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Pulsed laser diode based desktop photoacoustic tomography for monitoring wash-in and wash-out of dye in rat cortical vasculature

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To,
The Editor
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Dear Editor,

We wish to submit an original research article entitled "Second generation pulsed laser diode based photoacoustic tomography for monitoring wash-in and wash-out of dye in rat cortical vasculature" for consideration by Journal of Visualized Experiments. The authors are Sandeep Kumar Kalva, Paul Kumar Upputuri, Praveenbalaji Rajendran, Rhonnie Austria Dienzo, and Manojit Pramanik.

We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Photoacoustic tomography (PAT) is an emerging non-invasive biomedical imaging modality. With the advancement of laser technology, pulsed laser diodes (PLDs) are gaining prominence and are widely used for near-infrared wavelength excitation in PA imaging. These PLD lasers have the advantage of compactness and high pulse repetition rates (KHz) compared to conventional bulky Nd:YAG/OPO/Ti:Sapphire lasers with low repetition rates (10-100 Hz). Second-generation pulsed laser diode based PAT (PLD-PAT-G2) system was developed by using 8 acoustic reflector based single-element ultrasound transducers (SUTRs) and was demonstrated for high-speed dynamic *in vivo* imaging at 0.5 s acquisition scan time. This system provided 165µm spatial resolution high-quality images and an *in vitro* depth imaging up to 3 cm. In this work, by using this PLD-PAT-G2 system we are providing the visual demonstration of experiments for *in vivo* brain imaging and dynamic visualization of uptake and clearance process of Food and Drug Administration (FDA) approved indocyanine green (ICG) dye in rat brain vasculature.

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Thank you for your consideration of this manuscript.

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TITLE:

Pulsed Laser Diode-Based Desktop Photoacoustic Tomography for Monitoring Wash-In and Wash-Out of Dye in Rat Cortical Vasculature

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KEYWORDS:

acoustic reflector, single-element ultrasound transducer, photoacoustic imaging, photoacoustic tomography, pulsed laser diode, multiple ultrasound transducers, small animal imaging

SUMMARY:

A compact pulsed laser diode-based desktop photoacoustic tomography (PLD-PAT) system is demonstrated for high-speed dynamic in vivo imaging of small animal cortical vasculature.

ABSTRACT:

Photoacoustic (PA) tomography (PAT) imaging is an emerging biomedical imaging modality useful in various preclinical and clinical applications. Custom-made circular ring array-based transducers and conventional bulky Nd:YAG/OPO lasers inhibit translation of the PAT system to clinics. Ultra-compact pulsed laser diodes (PLDs) are currently being used as an alternative source of near-infrared excitation for PA imaging. High-speed dynamic in vivo imaging has been demonstrated using a compact PLD-based desktop PAT system (PLD-PAT). A visualized experimental protocol using the desktop PLD-PAT system is provided in this work for dynamic in vivo brain imaging. The protocol describes the desktop PLD-PAT system configuration, preparation of animal for brain vascular imaging, and procedure for dynamic visualization of indocyanine green (ICG) dye uptake and clearance process in rat cortical vasculature.

INTRODUCTION:

Photoacoustic computed tomography (PACT/PAT) is a promising non-invasive biomedical imaging modality combining rich, optical contrast with higher ultrasonic resolution[1-5]. When a nanosecond pulsed laser deposits energy onto light, absorbing chromophores present inside

any biological tissue, local temperature increases lead to thermoelastic expansion and contraction of the tissue, resulting in generation of pressure waves. These pressure waves are known as ultrasound waves or photoacoustic (PA) waves, which can be detected by ultrasound transducers around the sample. The detected PA signals are reconstructed using various reconstruction algorithms[6-9] to generate cross-sectional PA images. PA imaging provides structural and functional information from macroscopic organs to microscopic organelles due to the wavelength dependence of endogenous chromophores present inside the body[10]. PAT imaging has been successfully used for breast cancer detection[1], sentinel lymph node imaging[11], mapping of oxyhemoglobin (HbO_2), deoxyhemoglobin (HbR), total hemoglobin concentration (HbT), oxygen saturation (SO_2)[12,13], tumor angiogenesis[14], small animal whole body imaging[15], and other applications.

Nd:YAG/OPO lasers are conventional excitation sources for first generation PAT systems that are widely used in photoacoustic community for small animal imaging and deep tissue imaging[16]. These lasers provide ~100 mJ energy pulses at low repetition rates of ~10–100 Hz. The PAT imaging systems using these costly and bulky lasers are not suitable for high-speed imaging with single-element ultrasound transducers (SUTs), due to the limited pulse repetition rate. This inhibits real-time monitoring of physiological changes occurring at high speeds inside the animal. Using array-based transducers like linear, semi-circular, circular, and volumetric arrays with Nd:YAG laser excitation, high-speed imaging is possible. However, these array transducers are expensive and provide lower sensitivities compared to SUTs; yet, the imaging speed is limited by the low repetition rate of the laser. State-of-the-art single-impulse PACT systems with customized full-ring array transducer obtain the PA data at 50 Hz frame rates[17]. These array transducers need complex back-end receiving electronics and signal amplifiers, making the overall system more expensive and difficult for clinical use.

Their compact size, lower cost requirements, and higher pulse repetition rate (order of KHz) make pulsed laser diodes (PLDs) more promising for real-time imaging. Due to these advantages, PLDs are actively used as an alternate excitation source in second generation PAT systems. PLD-based PAT systems have been demonstrated successfully for high-frame rate imaging using array transducers[18], deep-tissue and brain imaging[19-21], cardiovascular disease diagnosis[22], and rheumatology diagnosis[23]. As SUTs are highly sensitive and less expensive compared to array transducers, they are still extensively used for PAT imaging. Fiber-based PLD system have been demonstrated for phantom imaging[24]. A portable PLD-PAT system has been demonstrated previously by mounting the PLD inside the PAT scanner[25]. With one SUT circular scanner, phantom imaging was performed during 3 s of scan time, and in vivo rat brain imaging was performed during a 5 s period using this PLD-PAT system[19].

