Quickly Alternating Green and Red Laser Source for Real-time Multispectral Photoacoustic Microscopy

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\textbf{A B S T R A C T}

Multispectral photoacoustic microscopy uses a wavelength-dependent absorption difference as a contrast mechanism to image the target molecule. In this paper, we present a novel multispectral pulsed fiber laser source, which selectively alternates the excitation wavelengths between green and red colors based on the stimulated Raman scattering (SRS) effect for imaging. This laser has a high pulse repetition rate (PRR) of 300 kHz and high pulse energy of more than 200 nJ meeting the real-time requirements of optical-resolution photoacoustic microscopy imaging. By switching the polarization state of the pump light and optical paths of the pump light, the operating wavelengths of the light source can be selectively alternated at the same fast PRR for any two SRS peak wavelengths between 545 and 655 nm. At 545 nm excitation wavelength, molecular photoacoustic signals from both blood vessels and gold nanorods were obtained simultaneously. However, at 655 nm, the photoacoustic signals of gold nanorods were dominant because the absorption of light by the blood vessels decreased drastically in the spectral region over 600 nm. Thus the multispectral photoacoustic system designed using the novel laser source implemented here could simultaneously monitor the time-dependent fast movement of two molecules independently, having different wavelength-dependent absorption properties at a high repetition rate of 0.49 frames per second (fps).

\textbf{1. Introduction}

The optical-resolution photoacoustic microscopy (OR-PAM) is a high-contrast and high spatial resolution biomedical imaging technique to image underlying biological tissues \cite{1,4}. The increase in temperature, owing to the absorption of short laser pulses in the endogenous chromophores or exogenous contrast agents of tissue, causes a thermelastic expansion of biological molecules leading to the generation of a pressure wave in the form of a photoacoustic wave. An ultrasound transducer detects these waves, and images are reconstructed to provide structural, functional, and molecular information of the underlying tissues \cite{5}. The OR-PAM is sensitive to the optical absorption contrast, while conventional high-resolution imaging techniques, such as optical coherence tomography, confocal microscopy, and two-photon excitation fluorescence microscopy, are sensitive to optical scattering and fluorescence contrast \cite{2,6,7}. To date, OR-PAM has been applied to many clinical applications based on endogenous biomolecules such as oxyhemoglobin, deoxyhemoglobin, lipids, and melanin. Applications include functional brain imaging, measurement of the oxygen consumption rate of a tumor, imaging of lipid-rich samples \cite{8}, and detection of circulating melanoma cells \cite{9}. Often, additional exogenous agents, such as metallic nanoparticles (gold nanoparticles, silver nanoparticles, iron oxide nanoparticles, carbon nanotubes, etc.), dyes (Evans blue, methylene blue, indocyanine green, etc.), and semiconducting polymers, are used for contrast enhancement, targeted imaging, drug delivery, and photothermal therapy \cite{10–15}. However, exogenous agents have different intrinsic optical absorption wavelengths as compared to endogenous biomolecules. Thus, to produce high

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signal-to-noise ratio (SNR) wavelength-dependent photoacoustic signals from various biomolecules as well as exogenous contrast agents, they need to be induced by a specific light source with an appropriate wavelength and pulse energy. Previous multispectral OR-PAM studies commonly used a dye laser or an optical parametric oscillator (OPO) because of the broad spectral range provided by these sources, i.e., from the visible to the near-infrared region. However, they suffer from a low pulse repetition rate (PRR) of 10 Hz – 10 kHz [16,17] and thus are not suitable for fast OR-PAM imaging, where PRR of over several hundred kHz is needed. Besides, OPO is bulky, expensive, and requires additional cooling. In this study, in addition to the pump laser module at 532 nm, we only used the optical fibers with different lengths for the wavelength tunability, which is very cost-effective compared to the OPO laser.

