Digital Optical Power and Energy Meter—Thorlabs PM 100D) M2, while the power P_1 in the main beam directed toward the target was monitored by a photodiode sensor (Thorlabs S121C) which was read out by meter M1. This provided calibration of the conversion factor C .

$$C = \frac{P_1}{P_2} \quad (3)$$

Knowing C allows P_2 to be used to determine P_1 (light intensity arriving at the brain target) during the surgical period, when P_1 cannot be directly measured, by using the formula

$$P_1 = CP_2 \quad (4)$$

As mentioned above, P_2 is continuously monitored in our laser system during operation (Fig. 1B).

2.2.3. Schemes for variable power intensity—As indicated in Eq. (1), the photochemical reaction rate leading to infarct induction is proportional to the intensity of the laser beam. This presents two major issues: first, the intensity must remain stable once set (discussed under 2.4.2) and second, the intensity of the beam used must be controllable (discussed below). We tested our system with two different lasers; the first laser had a polarized output beam, while the second was unpolarized. The polarization state of the output beam of a DPSS laser is set by the manufacturer and is typically not user-adjustable. Depending on manufacturing quality control, the type of laser crystal used (e.g. isotropic Nd:YAG (neodymium-doped yttrium aluminum garnet) vs. birefringent Nd:YVO₄ (neodymium-doped vanadate)), and the possible addition of polarizing optical elements inside the compact laser assembly, lasers that have nominally the same specifications may nonetheless have different output beam polarization properties. Therefore, two different schemes were explored to control the output beam intensity. The first scheme relied upon a polarized beam passing through a rotatable polarizer, whereas the second scheme employed a variable neutral density filter.

2.2.3.1. Scheme 1: Polarizer as intensity adjustment element: When the laser emitted a polarized output beam, a rotatable polarizer was used as the IA element in the laser system.

The intensity I of a polarized beam after passing through a polarizer with a transmission axis oriented at an angle θ to the E -field (e.g. electric field) polarization direction of the incident beam is given by

$$I=I_0\cos^2(\theta) \quad (5)$$

where I_0 is the intensity of the beam when the transmission axis of the polarizer (Edmund Optics, cat. no. NT43-786) is aligned with the E -field axis of the incident laser beam. Because of the simple sinusoidal geometric dependence embodied in Eq. (2), the use of a polarizer as an IA element is a popular method to control output beam intensity (Fig. 2A). There are some engineering caveats with respect to the use of this technique, however. First, the source laser beam must be polarized. As discussed above, this is not always the case. Therefore, it is important to check the polarization state of the output beam of any laser prior to use, independent of the manufacturer's claimed specifications. Second, the polarizer chosen must be able to handle the optical intensity of the laser being used; if the beam is too intense for the polarizer in question, damage to the polarizer may result. Finally, the beam incident on the target will be polarized if this technique is used. For polarization-sensitive targets such as anisotropic optical materials, this may be an issue; however it seems unlikely that biological targets such as rat brain vasculature are likely to be sensitive to the orientation of the electric field E of the laser beam.

It is worthwhile to note that polarization dependence of the reflection coefficients for dielectric interfaces (calculable via the Fresnel equations) (Born and Wolf, 1999) introduces certain complications in the beam monitoring situation when a polarizer is used to control the intensity of the beam arriving at the target. As discussed previously, we use the *pellicle beamsplitter* to sample the beam, in order to continuously monitor the laser beam intensity arriving at the target. While this appears to be a straightforward method for monitoring the beam power, the reflection from the *pellicle beamsplitter* is highly polarization dependent (as embodied in the Fresnel coefficients for the dielectric reflection). This leads to a somewhat non-intuitive polarizer angle dependence

$$C=C(\theta) \quad (6)$$

for the conversion factor

$$C=\frac{P_1}{P_2} \quad (7)$$

relating target beam power P_1 to monitored power P_2 (Fig. 2B).

