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SPIE.

Event: SPIE Photonics Europe, 2022, Strasbourg, France

# Evaluation of penetration depth of near-infrared irradiation generated by tunable ultra-short pulsed laser in *ex vivo* samples of mouse head

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## ABSTRACT

Optogenetic research has opened up the possibility to control neurons that will help detect and treat neurological diseases in the early stage. Treatment of dysfunctions requires exposure to a partial neural network accessible through the absorption of opsins or phytochromes expressed in the brain matter. The use of II-NIR USP lasers makes it possible to non-linear activate and deactivate photoactuators in neuronal cells through the skull. The possible obstacles for non-invasive stimulation are the limits in light penetration depth, scattering and absorption by biological tissues.

This research aimed to investigate light propagation and penetration depth in skin, skull and brain matter of mouse head. To evaluate the light transmittance in brain tissues, we developed an experimental setup with a tunable ultra-short pulsed laser source operating at the wavelength range of 1.1-1.2 μm. This spectrum range corresponds to the spectra of non-linear absorption of opsins/phytochromes and matches the second biological window where laser irradiation can penetrate the skin and skull bone without damaging and overheating them. The experimental results demonstrate that under certain conditions, the ultra-short pulsed laser radiation can reach a penetration depth with required power that will be sufficient for non-linear activation of opsins/phytochromes in the brain of living animals. These results could support applications of II-NIR USP laser in non-invasive optogenetics, photobiomodulation of the brain functioning and even neurological disorders diagnostics.

**Keywords:** tunable ultra-short pulsed laser, mouse head samples, light propagation

## 1. INTRODUCTION

Modern optogenetics is associated with the use of photoactuators excited by various wavelengths from the visible to the near-infrared range. Since optogenetic research is developing rapidly, opsins and phytochromes have opened up the possibility of precise modulation of neuronal activity. Light-sensitive proteins have proven advantages of their use in a treatment of sensorineural hearing loss[1,2], mood disorders[3], drug addiction[4], obsessive compulsive disorders[5], inherited retinal diseases[6] and Parkinson's diseases[7, 8].

On the other hand, medical *in vivo* applications require highly sensitive and non-immunogenic photoactuators. There are some problems associated with non-invasive neural stimulation. The main obstacle is the passage of a laser light through the skin, bones of the skull, and brain cortex tissues[9]. These biological samples significantly absorb visible light, which leads to their overheating and damage. This effect can be mitigated in two different ways. The first one is the development of photoactuators excited by wavelengths that are located in transparency windows. The so-called biological windows cover three near-infrared regions: (I) ~700-950 nm, (II) ~1000-1350 nm, (III) ~1550-1870 nm. Till date, the long-wavelength sensitive phytochromes developed are activate only in the first biological window[10].

The second solution is two-photon activation and deactivation of light-sensitive proteins[11]. This allows us to manipulate a photoreversible conversion, which can be driven by two-photon absorption in the second biological window[12].

To compare different approaches, we investigate the light-tissue interaction using the laser we developed, operating in first two biological windows. This study aims to assess the depth of light penetration into brain tissue, , taking into account scattering, reflection and absorption by the skin, skull and upper layers of the brain. In this investigation, we present the results of laser light transmittance through *ex vivo* samples of adolescent mouse head.

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Tissue Optics and Photonics II, edited by Valery V. Tuchin, Walter C. P. M. Blondel, Zeev Zalevsky, Proc. of SPIE Vol. 12147, 1214708
 · 0277-786X · doi: 10.1117/12.2621193

## 2. MATERIALS AND METHODS

### 2.1 Sample preparation

For all experiments, freshly prepared biological tissues of wild mice were used. The samples were obtained from four adolescent (postnatal P43) healthy male mice using standard protocol. Prior to sacrifice, mice were anesthetized with 5% isoflurane and nitrous oxide ($N_2O$) carried by compressed medical oxygen. The heads were gently shaved to remove fur from the skin covering frontal and parietal bones. Small incisions were made to cut a 15×15 mm square sample of scalp. The total thickness of scalp including skin, connective tissue, aponeurosis, loose areolar tissue was 0.5 mm. Figure 1 demonstrates schematic illustration of investigated mouse head tissues.