Recently, a diode-pumped fiber laser has been developed, which has a high PRR of several hundred kilohertz (kHz) to a few megahertz (MHz) and has sufficient pulse energy to produce a photoacoustic effect [18]. Also, a fast beam scanning system, with a galvanometer or a micro-electromechanical system (MEMS) mirror, and a high-speed signal processing system, such as a field-programmable gate array (FPGA) and a graphics processing unit (GPU), have been recently developed for fast real-time OR-PAM imaging at high PPR of over several hundred kHz. However, since diode-pumped fiber laser has a fixed operating wavelength, it could not be used for generating high-speed real-time multispectral OR-PAM images.

For facilitating wavelength tunability, several new techniques have been introduced in the development of fiber lasers. The generation of a supercontinuum using a nonlinear optical fiber is one of the methods for multispectral photoacoustic microscopy. In this method, a photonic crystal fiber was irradiated by a 1064 nm pump light with 1 kHz PRR to obtain a pulse light with a bandwidth ranging from 500 to 1600 nm. However, this supercontinuum source suffered from a low pulse energy density of 15 nJ/nm [19]. The second method consisted of a fiber laser based on a master oscillator power amplifier and was combined with a supercontinuum source and harmonic generation units. The supercontinuum pulse light of bandwidth 450 to 1100 nm was generated by irradiating a photonic crystal fiber with a 1064 nm pump light. The harmonic pulse lights of wavelengths 226, 355, and 532 nm were obtained using a nonlinear crystal. However, since the light in the visible region was obtained using the supercontinuum method, this technique also suffers from low pulse energy density [20]. The third method uses the stimulated Raman scattering (SRS) effect, which is a nonlinear optical effect, that produces one or more Stokes lights that were downshifted to the frequency of the pump light [21]. If the peak power of the pump light is strong, multiple-order Raman downshift will cause cascaded frequency shifts in a silica optical fiber with an interval of 13.2 THz. Recently, our research group reported the generation of multiple-wavelengths using SRS sources as one of the OR-PAM applications [22]. An increased number of SRS peaks up to the tenth Stokes order of 695 nm, with a pulse energy of up to 116 nJ, and a PRR of 300 kHz from a pump light of 532 nm was reported [23]. A 700 nm SRS laser, providing a pulse energy of up to 200 nJ and a PRR of 50 kHz, was used for photoacoustic imaging of Prussian blue nanoparticles in a mouse tumor [23]. Even though the SRS sources facilitated multispectral imaging, the real-time OR-PAM images at different excitation wavelengths, however, could not be obtained. It is difficult to manually adjust the optical power of the pump light to achieve the optimal condition for each Raman Stokes light of the desired excitation wavelength to acquire the wavelength-specific photoacoustic images [22].

Recently, new methods have been described to switch the excitation wavelengths of the SRS laser source by using a delay line [24], polarization modulation [6,25], and frequency-domain approaches [26]. In the delay-line method, light with multiple wavelengths of 532, 545, and 558 nm at PRR of 1 MHz was obtained by passively switching the wavelengths using an optical delay line composed of long optical fibers [24]. In this method, a delay time, which is shorter than the pulse repetition time between the multiple propagating lights, was required. However, the use of very long optical fiber for inducing the delay leads to a lowering of the maximum pulse energy of the low-Stokes-order light owing to the SRS effect. Thus, a weak photoacoustic signal was obtained using this method. The polarization modulation method uses an EOM for actively switching the unconverted 532 nm beam and the (2th Stokes order) 558 nm Raman beam at a PRR of 300 kHz [25]. The frequency-domain separation method was used to recover the partially overlapped photoacoustic signals when the multi-wavelength excitation time was shorter than the A-line duration [26]. These multi-wavelength switchable SRS laser studies demonstrated limited wavelength range from 532 to 560 nm (green to yellow colors), which is suitable for vascular morphology, detection of oxygen saturation, blood flow, and oxygen metabolism because oxyhemoglobin and deoxyhemoglobin have strong absorbance in this limited spectral region. Expanding the wavelength range beyond 560 nm would enable new imaging capability and applications, which is the focus of this study.