In order to set the “polarizer” angle for delivering output beam power, P_1 , sufficient to induce an infarct to the targeted area of the motor cortex, the relationship between these two factors was explored. In addition, the slope value ($m = Y/X$) for the P_1 curve was calculated for a selected segment of the polarizer, between 120 and 150°. The segment was selected based on the range of power that would be required for induction of stroke. The

slope value m was used as an index of “sensitivity” of the polarizer to deliver a precise power level. A smaller m value was considered as an advantage for the laser since a one-degree angle change on the polarizer would result in a smaller shift in beam power. Thus, a smaller “step size” would permit one to set the desired output power easily. Alternatively, a larger m value was considered a limitation of the laser system because it would produce a larger shift in beam power in response to a one-degree angle change.

2.2.3.2. Scheme 2: Variable neutral density (ND) filter as intensity adjustment

element: When the laser source is unpolarized, the rotatable polarizer scheme cannot be used to control the target beam intensity. In this situation, a ND filter can be used instead of a polarizer as the IA element. ND filters are optical elements with uniform absorption characteristics across the visible wavelength range. Commercially available variable transmission ND filters are made by coating the outer sectors of a transparent disk with a metallic coating of variable thickness that results in an angular dependence of the film transmission coefficient T , such that:

$$T=T(\theta) \quad (8)$$

In turn, this leads to the ability to dial a desired output beam intensity by setting the angle of the ND filter disk, as follows:

$$I=I_0T(\theta) \quad (9)$$

For this mode of operation, we used an angle-variable ND filter from Thorlabs (Model NDM2, O.D. range 0–2.0). The semitransparent metal film is made of Inconel (NiCrFe) and covers a total sector of 270° (i.e. 3/4 of a full rotation) with a continuously varying thickness, giving a variable optical density (O.D.) in the range from 0 to 2.0. The Inconel film is mechanically robust and also insensitive to beam heating.

To monitor the stability of P_1 during the surgery, both P_1 and P_2 (power for the sampled beam fraction reflected by the pellicle) were monitored by photodiode sensors. The relationship between P_1 and P_2 was established, and a conversion factor C was calculated using Eq. (7) above. During the induction of stroke, only P_2 was monitored and the actual P_1 was calculated by multiplying P_2 by C .

In order to select an angle on the ND filter disk for delivering an output beam with power (P_1) sufficient to induce an infarct to the targeted area of the motor cortex, the relationship between the angle set on the ND filter disk and P_1 was explored. In addition, the slope value m for the P_1 curve was calculated for a selected segment between 90 and 110°. A slope value m , as mentioned above, was used as an index of “sensitivity” of the ND to deliver precise power.

3. Results

3.1. Relationship between laser power stability and infarct size

Fig. 3 shows representative brains and brain sections from rats following photothrombotic stroke induced using a laser system without power stabilization circuitry. Although the stroke induction procedure was applied consistently across all animals and all brains were examined at 24 h after stroke, the protocol produced an infarct of the predetermined size only in some rats ($n = 5$) (Fig. 3A). Other rats had either a smaller infarct ($n = 5$) (Fig. 3B) or no signs of infarct ($n = 4$) (Fig. 3C). Since the observed high variability in infarct size (Table 1) was reasoned to be due to fluctuation in power output generated by the laser, the beam intensity was tested during its operation. As hypothesized, the beam intensity often dropped below the threshold intensity as shown in Fig. 4A. Thus, it was concluded that the DPSS lasers previously tested in our laboratory were lacking power stabilization circuitry that resulted in the fluctuation of output power. Indeed, when one of the manufacturers employed power stabilization circuitry at our request, the stability issue was resolved (Fig. 4B). Accordingly, the infarct size was then reproducible among rats ($n = 5$) after photothrombotic stroke (Table 1) when the stabilization circuitry was provided by the manufacturer.

