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*

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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)."

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

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."