In this study, we describe a novel multi-wavelength switchable SRS laser to implement multispectral OR-PAM images. Different excitation wavelengths were obtained in real-time for the much-needed expanded wavelength range of 532 to 655 nm (green to red colors). Owing to the optimal condition for efficient Raman Stokes generation, pulse energies of Raman Stokes peaks from the 1th (545 nm) to the 8th (655 nm) were measured to be greater than 200 nJ, which is the minimum required energy level for obtaining multispectral OR-PAM images. The polarization state of the pump light was switched using an EOM at 150 kHz to select an arbitrary path length for the corresponding Stokes order of Raman shift. The desired excitation wavelengths were rapidly alternated between 532 and 655 nm with a PRR of 300 kHz for capturing the multispectral images. The proposed method presented here is an advancement over our previous study [6], where an EOM method was used for alternating illumination at fixed wavelengths of 532 and 1064 nm. The current system achieves real-time photoacoustic image acquisition at 0.49 fps, while not generating any arbitrary SRS peaks. The selective alternating of the excitation wavelengths from 532 to 655 nm (green to red colors) enables the use of various endogenous biomolecules and exogenous agents that have different intrinsic optical absorption wavelengths in this expanded spectral region. In the present study, we present the multispectral capability of our system through an experimental demonstration where multispectral OR-PAM images of blood vessels and gold nanorods in mice were obtained by using two excitation wavelengths at 545 nm (green color) and 655 nm (red color), respectively, in real-time.

2. Materials and Methods

2.1. Multi-wavelength switchable SRS fiber laser source

The schematic of a high-speed multi-wavelength switchable SRS fiber laser source is shown in Fig. 1. The 532 nm pulse laser (GLP-510, IPG Photonics Corp.), used as a seed laser, has a PRR tuning range from 20 to 600 kHz and a pulse duration of 1 ns. The PRR of the 532 nm pulse laser was set to an external transistor-transistor logic (TTL) signal with a frequency of 300 kHz using a function generator (FG). A half-wave plate (HWP) and a polarization beam splitter (PBS) were used for adjusting the optical power and produce a linear polarization state, which is set to a p-polarized state. Linearly polarized light was passed through a pair of lenses (L1 and L2), which were used to reduce the beam size of the pump light to match with the entrance aperture size of the EOM (EO-AM-NR-C4, Thorlabs Inc.). The loss of pump light through the EOM was measured to be approximately 10%.

Light with the reduced spot size was passed through the EOM to switch the polarization state after changing the voltage between 0 and 168.2 V by the high-voltage amplifier (HVA200, Thorlabs Inc.). This voltage change between 0 and 168.2 V corresponds to phase-retardance of 0 and π/2, respectively. When the external TTL signal of 300 kHz, used for generating the laser pulses, and the sine wave signal of 150 kHz,
used for EOM voltage modulation, are in phase, the output beam from the EOM can be switched between the $p$-polarized and $s$-polarized state. The pair of lenses $L_2$ and $L_3$ expands the pump light that passes through the EOM. The expanded light was then split by PBS2 alternately into $s$-polarized light and $p$-polarized light.

The divided beams then travel through two Raman fibers of lengths 5 m and 30 m to generate the desired different Raman Stokes peaks. When the pump light has sufficient power to induce SRS in the silica optical fiber, the pump light gets downshifted in frequency by 13.2 THz, depending on the Raman gain coefficient ($g_R$) of silica. The Raman threshold power, $P_{th}$, for the SRS effect to occur can be described by the following equation: \[ P_{th} \approx \frac{16A_{eff}}{L_{eff} g_R} \] where $g_R$ is the Raman gain coefficient in the medium, $L_{eff}$ is the effective fiber length, and $A_{eff}$ is the effective core area. If $L_{eff}$ of the Raman fiber is increased or $A_{eff}$ is narrowed, $P_{th}$ for generating SRS can be lowered. We used Raman fibers of different $L_{eff}$ values to realize different Raman threshold power and used it to generate the Raman shift peaks of different orders.