Furthermore, improvements have been made to this PLD-PAT system to make it more compact and create a desktop model using eight acoustic reflector-based single-element ultrasound transducers (SUTRs)[26,27]. Here, SUTs were placed in a vertical instead of horizontal direction with the aid of a 90˚ acoustic reflector[28]. This system can be employed for scan times of up to 0.5 s and ~3 cm deep in tissue imaging and in vivo small animal brain imaging. In this work, this desktop PLD-PAT system is used to provide the visual demonstration of experiments for in vivo

brain imaging in small animals and for dynamic visualization of uptake and clearance process of Food and Drug Administration (FDA)-approved indocyanine green (ICG) dye in rat brains.

PROTOCOL:

All animal experiments were performed according to the guidelines and regulations approved by the Institutional Animal Care and Use Committee of Nanyang Technological University, Singapore (Animal Protocol Number ARF-SBS/NIE-A0331).

1. System description

1.1. Mount the PLD laser into the circular scanner and mount the optical diffuser (OD) in front of the PLD exit window to make the output beam homogeneous, as shown in **Figure 1A**. Connect the PLD to the laser driver unit (LDU).

NOTE: The PLD generates ~816 nm wavelengths, pulses of ~107 ns in duration, and up to a 2 KHz repetition rate with a maximum pulse energy of ~3.4 mJ. The LDU consists of chiller, 12 V power supply, variable high voltage power supply to control the laser power, and function generator to change the pulse repetition rate.

1.2. Mount all eight SUTRs on each SUTR holder one-by-one such that the surface of each acoustic reflector faces towards the center of the scanning area, as shown in **Figure 1B**. Connect each SUTR cable to the low-noise signal amplifier with the help of connecting cables.

NOTE: The central frequency of the ultrasound transducer is 5 MHz and has a 13 mm diameter active area. Two amplifiers each of 24 dB gain are connected in series for each channel.

1.3. Switch on the power supply of the chiller, then turn on the switch of the chiller to set the temperature between 20 °C and 25 °C.

1.4. Switch on the supply of the low voltage power supply and slowly turn the current control to set the current limit at 0.3 A. Set the voltage to 12 V. Verify that the current does not exceed 0.1 A.

1.5. Switch on the supply of the high voltage power supply. Press the “Preset” button and set the current to 1 A and voltage to 0 V. Enable the “Output” button: 0 V/0 A.

1.6. Switch on the power supply of the function generator. Press the “Recall” button and choose a 2 KHz configuration to generate the laser pulses at this repetition rate.

1.7. Place acrylic tank inside the scanner as shown in **Figure 1A** and fill the tank with water such that the detecting surface of the SUTRs are immersed completely inside water.

1.8 Make sure all the SUTRs detecting surfaces are inside the water medium. Switch on the power supply of the low-noise-signal amplifier.

2. Animal preparation for rat brain imaging

NOTE: Healthy female rats (see **Table of Materials**) were used to demonstrate the above described desktop PLD-PAT system for imaging small animal cortical vasculature.

2.1. Hold the animal on its back by arresting the head and body motion. Anesthetize the animal by intraperitoneal injection of a mixture of 2 mL of ketamine (100 mg/mL), 2 mL of xylazine (20 mg/mL), and 1 mL of saline (dosage of 0.2 mL/100 g).

NOTE: After the injection, the animal's toe is pinched to test for any positive reflexes such as leg or body movements, vocalization, or marked increases in respirations. An absence of such reflex actions confirms successful anesthetization of the animal.

2.2. Place the animal in prone position on the working bench and remove the fur on the scalp of the animal using a hair trimmer and gently apply hair removal cream to the shaved area and remove the fur completely.

2.2.1. After 4–5 min, remove the applied cream using a cotton swab.

2.2.2. To prevent dryness due to anesthesia and laser illumination, very carefully apply artificial tear ointment to the rat eyes.

2.3. Mount the custom-made animal holder (see **Table of Materials**) equipped with a breathing mask (see **Table of Materials**) on a lab-jack.

2.4. Place the animal in prone position on the holder so the head rests on the horizontal platform of the holder. To avoid movement of the animal during imaging, use surgical tape to secure the animal to the holder.

2.5. Ensure that the breathing mask covers the nose and mouth of the rat to deliver anesthesia mixture. The breathing mask is customized to suit the imaging window. 10% of the commercially available nose cone is cut and then connected to a piece of glove.

2.6. Connect the breathing mask to the anesthesia machine before switching it on.

2.7. Switch on the anesthesia machine and set it to deliver anesthetic mixture containing 1.0 L/min of oxygen with 0.75% isoflurane to the animal breathing mask.

2.7.1. Clamp the pulse oximeter to one of the animal's hind legs to monitor its physiological condition.

2.8. Apply a layer of colorless ultrasound gel to the scalp of the rat using a cotton tipped applicator. Adjust the lab-jack position to the center of the scanner and adjust the height of the

lab-jack manually so that the imaging plane is at the center of the acoustic reflector.

3. Dynamic in vivo imaging of uptake and clearance process of ICG in rat brain

3.1. Set the parameters in the data acquisition software (see **Table of Materials**) for a 360° acquisition scan.

3.2. Turn on the PLD laser emission by enabling the output of the function generator (laser emission will start). Then, slowly increase the voltage of the variable high voltage power supply to 120 V for maximum per pulse energy.

3.3. Run the data acquisition software (see the **Table of Materials**) program to rotate all eight SUTRs in 360° over a 4 s scan time.