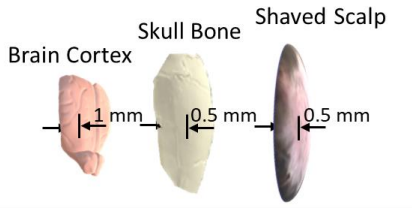


Figure 1. Illustration of mouse head tissues: brain slice, skull, shaved scalp (thickness of 1 mm, 0.5 mm, 0.5 mm, respectively).

For an undamaged extraction of the mouse brain, the cranial bone was dissected at the site of the interfrontal and mendosal sutures. The part, that contains frontal, parietal and interparietal bones, was used for optical measurements. The thickness of parietal bone was measured with a vernier caliper and varied from 0.48 to 0.52 mm for different mice samples. Since the brain was sliced at a thickness of 1 mm from cortex, gray matter was dominant compared to the amount of white matter in the sample. To prevent the brain from leaking or drying out, the sample and the 1 mm distance limiters were fixed between microscope slides.

### 2.2 Experimental setups

In our study, we investigated healthy mouse head samples using spectrophotometer and a developed laser source operating at the wavelength range of 1086-1183 nm. A Lambda 1050 UV/Vis/NIR spectrophotometer with a specialized integrating sphere module was used to record the transmittance and diffuse reflectance spectra[13].

For each sample, two types of spectrophotometer configurations were used. In the first case, a sample was located between integrating sphere and laser operating in a wavelength range of 300-2000 nm. The additional window in the sphere was closed by a reflectance standard. The transmitted light passed through biological tissues was collected by a photodetector. The second operating mode allows us to measure diffuse reflectance of mouse head tissues. A light source passed unhindered through the first window in the integrating sphere and then reflected by a sample, which closed the second window.

Additionally, transmission measurements of mouse head tissues were performed using the developed tunable laser system, which includes continuous-wave (CW) and pulsed modes. For an accurate comparison of the techniques, collimated beams of continuous-wave and pulsed lasers were directed to the same spot of sampling material. The experimental setup included three different lasers (Figure 2). The first one was a pulsed laser operating at 750-830 nm wavelength, while the other two were 1086-1183 nm CW and pulsed lasers.

The first laser was designed to measure the tissue transmittance in the first biological window. The 270 fs pulsed titanium-sapphire (Ti:Sa) laser (M squared Sprite XT) tunable in the 750-830 nm wavelength range was attenuated until 20 mW average power. The repetition rate was 78 MHz. The collimated laser beam with a diameter of 200 um was directed by a system of optical lenses. In the experiments, the beam was split by a plane-parallel plate to control the incident and transmitted power. An optical power meter (Thorlabs S145C) was closely attached to the measuring sample to detect the passing light.

The second part of the experimental setup included a synchronously pumped optical parametric oscillator pumped with the Ti:Sa laser. The Ti:Sa laser was tuned to emit a certain wavelength in the range of 750-830 nm. After passing through the optical parametric oscillator (OPO), it allowed us to obtain laser radiation with the corresponding

wavelength in the range of 1086-1183 nm. The average optical power passed to the sample was 20 mW, while the pulse duration was about 300 fs. The laser beam was collimated and focused into a spot with a diameter of 230 microns.

To compare the transmittance of mouse head tissues irradiated with pulsed and CW lasers, we developed a tunable continuous-wave laser operating at a wavelength of 1086-1183 nm. The third laser included a system of optical lenses and mirrors, a fiber coupled gain chip (Innolume GM-1140-120) and diffraction grating of 1200 lines/mm, that allowed us to change the wavelength with a step equivalent to the value of the pulsed laser. The optical system was established in such a way that the beam directions and spot sizes coincided for pulsed and continuous-wave lasers. The profile and beam diameter were controlled by a laser beam profiling digital camera.