As shown in Fig. 1, the $s$-polarized light from PBS2 passes through an HWP and enters the Raman fiber of length 30 m. The HWP controls the polarization state of the incident light at the entrance of the Raman fiber. The $p$-polarized light from PBS2 passes through a linear polarizer (LP) and enters the Raman fiber of length 5 m. Since the 5 m long Raman fiber requires less power to generate the SRS peak of wavelength 545 nm, an LP was used to adjust the peak power of the light. At the end of the 30 m long Raman fiber, the SRS peak with a 655 nm wavelength was generated. The generated Raman stokes lights are passed through a fiber collimator (FC) to generate a uniform beam size with a beam diameter of 4 mm. If the beam size is too small, the focused beam size increases, making it difficult to generate photoacoustic effects, and also reducing the lateral resolution. Both beams were filtered by optical bandpass filters (FT1 and FT2) and then combined again by a dichroic mirror (DM) into a single beam. The final output is thus a 300 kHz pulse light that alternates between the wavelengths of 545 and 655 nm, which is used as the excitation light to differentiate photoacoustic signals.

### 2.2. Real-time functional laser-scanning OR-PAM system

The schematic of the real-time laser-scanning OR-PAM system is shown in Fig. 2. The output light from the collimated multi-wavelength switchable SRS fiber laser source, having a beam diameter of 4 mm, was...
scanned by a 2D galvanometer scanning mirror (GVS002, Thorlabs Inc.). The B-san (fast-axis) rate was 240 Hz. The light was focused by an objective lens (49-356, Edmund Optics Inc., Barrington, NJ) having a focal length of 50 mm and a numerical aperture of 0.25. For detecting the acoustic waves in reflection mode imaging, a glass plate with a thickness of 1 mm was used in a water cube at 45° [6]. The photoacoustic signals generated from the sample were detected by the focused ultrasound transducer having a focal length of 50.8 mm, a center frequency of 10 MHz, and −6 dB bandwidth of 74.96 % (V312-N-SU, Olympus-NDT, Japan). The detected photoacoustic signals were then amplified by a 47 dB amplifier (5072PR, Olympus-NDT, Japan). These were finally converted by a digitizer with a 12-bit resolution and a sampling rate of 250 Msamples/s (AT9250, AlazarTech Inc.). The analog output board generated synchronized signals for a frame trigger of 240 Hz and generated the triangular waveform for the fast-axis galvanometer mirror. For real-time OR-PAM image acquisition, parallel signal processing was performed using the GPU described in our previous study [6,28].

2.3. Animal preparation

All the experimental procedures were approved by the Animal Care Committee of the Korea Research Institute of Bioscience & Biotechnology (KRIBB). In this study, we used one 8-weeks old BALB/c-nude mouse. The mice were anesthetized with a 300 μL 2.5 % Avertin during treatment and imaging. At the end of the experiment, the mice were euthanized.

3. Results and discussion

3.1. Multi-wavelength alternating based on polarization modulation

For real-time multispectral OR-PAM images, two excitation wavelengths, with the sufficient energy level and repetition speed, were selectively alternated. The polarization modulation was carried out using the EOM, as the multi-wavelength switching method, while preserving the incident pulse energy. Unlike the delay line method [24], since this switching method can preserve the total peak power of pump light, it is suitable for generating higher Stokes order light having wavelengths over 600 nm. By adjusting the phase retardation condition, the polarization state of the incident light can be determined to be either a p- or an s-polarized state. The EOM was driven by a sine-wave signal, modulated between 0 V to 168.2 V, in which the phase retardance of 0 to π/2 was attained. Fig. 3 shows the 532 nm light pulse signal detected by a photodiode after passing through the PBS.