NOTE: For example, if the SUTRs are rotated for 4s, the PLD delivers 8,000 (= 4 x 2,000) pulses and each SUTR collects 8000 A-lines. These 8,000 A-lines are reduced to 400 by averaging over 20 signals (after averaging A-lines = 8,000/20 = 400). A reconstruction program based on delay-and-sum back projection algorithm is used to find out the scanning radius of each SUTR.

3.4. Disable the output of the function generator to turn off the laser emission.

3.5. Using the reconstruction algorithm in data processing software (see **Table of Materials**) find out the scanning radius of all eight SUTRs by trial-and-error, using the back-projection algorithm.

3.6. Set the parameters in the data acquisition software (see **Table of Materials**) for 45° acquisition over a 0.5 s scan time.

NOTE: For example, if the SUTRs are rotated for 0.5s, the PLD delivers 1,000 (= 0.5 x 2,000) pulses and each SUTR collects 1000 A-lines. These 1,000 A-lines are reduced to 400 by averaging over 20 signals (after averaging A-lines = 1,000/20 = 50).

3.7. Enable the output of the function generator to turn on the laser emission.

3.8. Run the data acquisition software (see **Table of Materials**) program to rotate all eight SUTRs in 45° to obtain initial control data before administering ICG.

3.9. Disable the output of the function generator to turn off the laser emission.

3.10. Identify the tail vein of the animal and inject 0.3 mL of ICG (see **Table of Materials**) (323 µM) into the tail vein of the rat.

4. NOTE: 1.25 mg of ICG powder was weighed using a micro-weighing machine and mixed with 5 mL of distilled water to obtain a concentration of 323 µM for the ICG solution.

4.1. Enable the output of the function generator to turn on the laser emission.

4.2. Run the data acquisition software (see **Table of Materials**) program to acquire A-lines over a 0.5 s scan time in 45° rotation.

5. NOTE: A-lines acquired during a 0.5 s scan time are used to generate one cross-sectional image. There is time gap of ~0.4–0.6 s between each scan.

5.1. After the data acquisition is over, using the back-projection algorithm in data processing software (see **Table of Materials**), reconstruct the cross-sectional brain image from the saved A-lines.

5.2. Turn off the laser and then turn off anesthesia machine, lower the lab-jack and remove the animal from the stage. Return the animal to the cage and monitor until it regains consciousness.

[Place **Figure 1** here]

REPRESENTATIVE RESULTS:

The potentiality of the described desktop PLD-PAT system for dynamic in vivo brain imaging has been showcased in this protocol with corresponding results. High-speed imaging capability of the desktop PLD-PAT system was demonstrated by performing in vivo brain imaging of healthy female rats. PA signals were collected using eight SUTRs rotating in 360° and 45° around the rat brain at scan speeds of 4 s and 0.5 s, respectively. **Figure 2A,B** show brain images of a female rat (98 g) at scan speeds of 4 s and 0.5 s, respectively. Sagittal sinus (SS) and transverse sinus (TS) are clearly visible in both the images. **Figure 2C,D** show photographs of the rat brain before and after removing the scalp over the brain area, respectively. PAT imaging was done non-invasively with intact skin and skull.

[Place **Figure 2** here]

Before injecting ICG into the tail vein of the same rat, control data was acquired. After injecting ICG, PA data was acquired continuously for first 5 min with a 0.5 scan time. Then, PA data was acquired at ~2–3 min intervals with 0.5 s scan times each for the next 15–20 min. **Figure 3** shows the plot representing the increases in average PA signal in the sagittal sinus (SS) due to increases in optical absorption by ICG at 816 nm wavelengths, and subsequently, decreases over time.

[Place **Figure 3** here]

FIGURE LEGENDS:

Figure 1: Schematic of the desktop PLD-PAT system. (**A**) Schematic of the desktop PLD-PAT set

up. PLD: pulsed laser diode, OD: optical diffuser, SUTR: acoustic reflector based single-element ultrasound transducer, AM: anesthesia machine, CSP: circular scanning plate, SM: stepper motor, LDU: laser driving unit, AMP: amplifier, DAQ: data acquisition card. (**B**) Circular arrangement of eight SUTRs around the scanning center.

Figure 2: Non-invasive in vivo desktop PLD-PAT images. In vivo images of cortical vasculature at scan times of (**A**) 4 s and (**B**) 0.5 s. SS: sagittal sinus, TS: transverse sinus. (**C**) and (**D**) are photographs of the rat brain before and after removing the scalp, respectively.

Figure 3: Pharmacokinetics of ICG. Pharmacokinetics of ICG showing the uptake and clearance process. The red arrow mark shows the time of injection of ICG into the tail vein.

DISCUSSION:

This work presents a protocol to use a desktop PLD-PAT system for conducting experiments on small animals like rats for in vivo brain imaging and dynamic fast-uptake and clearance process of contrast agents like ICG. Bulky, expensive OPO-PAT systems take several minutes (2–5 min) to acquire a single cross-sectional in vivo image. A compact, low-cost, first generation portable PLD-PAT system provides single cross-sectional in vivo images in 5 s. In contrast, a high-speed, compact, low-cost desktop PLD-PAT system renders a high quality 2D cross-sectional in vivo image in just 0.5 s[26]. Here, the same desktop PLD-PAT system was demonstrated for fast in vivo dynamic brain imaging. Using this system, continuous monitoring of rapidly changing physiological phenomena is performed inside small animals for a fast rise and fall of PA signals due to ICG uptake and clearance processes. However, PLDs have a few limitations such as single wavelength generation, which forbids functional imaging. Additionally, multiple wavelength illumination is needed for acquiring the functional information. Also, imaging depth is limited due to a low per-pulse energy of PLD, which can be circumvented using exogenous photoacoustic contrast agents for enhancing the imaging depth.