3.2. Relationship between intensity adjustment element and infarct size

3.2.1. Polarizer as intensity adjustment element—Fig. 5A shows the power of the laser beam (P_1) emitted by a Nd:YAG DPSS laser as a function of the angle between the light's initial polarization direction and the axis of the polarizer (θ). A sinusoidal wave pattern is a manifestation of Eq. (5) (the “law of Malus”) (Born and Wolf, 1999), and maximum amplitude is exhibited at $\approx 150^\circ$ and 330° . Laser output to obtain a power P_1 of $\sim 36 \pm 0.6$ mW and intensity $I = 285 \pm 5$ mW/cm², that is sufficient to induce a stroke in the targeted area, was observed at a polarization angle θ between 126° and 128° (Fig. 5B). Therefore, during induction of photothrombotic stroke, the “polarizer” angle was set at $127 \pm 1^\circ$, and the laser beam was delivered to the targeted area. When laser power was monitored, and stability of the power was confirmed, the light beam in combination with the photosensitive dye produced reproducible infarcts of the predetermined size. Additionally, our system was flexible for manipulating infarct size by tuning the beam intensity. For example, when beam intensity was increased up to 300 mW/cm² by setting the polarizer at 130° , the brain injury was larger and spanned up to the corpus callosum ($n = 5$) (Fig. 6B). Thus, the laser system with the polarizer as IA element can precisely control infarct size (Table 1).

The stability of output beam power P_1 during the surgery was monitored by continuous measuring of P_2 and multiplying P_2 by C . One disadvantage was that the relationship between P_1 and P_2 was complex and required an experienced researcher for optimal operation of the laser system. Both P_1 and P_2 curves in relation to “polarizer” angle had a sinusoidal waveform, but there was a phase shift by $\approx 50^\circ$ (Fig. 5A). As a result, P_1/P_2 (Fig. 2B) and P_1 (Fig. 2A) curves reached local maxima at different angles, $\approx 132^\circ$ and $\approx 150^\circ$ for P_1/P_2 and P_1 , respectively. This mismatch was confusing for inexperienced operators who required intensive training. The advantage of using a polarizer was its high sensitivity. When

an operator set the desired power, the change in laser beam power relative to the polarizer angle between 120 and 150° was 0.163 mW for a 1° angle change (Fig. 5A).

3.2.2. Variable neutral density filter as intensity adjustment element—The laser system with a variable ND filter also allowed the operator to adjust and deliver a beam of sufficient and stable power to induce a stroke in the targeted region of the motor cortex in the rat by setting an angle on the ND filter disk. Fig. 7 characterizes the beam power emitted by a Nd:VO₄ DPSS laser as a function of the angle set on the ND filter disk. Under our conditions, setting the angle on the ND filter disk at $102 \pm 0.2^\circ$ induced the desired infarct in the targeted area since it delivered a beam power P_1 of $\sim 36 \pm 0.6$ corresponding to intensity $I = 285 \pm 5$ mW/cm². The laser system utilizing the ND filter as IA element also allowed precise control of the infarct size when P_1 was stable throughout the surgery. First, the infarct size was comparable among the rats (Table 1). Second, the system permitted manipulation of the infarct size via manipulation of P_1 by altering the angle on the ND filter disk.

The stability of output beam power P_1 during the surgery was monitored by continuous measurements of P_2 and multiplying P_2 by C . The measured ratio of meter readings M1 and M2 was $C = P_1/P_2 = 6.62 \pm 0.31$ across the entire angular range (Fig. 7). Thus, P_2 and P_1 exhibited a linear relationship. As a result, conversion of the monitored power P_2 to the actual power P_1 delivered to the target required only a simple multiplication by a constant factor $C = 6.62$. Therefore, utilizing the ND filter as the IA element in the laser system was user friendly when the operator monitored the beam stability during the induction of the stroke. A disadvantage was the lower sensitivity when using the ND filter; the laser beam power changed by 2.16 mW per 1° increment. Therefore, setting an angle on the ND filter required extremely fine tuning to set a desirable power P_1 .

4. Discussion

The degree of functional abnormalities and improvement after stroke is highly dependent on both the initial size of the brain injury and neuronal and vascular integrity of the peri-infarct region. Therefore, stratifying stroke survivors by initial infarct size assists with the development and tailoring of treatment strategies to promote brain repair and functional recovery. Monitoring of the infarction size acutely after stroke is advocated for both clinical and pre-clinical stroke research. Although clinical researchers have access to MRI facilities, one of the challenges they face is generalization of the findings from one case to another due to high inter-individual variability. In contrast, whereas preclinical stroke researchers do not always have access to animal MRI facilities acutely after stroke, rodent stroke models can be carefully controlled to induce reproducible infarcts. The rodent model of photothrombotic stroke, which relies on photochemically-induced vascular thrombosis, is a widely used pre-clinical stroke model because of its reputation for producing highly reproducible infarcts (Murphy and Corbett, 2009).