The unmatched phases of the EOM modulation signal and laser pulse trigger (TTL) are shown in Fig. 3(a). In this condition, since the mismatched phase retardance of EOM prevents the polarization state to be changed linearly to a p- or an s-polarized state at every 150 kHz, the laser pulse signal could not be switched at 150 kHz. However, when the phase retardance of EOM matches well with the TTL trigger signal, the laser pulse signal can be switched at 150 kHz. The results at the positions of PBS transmission (p-pol) and PBS reflection (s-pol) are shown in Figs. 3(b) and (c), respectively. The SNR was high and measured to be approximately 22 dB. The obtained 150 kHz of the switching pulse light can be irradiated alternately onto the Raman fibers of different lengths, generating different wavelengths.

3.2. Characteristics of multi-wavelength switchable SRS fiber laser

The full spectrum containing the Raman Stokes peaks obtained by passing the beam through the Raman fibers (PM-8405-xp, Thorlabs Inc.) of lengths 5 and 30 m are shown in Fig. 4(a) and (b), respectively, and are measured by a spectrometer. The maximum Stokes order and maximum pulse energy were determined by the combination of the length of the Raman fiber and the peak power of the incident light obtained through experiments [22]. The 5 and 30 m Raman fibers, chosen here, obtain the maximum pulse energy at the wavelengths of 545 nm (1st order Stokes peak) and 655 nm (8th order Stokes peak), respectively [22]. The coupling efficiency of the Raman fiber was measured to be approximately 50 %. When the peak power of the pump light was approximately 1.5 kW, the maximum pulse energy was obtained at 655 nm. Thus, specific excitation wavelengths needed for photoacoustic imaging between 532 and 655 nm can be selected from multiple Stokes peaks by simply controlling the length of the Raman fiber.

When a strong nanosecond laser pulse propagates through an optical fiber, additional wavelengths are generated owing to SRS and four-wave mixing (FWM) [22,29]. As shown in Fig. 1, HWP was used in front of the 30 m Raman fiber for adjusting the polarization of the incident light. We measured the spectrum at different angles of the HWP to produce stable high Stokes order peaks while suppressing the FWM effect. As shown in Fig. 4(b), when the HWP angle is 0°, the FWM effect is highly suppressed, and discrete SRS peaks can be observed. On the other hand, when the HWP angles are 20° and 30°, a slight misalignment in peaks is observed, as compared to 0°, due to the polarization of the incident light after passing through the Raman fiber.

We observed that the FWM effect occurs at the pump light wavelength of 532 nm, which interrupts the discrete SRS shift. The FWM makes the lower Stokes order peaks broader, and the generation of higher Stokes order peaks difficult. For minimizing the effect of FWM in the spectrum, it is necessary to adjust the polarization state of the incident light using the HWP more so in the 30 m Raman fiber arm because the higher Stokes order is affected more by the FWM conditions. As a result, for generating higher Stokes order light longer than 600 nm, it is necessary to control the polarization state of the incident pump light using the HWP.

The long-term stability, i.e., for 60 minutes, of the filtered output pulses at wavelengths 545 and 655 are shown in Fig. 4(c). The average power was monitored using an optical power meter (S121C, Thorlabs Inc.) to verify whether the filtered wavelength in the Stokes light,
generated by the Raman fiber, has sufficient pulse energy and stability needed for the PAM system. The pulse energy of the output light at both 545 and 655 nm was measured to be above 200 nJ, which is sufficient for generating the photoacoustic signal. The relative standard deviation of measured average power at 545 and 655 nm wavelengths was 1.60 % and 2.62 %, respectively. After the polarization switching of the pump

Fig. 4. (a) SRS spectra obtained from the Raman fibers of different lengths and peak power of the pump light: SRS spectrum obtained from the 5 m Raman fiber and SRS spectrum obtained from the 30 m Raman fiber at 1.5 kW of peak power. (b) SRS spectra (30 m Raman fiber) affected by the rotation angle of the HWP kept at 0°, 20°, and 30°. The spectrum of 0° is a reference spectrum at the reference angle when the FWM phenomenon is observed to be highly suppressed. (c) Long-term stability of the filtered output pulse at 545 nm and 655 nm for 60 min.