While conducting the experiments using the desktop PLD-PAT system, certain precautions need to be taken: (a) due to the non-uniform beam profile of the PLD laser, an optical diffuser should be used at the laser output window, (b) it should be ensured that PLD laser beam is at the scanning center and that all SUTRs are facing towards the center of the PAT scanner, (c) care should be taken during anesthesia injection so that the surrounding organs like urinary bladder, kidneys, and intestines are not affected, (d) a proper amount of anesthesia mixture must be injected according to the weight of the animal, (e) during the procedure of trimming hair on the animal head, scratches on the scalp must be avoided, and (f) it must be ensured that the imaging plane of the rat brain is at the center of the acoustic reflector of the SUTRs. Troubleshooting may be needed if the image quality is low. Major applications of this system include high frame rate imaging (1 frame in 0.5 s), small animal brain tumor imaging, subcutaneous tumor imaging, and investigating biomaterials for potential PA contrast agents and therapeutic applications.

The maximum permissible exposure (MPE) safety limit for in vivo imaging is governed by the American National Standards Institute (ANSI) laser safety standards[29]. These safety limitations

are dependent on laser pulse width, illumination area, exposure time, and illumination wavelength, as well as several other factors. Higher than a 700–1,050 nm wavelength range and maximum per pulse energy density on the skin surface should not exceed 20 x $10^{2(\lambda-700)/1,000}$ mJ/cm^2, where λ (in nm) is the illumination wavelength. So, the MPE safety limit at a 816 nm wavelength of PLD laser used is ~34.12 mJ/cm^2. For continuous illumination of the laser over a period of t = 0.5 s, the MPE safety limit becomes 1.1 x $10^{2(\lambda-700)/1000} \times t^{0.25}$ J/cm^2 (= 1.58 J/cm^2). The pulse repetition rate of the PLD was maintained at 2,000 Hz in all experiments. Over the course of a 0.5 s scan time, a total of 1,000 (0.5 x 2,000) pulses were delivered to the sample. This implies that per pulse, the MPE was 1.58 mJ/cm^2. The desktop PLD-PAT system delivers a per pulse energy of ~3.4 mJ. The laser energy density was maintained at ~0.17 mJ/cm^2 on the brain area as the laser beam expanded over a ~20 cm^2 area. This laser energy density was well below the ANSI safety limit over a period of 0.5 s. By reducing the pulse repetition rate, reducing the laser power, or expanding the laser beam, the ANSI laser safety limit for the desktop PLD-PAT system can be changed.

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DISCLOSURES:

The authors have no relevant financial interests or potential conflicts of interest to disclose.

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Figure 1

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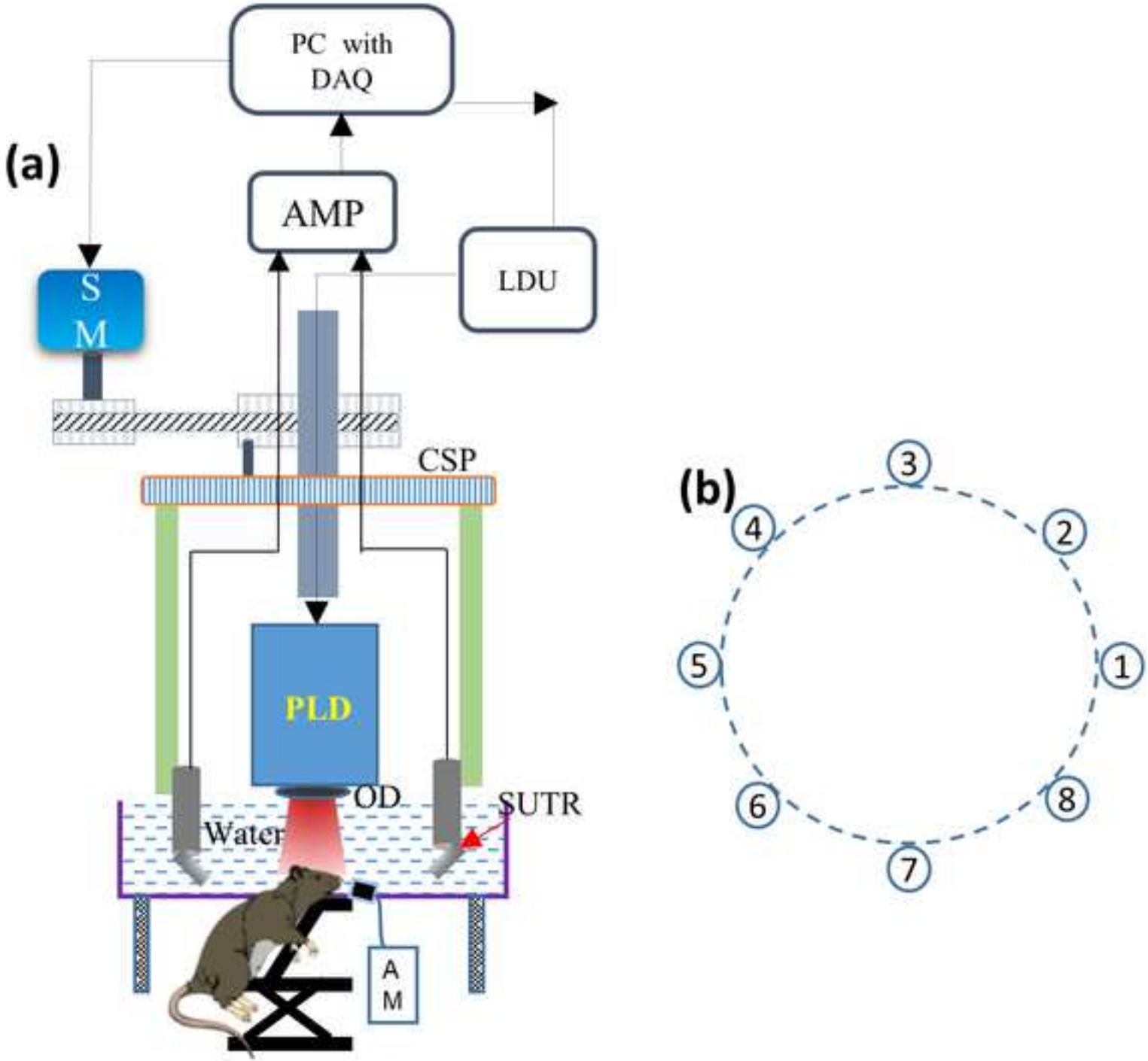