Despite following a rigorous protocol that controlled the major determinants of infarct volume in this model, our research group observed high variability in infarct size in a group of rats subjected to cortical photothrombosis. We suspected that fluctuations in power output

generated by the laser accounted for this. Therefore, in this study we explored the properties of DPSS lasers emitting 532 nm (green) light, and the relationship between power output and infarction size. We also provide methodological refinements in induction of focal cortical ischemia in rats to prevent this important non-biological source of inter-individual variability in initial infarct size.

The photothrombotic stroke model in rats in its original design utilized lamp-pumped lasers as a light source to excite a photosensitive dye to induce brain infarction in the rat (Watson et al., 1985). The major limitation of this approach was heat generation and associated heat-induced brain injury that confounded evaluation of stroke-associated pathophysiological processes and functional outcomes. Heat was generated due to inefficient pumping to convert electricity into pump light and inefficient absorption of the light by the laser crystal since the lamps produce broadband emission throughout the visible and infrared light spectrum. Excessive heat generation required cooling of the surface of the skull and the system itself. Another drawback was the short lifetime of lamp-pumped lasers, which was 200 to 600 h. In turn, this frequent routine maintenance concealed another limitation, which is that optical alignment tends to drift over time and would require periodic realignment irrespective of any lamp change (Byer, 1988). Additionally, lamp-pumped lasers required a strong power supply (multi KW). Since the diode pumping eliminates these drawbacks, neuroscientists were advised to use DPSS lasers to induce photothrombotic strokes (Watson et al., 1985; Watson and Prado, 2009).

We found that some commercially available and commonly used DPSS lasers in the price range of \$1500 USD or less gave inconsistent results in induction of focal ischemia in the forelimb region of the motor cortex in the rat. Based on the findings reported here, it is clear that, during induction of stroke, the beam intensity delivered by a DPSS laser that has no power stabilization circuitry can fall below the threshold intensity required to induce an infarction of the desired size. This can even occur when the intensity of the beam is carefully selected and set before surgery. Our observation on the output beam intensity fluctuation is in agreement with previous findings on the limitations of DPSS lasers that describe strong temperature sensitivity of the basic pump process that can alter their operation (Csele, 2014a). While accurate temperature measurement of the packaged laser crystal may be difficult or impractical, depending on the configuration, we did find that an additional cooling fan could in some cases improve the laser power stability. Nevertheless, additional fans do present some vibrational issues which may affect the stability of the optical system, and there can be space, safety, and noise issues associated with the use of an external fan as well. A properly temperature-stabilized laser system is to be preferred if the manufacturer can provide this.

The DPSS lasers reported on in the present study utilize the widely used Nd:YAG or Nd:YVO₄ crystals to generate 532 nm green light. They rely on a semiconductor diode laser to optically pump a crystal of Nd:YAG or Nd:YVO₄, respectively. These crystals then lase at the infrared wavelength of 1064 nm. A subsequent nonlinear optical crystal (typically potassium titanyl phosphate, commonly referred to as KTP) is then used to double the frequency of the light and thereby halve the wavelength to 532 nm. Because of the nonlinearity used for doubling and other temperature-related issues such as phase matching

and to a lesser extent alignment, frequency doubled solid-state lasers are inherently prone to sometimes large output power fluctuations (Csele, 2014b). Therefore, stabilization circuitry should be employed by manufacturers, and laser intensity stability within a $\pm 2\%$ range should be guaranteed. Otherwise, such fluctuations could easily lead to spurious experimental results because of the strict threshold intensity requirement for induction of complete cortical infarctions.