Fig. 5. Time- and spectral-domain characteristics of the multi-wavelength high-speed switchable pulsed SRS fiber laser. (a) and (b) are time- and spectral-domain spectra obtained at 545 nm, whereas those in (c) and (d) are the spectra obtained at 655 nm.
light, the output spectrum of each arm and the beam stability was optimized experimentally. The two beams were then combined in synchronization to be used in high-speed multispectral OR-PAM imaging system.

The time-domain characteristics of the 545 and 655 nm pulse lights are shown in Fig. 5(a) and (c), respectively. Each wavelength was selected using the spectral bandpass filter, and they were measured using a high-speed photodetector. The light’s pulse period at 545 or 655 nm was measured to be 6.66 μs (corresponding to a PRR of 150 kHz), which is twice the laser pulse period of 300 kHz pulse rate. The pulse period of the combined output was 3.33 μs, which corresponds to a PRR of 300 kHz. The output wavelength spectra measured by a spectrometer are shown in Fig. 5(b) and (d). To confirm that selectively alternating excitation wavelengths of 545 nm and 655 nm can be used in real-time multispectral PAM, we injected gold nanorods (having an absorption peak of 650 nm) into the mouse ear. An in vivo photoacoustic imaging experiment was performed on the mouse ear using our proposed laser source to distinguish the nanorods from the blood vessels. Blood vessels have a high absorption peak around 540–580 nm and a drastically lower absorption over 600 nm. For distinguishing blood vessels from gold nanorods in the multispectral PAM images, two-wavelength windows were selected by applying two bandpass filters. The bandwidths of the 545 and 655 nm SRS peaks were measured to be 3.04 nm and 6.42 nm, respectively. The higher the order of the SRS peak, the wider is its bandwidth owing to the nonlinear optical phenomenon. In the 5 m Raman fiber, the SRS spectrum was filtered to obtain the 545 nm peak using a 10 nm bandpass filter. In the 30 m Raman fiber, the SRS spectrum was filtered to obtain 655 nm peak using a 50 nm bandpass filter. Even though the residual energies from the 6th (619 nm), 7th (636 nm) and 9th (674 nm) Stokes peaks were included in the wide bandwidth ranging from 619 to 674 nm, with the peak centered at the strong 8th (655 nm) Stokes, it was still possible to distinguish the strong absorption of light by the gold nanorods around 650 nm, from the lower absorption spectral region of blood vessels around 600 nm.

Fig. 6 shows the lateral resolution of the proposed OR-PAM system. The lateral resolution was obtained by calculating the edge spread function (ESF) and line spread function (LSF) in the raw photoacoustic data, which was acquired by scanning the edges of element 3 group 4 of the 1951 USAF resolution target. The edge spread function (ESF) was obtained from scanning the edge in steps of 1.25 μm and by using the nonlinear Boltzmann function in OriginPro 9. The line spread function (LSF) was calculated by differentiating ESF. The lateral resolution was obtained by measuring the full width at half-maximum (FWHM) of the LSF. The lateral resolution of the proposed PAM system is about 6.5 and 6.8 μm, corresponding to each wavelength of 545 and 655 nm, respectively.

3.3. Real-time molecular photoacoustic image with blood vessels and gold nanorods

Conventional multi-wavelength switchable SRS laser sources with a wavelength of 532, 545, and 558 (green to yellow colors) have been used to spectrally limited applications such as measuring oxygen saturation in hemoglobin, the concentration of hemoglobin, and cerebral blood flow [24–26,30]. In addition, these conventional laser sources have also been used in broader spectral applications with Prussian blue and gold nanoparticles that exhibit the highest absorption peaks beyond 600 nm. However, to the best of our knowledge, no investigation has been conducted so far to implement a wider spectral multi-wavelength SRS switching over 100 nm owing to the lower efficiency of Raman shift caused by the 532 nm pump light [24,25].