Figure 2

Click here to access/download;Figure;figure2.TIF

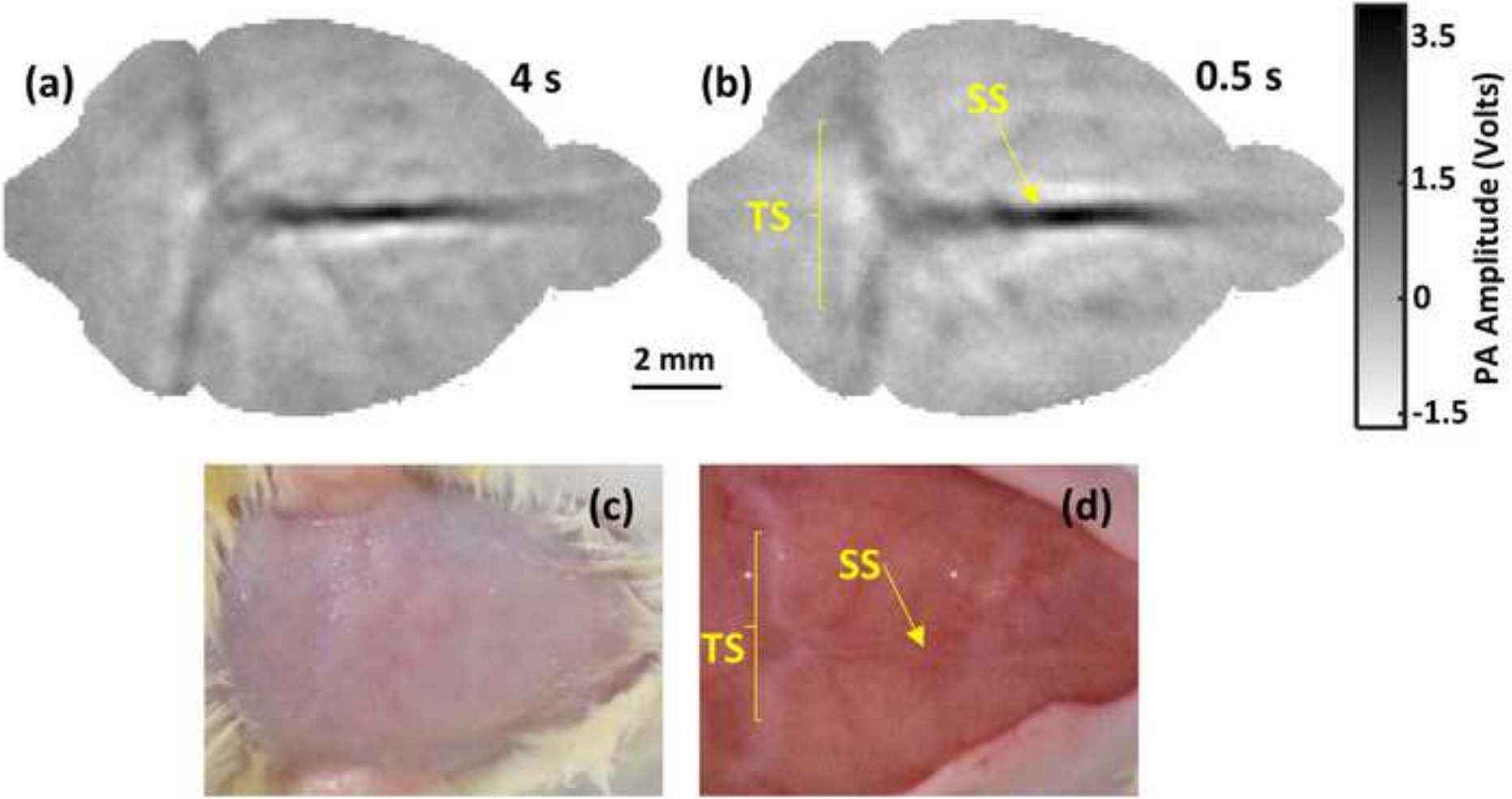
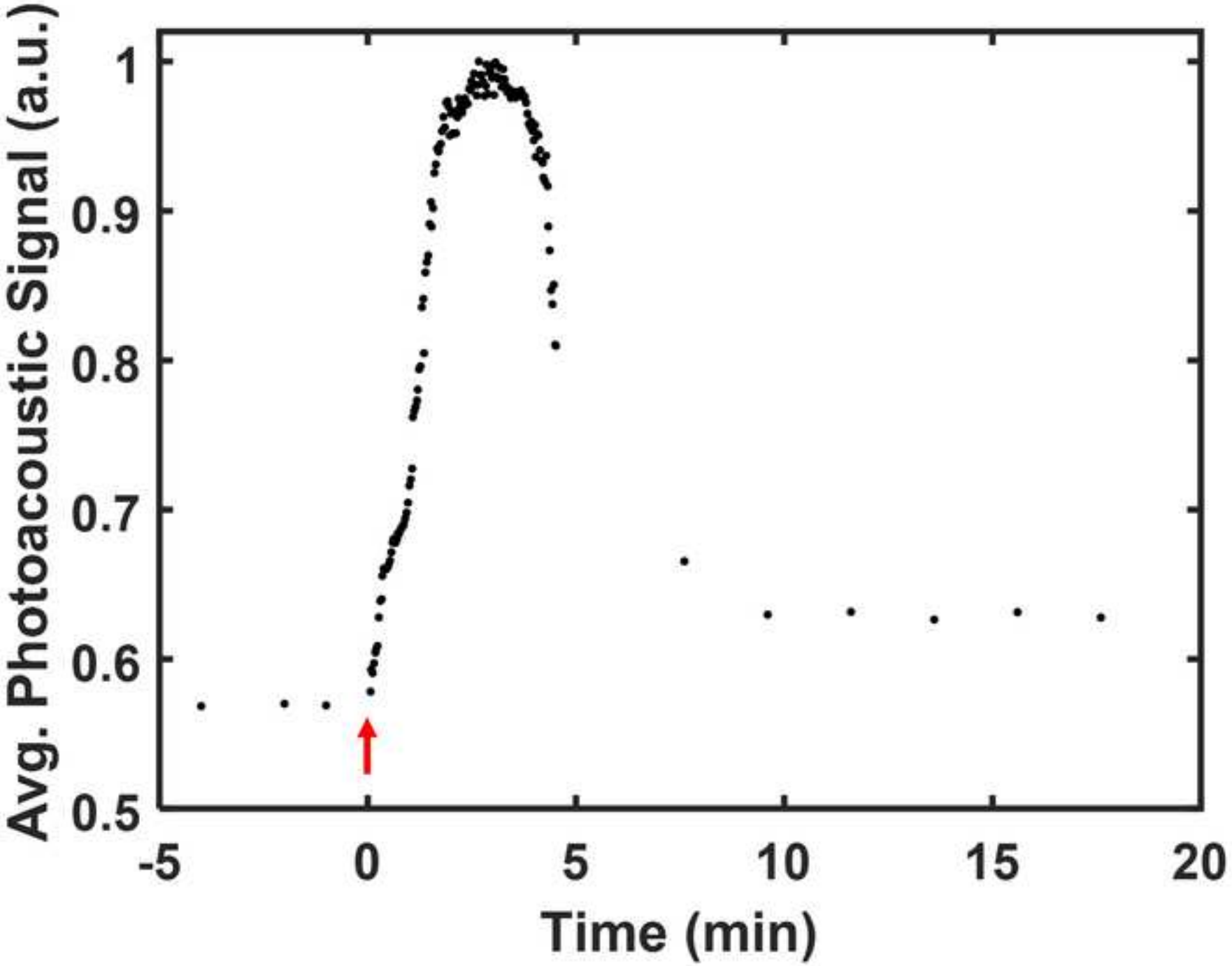