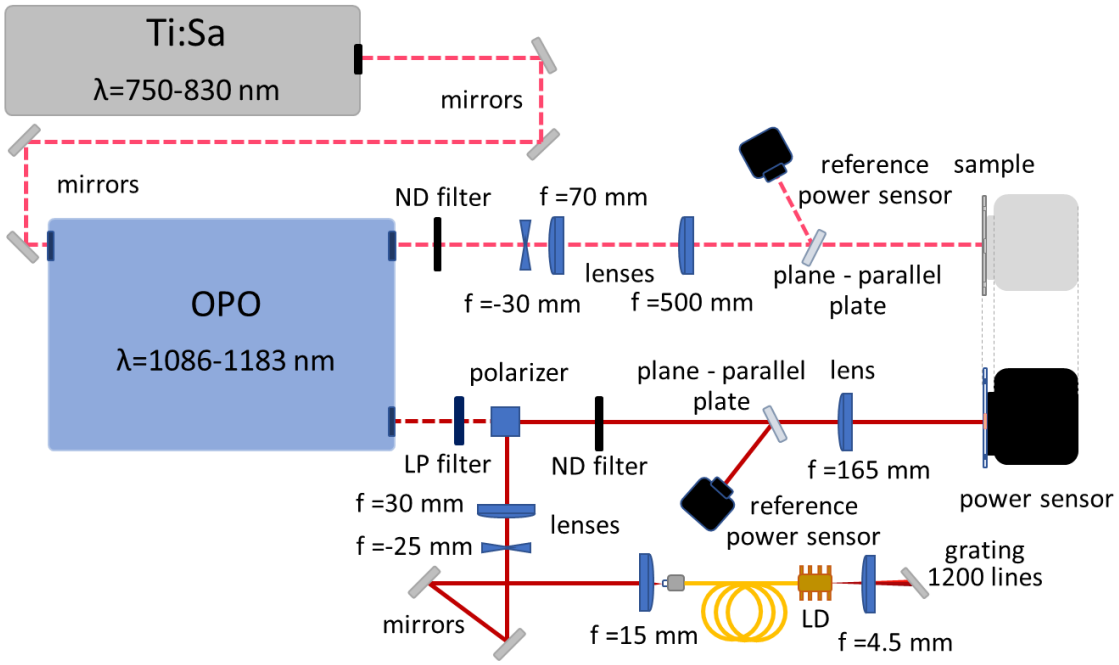


Figure 2. Schematic view of an experimental setup for measuring the transmittance of mouse head tissues irradiated with CW and pulsed lasers at the same point.

## 3. RESULTS

### 3.1 Spectrophotometry

The optical properties of freshly prepared mouse head samples (0.5 mm scalp, 0.5 mm skull and 1 mm brain) were measured using UV/Vis/NIR spectrophotometer. Figure 3 demonstrates transmittance and diffuse reflectance spectra with three biological transparency windows separated by water absorption peaks (1490 nm, 1950 nm).

The transmittance spectra clearly show three biological windows in the near-infrared region. The first (I) one covers wavelength region of ~700-970 nm, while the second (II) and third (III) windows can be identified in ~1000-1350 nm, ~1550-1870 nm, respectively.

Considering the light transmittance in windows, (III) near-infrared region demonstrates the lowest transmittance for all biological tissues, but this ratio depends on specific type of mouse samples, age and sample preparation. The highest transmittance values for both scalp and brain of wild type mice of C57BL/6J genetic background are in the wavelength range of ~1040-1190 nm, which is in the second biological window. In this region, all spectra have a noticeable trough at the wavelength of about 1195 nm that can be characterized by the light absorption of water[14]. Besides the trough at 1190 nm, water absorption can be observed at 975 nm, 1450 nm and 1760 nm.