For the first time, we demonstrate a real-time multi-wavelength switchable SRS fiber laser source that alternates selectively between two excitation wavelengths within the visible green-to-red region SRS peaks. To show that our proposed laser can simultaneously distinguish endogenous blood and exogenous targets, we selected gold nanorods with high absorbance beyond 600 nm wavelengths as exogenous contrast agents. The gold nanorods (C12-10-650-PEG-DIH-50, Nanopartz Inc.) of diameter 10 nm and length 24 nm were chosen due to their highest absorbance centered at 650 nm and half absorbance at 545 nm; the concentration was $5.55 \times 10^{13}$ nps/mL.

The normalized absorbance spectrum of gold nanorods, oxyhemoglobin, and deoxyhemoglobin, are shown Fig. 7. In this figure, the absorbance spectrum of hemoglobin is adapted from Prahl [31] and Kollias et al. [32], where this spectrum was obtained from 150 g

![Fig. 6. Measurement of the lateral resolution using the edge of an element (element 3 group 4) in a 1951 USAF resolution target: (a) 545 nm and (b) 655 nm. ESF: edge-spread function, LSF: line-spread function.](image)
hemoglobin in 1 L of blood with 90% and 10% oxygen saturation, respectively. The absorbance spectrum of gold nanorods was measured at a concentration of 2.4 mg/ml. The gold nanorods have the highest absorption at 646 nm and broad absorption bands ranging from 500 to 700 nm. They have relatively lower absorbance in the wavelength range of 532, 545, and 558 nm, which are mainly Raman Stoke light wavelengths for blood vessel measurement. In this study, we selected 545 nm and 655 nm Raman stokes light wavelengths to confirm the feasibility of the multispectral photoacoustic imaging of blood vessels and gold nanorods at two specific excitation wavelengths.

For monitoring the time-dependent movement of the exogenous contrast agents in vivo, images of both the target agents and the background blood vessels are needed repeatedly to compare the spectral information in the two images. In this study, the proposed SRS source and laser scanning OR-PAM system were used simultaneously for real-time display of photoacoustic images of the two contrast agents.

Fig. 8(a) shows the process of acquiring a series of photoacoustic images of size 500 × 500 pixels using alternating excitation wavelengths of 545 and 655 nm at each pixel in the images. One of the 2D galvanometers was used to scan the laser beam in the x-axis direction (500 pixels) at a B-scan rate of 240 Hz, and the other 2D galvanometer was used to scan along the y-axis (500 lines) at a C-scan rate of 2.04 Hz. A field of view of 3.5 mm × 3.5 mm, which corresponds to 500 pixels × 500 pixels, was obtained by scanning a 2D galvanometer with the applied amplitude of 0.8 V. The high-speed digitizer collects depth data in the z-axis direction for 6.66 μs, which corresponds to 1536 samples, at an A-scan rate of 150 kHz. Since the alternating excitation wavelengths are distributed at equal intervals within 150 kHz, the photoacoustic signals are collected for excitation wavelengths of 545 and 655 nm by dividing the collected data into two channels of 736 samples each. In

Fig. 8. (a) Process of acquiring a series of photoacoustic images using alternating excitation wavelengths of 545 and 655 nm in real-time. (b) molecular MAP PAM images of the blood vessels and gold nanorods in the mouse’s ear at the excitation wavelengths of 545 nm and 655 nm.
general, the pulse repetition rate is used to adjust the image range to avoid overlapping the photoacoustic signal. In our method, since A-scan is divided into two channels to obtain a photoacoustic signal, the image range is reduced. For a pulse repetition rate of 300 kHz, the image range in our system was calculated to be about 5 mm. Since the photoacoustic system have trade-off between the lateral-resolution and the imaging range, in our OR-PAM system, the achieved range of 5 mm was sufficient when using an objective lens with high NA for high lateral resolution.