Figure 3

Click here to access/download;Figure;figure3.TIF



Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Reagent/ Equipment	Company
12 V power supply	Voltcraft
Acoustic reflector	Olympus
Acrylic water tank	NTU workshop
Anesthetic Machine	Medical plus pte ltd
Animal distributor	In Vivos Pte Ltd, Singapore
Animal holder	NTU workshop
Breathing mask	NTU workshop
Circular Scanner	NTU workshop
DAQ (Data acquisition) Card	Spectrum
Data acqusition software	National Instruments Corporation,Austin,TX,USA)
Data processing software	Matlab (Mathworks, Natick, MA, USA)
Function generator	RIGOL
Low noise signal amplifier	Genetron
Optical diffuser	Thorlabs
Pulsed laser diode	Quantel, France
Rats	In Vivos Pte Ltd, Singapore
Stepper motor with gearbox	LIN Engineering (Servo Dynamics)
Ultrasound gel	Progress/parker acquasonic gel
Ultrasound Transducer	Olympus
Variable high voltage power supply	Elektro-Automatik

Catalog Number

PPS-11810
F102

Custom-made

Non-Rebreathing Anaesthesia machine with oxygen concentrator.

Custom-made
Custom-made
Custom-made
M2i.4932-exp

NI LabVIEW 2015 SP1 (32 bit)

Matlab R2015b

DG1022
Custom-made using Mini-circuits, ZFL-500LN-BNC
DG-120

QD-Q1924-ILO-WATER
NTac:SD, Sprague Dawley / SD

Motor: CO-5718L-01P-RO, Gearbox: DPL64/1; Power supply PW-100-24

PA-GEL-CLEA-5000

V309-SU/ U8423013
EA-PS 8160-04 T

Comments/Description

To supply operating voltage for PLD
45 degree reflector augmented to the ultrasound transducer
It is used to hold water that acts as an acoustic coupling medium between animal brain and detector

Supplies oxygen and isoflurane to animal
Animal distributor that supplies small animals for research purpose
Used for holding animal on its abdomen
Used along with animal holder to supply anesthesia mixture to the animal
Scanner is made out of aluminum
16 bit, 30 Ms/s, 8 channels, 1 Gs, PCIe
LabVIEW based program developed in our laboratory for controlling the stepper motor and acquring the PA singnals from the detector
Matlab code developed in our laboratory for reconstructing cross-sectional PA images
To change the repetition rate of the PLD. It will provide TTL signal to synchronize the DAQ with the laser excitation.
To receive, and amplify the PA signal from SUTR. Its gain is 24 dB.
Used to to make the laser beam homogeneous
It is the excitation laser source with specifications of 816 nm wavelength, 3.4 mJ per pulse energy, 107 ns pulse width, 2 KHz maximum pulse repitition rate, dimensions : 13.0 x 7.6 x 5.0 cm
Female, weight 100±10g, strain of rats: Sprague Dawley, age: 4-5 weeks
To move the detector holder in a circular geometry. Torque: 2.08 N-m, Rotor inertia: 2.6 kg-cm2