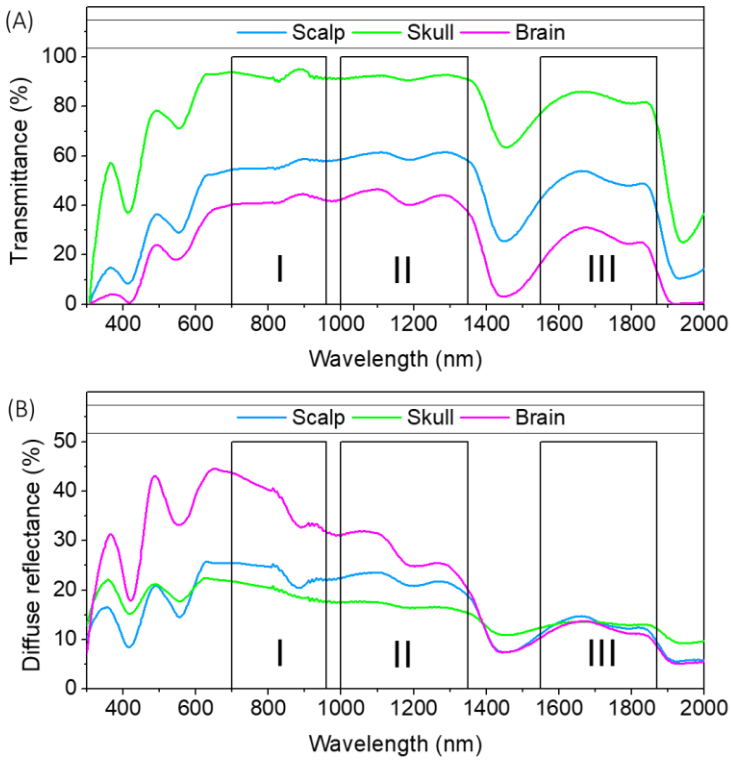


Figure 3. (A) Transmittance and (B) diffuse reflectance spectra of mouse head tissues measured with UV/Vis/NIR spectrophotometer. Thickness of mouse scalp, skull and brain are 0.5 mm, 0.5 mm and 1 mm, respectively. Three biological windows are indicated: (I) ~700-970 nm, (II) ~1000-1350 nm, (III) ~1550-1870 nm.

In general, main transmittance troughs in spectra are associated with absorption of blood, water, collagen, other proteins and lipids. In the second near-infrared region, the influence of these components on the tissue transmittance is significantly lower. It is quite important that there is a significant decrease in scattering in this area. This fact confirms the advantages of using the second biological window in comparison to the first and third ones. The lasers operating at 1040-1190 nm wavelength potentially can be used for non-linear absorption of opsins/phytochromes in brain tissues without damaging and overheating skin and skull bone.

### 3.2 Transmission measurements

We investigated *ex vivo* mouse samples using continuous-wave and pulsed lasers operating in the second biological window and compared results with transmittance in the first near-infrared region. The thickness of freshly prepared brain cortex, skull bone and scalp were 1 mm, 0.5 mm and 0.5 mm, respectively.

Since phytochromes can be activated by absorbing low-intensity light in the far-red and near-infrared regions[15,16], we have used fs pulsed laser in the wavelength range of 750-830 nm, which is covered by the first biological window[17]. Figure 4a demonstrates the transmittance spectra of biological tissues irradiated by 750-830 nm ultra-short pulsed laser.

The investigation of the second tissue transparent window is attractive for bioimaging[18] and two-photon absorption of opsins and phytochromes[15,19,20]. Transmittance measurements in this biological window were carried out using continuous-wave and pulsed lasers operating in the wavelength range of 1086-1183 nm. To make a comparison, the parameters of incident beams were matched for both types of lasers.

Each measurement was carried out in less than 5 seconds to avoid damage to the sample. During the experiments, the tissue temperature did not increase by more than 1.5 °C that was controlled by a thermocouple. Transmittance measurements were repeated three times for each sample. Figure 4b demonstrates the average transmittance values depending on a laser wavelength. A solid line shows the results of measurements using a continuous-wave laser, while a dotted line depicts the transmittance values of mouse head tissues irradiated with an ultra-short pulsed laser.