The novelty of this study is the finding that one should monitor and control DPSS laser intensity, using special sensors, continuously during the surgery to induce reproducible infarct size after photothrombotic stroke in the rat. It is essential to monitor the output laser intensity in real time to verify the stability of the laser operation due to the possibility of unexpected fluctuation in the laser power. Our innovative approach of continuous monitoring of laser intensity via dielectric reflection of a small fraction of the incident power using the *pellicle beamsplitter* and the *photodiode sensor* mounted opposite to the *beamsplitter* provides assurance that the beam intensity remains at the desired level. That, in turn, will limit variability in the infarct size to that due to inherent biological variation. Thus, the susceptibility of DPSS lasers to power and temperature instabilities documented recently (Csele, 2014a) combined with our own observations lead us to urge prospective biomedical researchers using inexpensive 532 nm lasers to also include continuous power monitoring in their systems. This is an essential component of a laser apparatus utilized for inducing photothrombotic stroke in rats, even if manufacturers claim *stabilization circuitry has been employed to limit power fluctuation*. It is noteworthy that continuous monitoring of laser intensity may not be necessary in laser systems used to induce photothrombotic stroke in mice. Lower laser power (~ 24 mW) is sufficient to induce cortical photothrombosis in mice, and therefore the output beam is likely not prone to the fluctuation observed with the lasers of ~ 100 mW or higher power used in studies in rats.

The selection of a method for adjusting power intensity presents another challenge in designing a stroke-inducing system utilizing a 532 nm DPSS laser. As we observed, the efficacy of this element will depend on the birefringence properties of the crystals used in the DPSS laser and the possible addition of polarizing optical elements inside the compact laser assembly. Therefore, we advise biomedical stroke researchers to evaluate the beam polarization characteristics before installing an IA element. If the laser beam is polarized, either a polarizer or a variable neutral density filter can be used to adjust beam intensity to the desired level. If the beam is unpolarized, then the experimenter is restricted to using a variable neutral density filter for this purpose.

The capacity to both adjust power intensity and monitor power continuously requires simultaneous operation of two optic elements, an intensity adjustment element and a beamsplitter. It also requires a linkage, that is, the ability to convert the monitored beam power (P_2) to the beam power delivered to the target area (P_1). When a ND filter is used in the system, this conversion requires only a simple multiplication by a constant factor, which can be a significant engineering advantage in the environment of a busy surgical suite. In contrast, a more complex conversion procedure is required for the polarizer method. Whereas a laser system employing a ND filter is a more user-friendly instrument for

operators with limited experience in laser use, utilizing a polarizer as the intensity adjustment element is superior for fine-tuning laser intensity because of its added sensitivity.

Finally, we highly recommend that scientists utilizing laser systems to induce photothrombotic stroke in rodents report, in addition to other key variables contributing to infarct size, either laser intensity (power density expressed as power per unit area of the beam) or the laser power and beam diameter directed to the target area. Unfortunately, most of the literature neglects this important factor, which limits both reproducibility of reported methods and dissemination of knowledge collected both on stroke induction and related functional and neural processes.

In summary, our refined approach to induce photothrombotic stroke in rats includes continuous monitoring of laser intensity via the dielectric reflection of a small fraction of the incident power using a *pellicle beamsplitter* and *photodiode sensor*. Such an approach guarantees monitoring of the beam intensity delivered to the targeted brain area in real time during stroke induction, with the intensity controlled either through a polarizer or variable neutral density filter. Continuous laser intensity monitoring is critical for limiting variability in the infarct size induced by photothrombotic stroke to within the inherent biological range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DPSS	diode pumped solid state
Nd:YAG	neodymium-doped yttrium aluminium garnet
Nd:YVO₄	neodymium-doped yttrium orthovanadate

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jneumeth.2015.03.029>.

HIGHLIGHTS

- Reproducible photothrombosis requires monitoring and control of laser intensity.
- Intensity of a polarized DPSS laser beam can be varied by a rotatable polarizer.
- Laser beam intensity can also be varied using a variable neutral density filter.
- A beamsplitter allows continuous beam intensity monitoring during stroke induction.

Fig. 1.

(A) Schematic of the laser system. L—lens; IA—intensity adjustment module. Note: a power meter is mounted beside the pellicle beamsplitter to monitor the sampled beam. (B) Photograph of an assembled laser system with a variable neutral density (ND) filter mounted as an “intensity adjustment” (IA) element. ND cage—the cage for a ND filter; D. Mirror—dielectric mirror; RP cage—the cage for a rotational polarizer (RP).

Fig. 2.

(A) Output beam power P_1 as a function of the polarizer angle (θ_p). C —conversion factor; P_1 —a fraction of the beam targeting the brain during stroke induction. (B) P_1/P_2 ratio as a function of θ_p . P_2 —a fraction of the beam (split into fractions by a beamsplitter) continuously monitored by a sensor during the stroke induction.