Clear ultrasound gel
Ultrasonic sensors used for photoacoustic detection. Central freqency 5 MHz, 0.5 in
To change the laser output power

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Title of Article: Second generation pulsed laser diode based photoacoustic tomography for monitoring of wash in and wash out of dye in rat cortical vasculature

Author(s): Kalva, Sandeep Kumar; Upputuri, Paul Kumar; Rajendran, Praveenbabji; Dienzo, Rhonnie Austria; Pramanik, Manojit;

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Institution:	Nanyang Technological University
Title:	Asst. Prof.
Signature:	Manojit Pramanik
Date:	31 - January - 2019

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Rebuttal Letter
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Manuscript ID: JoVE59764

Manuscript title: *Pulsed laser diode based desktop photoacoustic tomography for monitoring wash-in and wash-out of dye in rat cortical vasculature*

Authors: Sandeep Kumar Kalva, Paul Kumar Upputuri, Praveenbalaji Rajendran, Rhonnie Austria Dienzo and Manojit Pramanik

Private comments to the Editor

Dear editor,

Thank you for coordinating the review of our manuscript. We also thank the reviewers for their comprehensive and critical evaluation. We have addressed the reviewers' comments and made appropriate revisions to the manuscript as needed.

An optional red-lined version of the revised manuscript is also uploaded.

Below please find our responses to the reviewers' specific comments. The reviewers' comments are in *italics*, and our responses are in normal font.

Sincerely,
The Authors

Reply to editor's comments:

Reply: We would you like to offer our sincere thanks for your encouraging and constructive comments. The manuscript has been revised to address these comments.

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply: Thank you for your suggestion. We have checked the revised manuscript and corrected them.

2. Please revise lines 47-51, 162-170, and 295-302 to avoid overlap with previously-published text.

Reply: Thank you for pointing this. The corresponding text has been modified in the revised manuscript.

"High-speed dynamic *in vivo* imaging has been demonstrated using a compact PLD based desktop PAT system (PLD-PAT). A visualized experimental protocol on using the desktop PLD-PAT system has been provided in this work for dynamic *in vivo* brain imaging. The protocol describes the desktop PLD-PAT system configuration, preparation of animal for brain vascular imaging, and an experimental procedure for dynamic visualization of indocyanine green (ICG) dye uptake and clearance process in rat cortical vasculature."

"2.6. Connect the breathing mask to the anesthesia machine before switching it on.

2.7. Switch on the anesthesia machine and set it to deliver anesthetic mixture containing 1.0 L/min of oxygen with 0.75% isoflurane to the animal breathing mask.

2.7.1. Clamp the pulse oximeter to one of the animal's hind legs to monitor its physiological condition."

"Laser safety standards for *in vivo* imaging

The maximum permissible exposure (MPE) safety limit for *in vivo* imaging is governed by the American National Standards Institute (ANSI) laser safety standards.[27] These safety limitations are dependent on laser pulse width, illumination area, exposure time, illumination wavelength, etc. Over 700 to 1,050 nm wavelength range, the maximum per pulse energy density on the skin surface should not exceed $20 \times 10^{2(\lambda-700)/1,000}$ mJ/cm^2, where λ (in nm) is the illumination wavelength. So the MPE safety limit at 816 nm wavelength of PLD laser used is ~34.12 mJ/cm^2. For continuous

illumination of the laser over a period of t = 0.5 s, the MPE safety limit becomes $1.1 \times 10^{2(\lambda-700)/1000} \times t^{0.25}$ J/cm^2 (= 1.58 J/cm^2)."

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For example: LabVIEW

Reply: Thank you for your suggestion. We have gone through the manuscript and corrected in the revised manuscript.

Protocol:

1. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please mention how proper anesthetization is confirmed.

Reply: Thank you for your suggestion. The corresponding text has been added in the revised manuscript after step 2.1.

"Note: After the injection, the animal cannot move and it stays in the same place without any motion. This confirms the animal is anesthetized."

b) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia

Reply: Thank you for your suggestion. The corresponding text has been added in the revised manuscript after step 2.2.2.

"Note: Due to anesthesia, the animal's eyes get dried. The laser light might fall on animal's eyes during experiment. In order to prevent all these, we need to use vet ointment on animal's eyes before the experiment."

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Reply: Thank you for suggesting this. The steps 2.1, 2.2, 2.4, and 2.8 are modified and step 1.7 split into two steps in the revised manuscript.

Specific Protocol steps:

1. 2: What are the strain and age of the rats?

Reply: The strain of the rats is Sprague Dawley and the rats are of 4-5 weeks age. These details are mentioned in the revised table of materials.

2. 3: Please provide more information regarding setting of parameters in LabView (if these steps are to be filmed). Please also provide more information on using MATLAB for reconstruction. Thin information can include supplemental material.

Reply: Thank you for asking this. The setting of parameters in LabView will not be filmed. So, we did not provide information about these steps. We have used conventional delay-and-sum algorithm implementing back-projection technique for reconstructing the photoacoustic images, which is very common in the photoacoustic community. So, we are not providing this information as well.

Figures:

1. Figure 2: What is the scale on the right measuring?

Reply: The scale on the right is measuring the PA amplitude (volts). We have mentioned this information in Fig. 2 in the color bar.

2. Figure 3: What exactly is the 'Fitted data' here?

Reply: The fitted data is an approximation curve for the experimental data obtained for uptake and clearance process of ICG in rat cortical vasculature. The fitted data was obtained from the experimental data using the polynomial fit with a degree of 17.

References:

1. Please ensure references have a consistent format.

Reply: Thank you. We have gone through the references and made sure of a consistent format in the revised manuscript.

Table of Materials:

1. *Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.*

Reply: Thank you for your suggestion. We have gone through the table of materials information and revised accordingly.