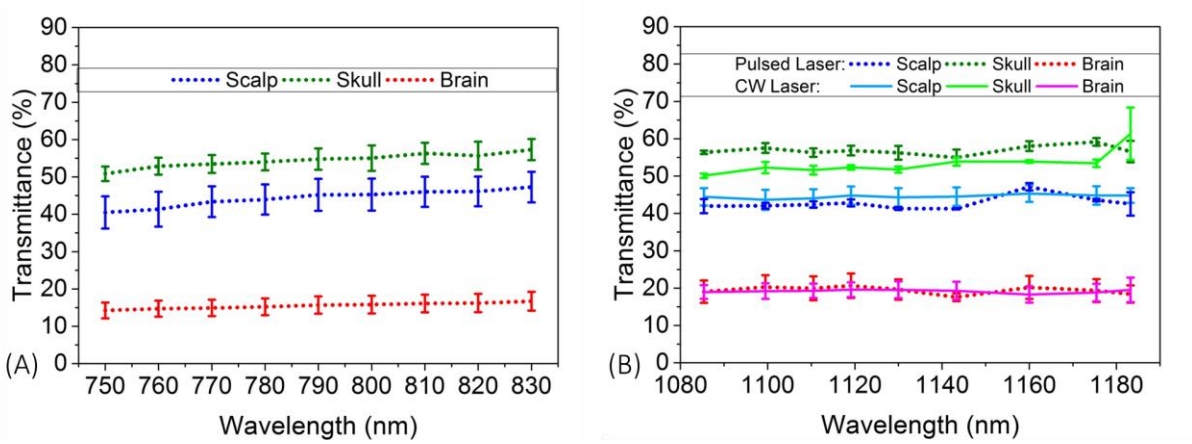


Figure 4. Spectra of the transmittance for the freshly prepared mouse head tissues irradiated with continuous-wave and pulsed lasers at the same point.

According to experimental results, the transmittance of scalp irradiated by the pulsed laser is increasing by 7% in the wavelength range of 750-830 nm. In the region of 1086-1183 nm, the transmission value is actually constant and is about 43%. Similar values are observed for CW radiation.

The green and red curves show the transmission spectra of skull bone and brain cortex, respectively. In the case of pulsed laser irradiation, both curves demonstrate the growth of light transmittance with increasing wavelength in the range of 750-830 nm. The reason is a decrease in the absorption of light by blood hemoglobin in this range.
Since the proportion of red blood cells in the vessels of skull bone and brain cortex changes significantly after eight hours[21], the optical properties of the samples were measured no later than an hour after sample preparation. The difference between a freshly prepared skull sample and one stored on ice for 24 hours is noticeable in Figure 5.

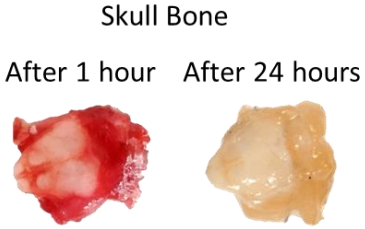


Figure 5. Blood vessels of skull bones: 1 hour and 24 hours after sample preparation.

The comparison of transmittance spectra of skull irradiated with CW laser (green solid line) and pulsed laser (green dotted line) in the 1086-1183 nm region shows that the CW beam is more reflected from the surface of the bone, while the pulsed laser light penetrates deeper to the sample. In the case of brain transmittance spectra, the difference between CW and pulsed laser beams is not significant. However, it is important to note that with the help of pulsed radiation, it is possible to deliver more power deeper into the tissue, while not causing overheating.

The ultra-short pulsed lasers operating in the second near-infrared region proved their efficient to deliver high power to brain cortex through scalp and skull bone without damaging. Since 1190 nm lasers can successfully activate and deactivate bacterial phytochromes[15], ultra-short pulsed lasers operating at this wavelength can reach a penetration depth with required power that will be sufficient for non-linear absorption of opsins/phytochromes in the brain of living animals.

## 4. CONCLUSION

The laser light transmittance through *ex vivo* samples of adolescent mouse head such as skin, bone skull and brain cortex has been investigated. The transmission and diffuse reflectance spectral measurements were carried for all mouse head samples. The investigation was performed in a broad wavelength range of 350-2000 nm with analysis and comparison of

three near-infrared biological windows. All samples show high values of transmittance in the region of 1040-1190 nm wavelength.

To investigate laser-tissue interaction with skin, skull and brain, we developed a tunable source with 1.1-1.2 μm operating in the continuous-wave and pulsed mode. The samples, consisting of a skull up to 0.5 mm thick and a brain tissues up to 1 mm thick, demonstrated sufficiently high transmission of II-NIR ultrashort pulses. Approximately 22-23% of initial radiation passed through scalp and skull samples while the temperature rise was limited to 1.5 °C.

The results show that the radiation of the developed tunable laser source passes through mouse head tissues efficiently. This amount of irradiation should be sufficient[15] for nonlinear phytochrome photo-conversion. The results suggest the possibility of *in vivo* optogenetics in living animals and can be used for further research for non-invasive applications in photobiomodulation of the neurons.

## ACKNOWLEDGMENTS

This work has been supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No.863214 – NEUROPA project. D.G. gratefully acknowledges funding by the Return to Research grant, Rank Prize.

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