Fig. 3.

Representative images of a dorsal view of the rat brain (top panel) and a coronal brain section at 0.7 mm anterior from bregma (bottom panel) after photothrombotic stroke to the caudal area of the forelimb motor cortex. Cortical infarcts were induced by transcranial illumination with a green laser (532 nm) with a power density of 280 mW/cm^2 in combination with 10 mg/kg of Rose Bengal. The high variability in infarct size among rats is illustrated by (A) an infarct of the desired size, (B) a smaller than desired infarct and (C) no signs of infarct.

Fig. 4.

Temporal profile of output laser power. (A) The beam was generated by a Diode Pumped Solid State (DPSS) laser without stabilization circuitry. Note the spontaneous drop from 103 mW (desired operation point) to 85 mW followed by recovery. (B) The beam was generated by a DPSS laser with stabilization circuitry. Note intensity stability throughout the experiment.

Fig. 5.

(A) Output beam power as a function of polarizer angle (θ_p). P_1 —a fraction of the beam targeting the brain during stroke induction. P_2 —a fraction of the beam (split into fractions by a beamsplitter) continuously monitored by a power meter during the stroke induction. (B) Output beam intensity (measured at the skull) as a function of polarizer angle (θ_p). The beam is 4 mm in diameter at the skull surface.

Fig. 6. Representative images of a dorsal view of the rat brain (top panel) and a coronal section at 0.7 mm anterior from bregma (bottom panel) after a photothrombotic stroke to the caudal area of the forelimb motor cortex. (A) 280 mW/cm² beam and (B) 300 mW/cm² targeted the skull and underlying brain tissue. A higher beam intensity induced a bigger infarction.

Fig. 7.

Output beam power as a function of a variable neutral density (ND) filter angle (θ_{ND}). P_1 —a fraction of the beam targeting the brain during the stroke induction. P_2 —a fraction of the beam (split into fractions by a beamsplitter) continuously monitored by a sensor during the stroke induction.

Table 1

The influence of laser system stability on infarct size following photothrombotic stroke.

Group	Laser intensity (mW/cm ²)	Infarct quality	Rat (n)	Infarct area (mm ²)			CV (%)		
				Min	Max	Mean	SD	Mean	CV (%)
Unstable laser system	285 *	F	4	0.00	0.00	0.00	NA	NA	NA
		P	5	3.00	12.56	8.08	4.13	51.10	51.10
		S	5	15.90	19.63	17.51	1.44	8.20	8.20
		Total	14	0.00	19.63	9.14	7.68	84.01	84.01
		S	5	15.20	19.63	17.68	1.94	10.95	10.95
Stable laser with polarizer	285	S	5	15.20	19.63	17.68	1.94	10.95	10.95
Stable laser with ND filter	285	S	8	12.56	17.34	14.98	1.63	10.88	10.88
Stable laser with polarizer	300	S	5	19.63	23.75	21.17	1.71	8.05	8.05
Infarct depth (mm)									
Unstable laser system	285 *	F	4	0.00	0.00	0.00	NA	NA	NA
		P	5	0.10	2.40	1.16	0.99	85.04	85.04
		S	5	1.70	3.00	2.33	0.39	16.86	16.86
		Total	14	0.00	2.80	1.19	1.14	90.10	90.10
		S	5	1.80	2.10	1.98	0.11	5.53	5.53
Stable laser with polarizer	285	S	5	1.80	2.10	1.98	0.11	5.53	5.53
Stable laser with ND filter	285	S	8	1.70	3.00	2.33	0.39	16.86	16.86
Stable laser with polarizer	300	S	5	2.90	3.50	3.06	0.25	8.20	8.20

Min—minimum; Max—maximum; SD—standard deviation. CV—coefficient of variation. Unstable laser system—the laser system without stabilization circuitry; stable laser—the laser system with stabilization circuitry. ND—neutral density; F—failure (no signs of infarct); P—partial infarct; S—success (complete infarct).

* The desired laser beam intensity. It is suspected that the beam intensity dropped below the threshold intensity sporadically, resulting in a partial or no infarct.