Reply to the reviewer's comments

Reviewer#1

Manuscript Summary:

In this work, Kalva and colleagues demonstrate photoacoustic imaging with a pulsed laser diode for monitoring dye wash in and wash out from rat cortical vasculature. This is impressive work and will certainly be of interest to the research community. Some minor concerns/suggestions are mentioned below.

Reply: We would you like to offer our sincere thanks for your encouraging and constructive comments. The manuscript has been revised to address these comments.

1. The distinction between "First-generation" and "Second-generation" may be a point of confusion. The term "first-generation" in the title to me would imply that a prior design had been adopted by many groups. However, it is not clear if "first-generation" refers to the Pramanik group's prior design only in this topic. In that case, it seems a bit heavy to use the term "second generation" in this context. Will the next design modification/improvement be called "third-generation? The authors may consider to remove the term "second-generation" from the title.

Reply: Thank you bringing this discussion. Nd:YAG/OPO based PACT/PAT systems can be considered as first generation PACT systems. These lasers are bulky, expensive and occupy large space. Their repetition rates are also very low (below 100 Hz). Whereas the PLD lasers are compact, low-cost, portable and have high repetition rates in the order of KHz. Therefore, we consider the pulsed laser diode (PLD) based PAT systems as second generation systems.

The corresponding text has been modified in the revised manuscript.

"Nd:YAG/OPO lasers are conventional excitation sources for first generation PAT systems that are widely used in photoacoustic community for small animal imaging, deep tissue imaging[16] etc."

"Compact in size, lower cost and higher pulse repetition rate (order of KHz) of the pulsed laser diodes (PLDs) makes them more promising for real-time imaging. Due to these advantages, PLDs are actively used as alternate excitation source in second generation PAT systems."

"Portable PLD-PAT system was demonstrated previously by mounting the PLD inside the PAT scanner.[24] With one SUT circular scanner, phantom imaging was done in 3 s scan time and in vivo rat brain imaging was done in 5 s using this PLD-PAT system.[18] Further, improvements are done to this PLD-PAT system to make it more compact and desktop model by using 8 acoustic reflector based single-element ultrasound transducers (SUTRs).[25, 26]"

To avoid confusion, we have removed “second generation” from the title and have referred to our new design as “desktop PLD-PAT system” throughout the revised manuscript.

2. The authors should mention how this design compares to other similar implementations with compact laser and ring or 3/4 ring transducer such as Wang et al., Biomedical Optics Express Vol. 8, pp. 112-123 (2017).

Reply: Thank you for raising this point. By mounting the PLD laser inside the PAT scanner, we were able to directly illuminate the sample without use of any optical fiber. Use of any optical fiber results in loss of amount of energy delivered on to the sample. Also, we were able to make the whole PAT system compact by saving lot of space in case of laser being outside the scanner. Though the PLD has lesser energy (few mJ), due to high repetition rates in the order of KHz compared to Nd:YAG lasers (1-50 Hz), we are able to achieve high resolution images and able to image till 3 cm deep inside the tissue. We have used commercially available cheap single-element ultrasound transducers and augmented them with 45 degree acoustic reflectors (SUTRs) for PA signal detection. These acoustic reflectors are also available in the market at cheaper price. Whereas the ¾ ring array transducer used by Wang et al., are very expensive, being custom-made. Also, it has limited view problem since it can collect the PA data only 270 degree around the imaging object for a single cross-sectional scan. Whereas in desktop PLD-PAT system, by scanning the 8 SUTRs in 45 degree within 0.5 s scan time, we were able to collect the PA data in full 360 degree around the target object eliminating the limited view problem.

3. In mentioning deep tissue imaging in the introduction, the authors may include the reference Zhou et al., Theranostics. 2016; 6(5): 688-697, which demonstrated photoacoustic imaging through 11.6 cm of chicken breast.

Reply: The suggested reference has been added in the second paragraph of introduction section of the revised manuscript.

“Nd:YAG/OPO lasers are conventional excitation sources for first generation PAT systems that are widely used in photoacoustic community for small animal imaging, deep tissue imaging[16] etc.”.

Reviewer#2

Major Concerns:

The abstract states "in vivo imaging of small animal cortical vasculature", however not any solid result supports that. The introduction does not mention many existing full-ring PACT systems that are similar instead many self-citation. The figures are too simplistic, e.g., Fig 2 does not make any scientific point, Fig 3 does not have any statistical analysis to show that the results are reproducible. More experimental results will be appreciated.

Reply: We would you like to offer our sincere thanks for your encouraging and constructive comments. The manuscript has been revised to address these comments.

Figures 2 (a) and 2(b) show the reconstructed PA images of *in vivo* small animal cortical vasculature for 4 s and 0.5 s scan time respectively. The reconstructed image for 0.5 s scan time was obtained by using 8 SUTRs rotating for 45 degree around the target object whereas the reconstructed image for 4 s was obtained by using 1 SUTR rotating for 360 degree around the target object. We need to rotate all 8 SUTRs once around 360 degree in order to obtain the scanning radius for each of them before conducting the dynamic imaging of ICG dye wash in and out process.

Figure 3 is a representation of ICG dye wash-in and wash-out process. We had obtained the similar rise and fall pattern in the previously published article on this work[1]. This shows that the results are reproducible.

[1] S. K. Kalva, P. K. Upputuri, and M. Pramanik, "High-speed, low-cost, pulsed-laser-diode-based second-generation desktop photoacoustic tomography system," Optics Letters 44(1), 81-84 (2019).

We have modified the text in the revised manuscript mentioning about existing full-ring PACT system.

"State-of-the-art single-impulse PACT system with customized full-ring array transducer obtains the PA data at 50 Hz frame rate[17]. These array transducers need complex back-end receiving electronics and signal amplifiers making the overall system more expensive and difficult for clinical translation."