The proposed laser can be used in the acoustic-resolution PAM (AR-PAM) system for deeper images by changing the pulse repetition rate from 20 to 600 kHz. The system would also need EOM modulation frequency to be adjusted accordingly to reduce the imaging speed. Thus, our system is flexible to both superficial and deeper structures. The photoacoustic image was then constructed in each channel by measuring

Fig. 9. (a) In vivo time-lapse molecular overlapped MAP photoacoustic images of the blood vessels and gold nanorods injected in the mouse’s ear for 100 seconds. (Supplementary Movie S1) (b) In vivo 3D photoacoustic images of blood vessels and gold nanorods at 545 nm and 655 nm using 3D Photoacoustic Visualization Studio (3D PHOVIS) [33].
the maximum amplitude signal among 736 sampling points per line. The pulse energy irradiated onto the mouse ear, for obtaining the photoacoustic image, was approximately 200 nJ for both 545 and 655 nm excitation beams.

The maximum amplitude projection (MAP) images of blood vessels and gold nanorods in mouse ear, obtained using OR-PAM with multi-wavelength switchable SRS fiber laser, are shown in Fig. 8(b). The 545 nm photoacoustic image shows that the blood vessels and gold nanorods were imaged together. In the 655 nm image, gold nanorods are imaged dominantly. This is because the 545 nm wavelength has the highest absorption for oxy- and deoxyhemoglobin as isosbestic points, and the 655 nm wavelength has the highest absorption for gold nanorods. The normalized absorbance value of hemoglobin at 545 nm is 0.920, while the absorbance of oxy- and deoxyhemoglobin at 655 nm are 0.001 and 0.050, respectively, which are negligibly low. As shown in Fig. 8(b), the photoacoustic signals from the blood vessel network are clearly shown in the wavelength 545 nm image, but not in the 655 nm image. The normalized absorbance values of gold nanorods are 0.376 and 0.961 at 545 and 655 nm, respectively. Since the difference in absorbance at the two alternating excitation wavelengths are not different for gold nanorods, unlike hemoglobin, photoacoustic signals from gold nanorods were measured at both 545 and 655 nm. The resulting overlapped photoacoustic images, with excitation wavelengths of 545 and 655 nm, distinguish both spatial distributions of blood vessels and gold nanorods.

The time-lapse multispectral photoacoustic images of blood vessels and gold nanorods are shown in Fig. 9(a) and Movie S1. A total of 50 images were gathered over a period of 102 seconds by overlapping the photoacoustic images at 545 and 655 nm at a frame rate of 0.49 frames per second (fps). Fig. 9(b) shows three-dimensional (3D) photoacoustic images of blood vessels and gold nanorods at 545 nm and 655 nm, respectively, obtained using 3D Photoacoustic Visualization Studio (3D PHOVIS) software. The 3D PHOVIS is a freely available special 3D visualization software package developed by Cho et al. for visualization and rapid processing of photoacoustic data [33]. We used 3D PHOVIS’s volume renderer function to create 3D images of photoacoustic data acquired with excitation wavelengths of 545 nm and 655 nm. To improve image processing speed, among the 1536 samples obtained in the z-direction, only the regions where photoacoustic signals of 545 nm and 655 nm existed were selected for depth. The 3D images of 545 nm were obtained using 250 samples from 101 to 350. For the 655 nm, samples from 901 to 1150 were used, i.e., 250 samples. These images show that the proposed laser and system not only distinguish blood vessels from gold nanorods but also provide 3D structural information. These results demonstrate that our rapidly alternating excitation wavelengths of the pump light, from the SRS laser source, can monitor the time-dependent fast movement of various contrast agents in real-time and thus is useful for imaging tumor cells, blood vessels, and nanoparticles. The main limitation of our multi-spectral photoacoustic microscopy system is that the number of wavelengths available is two since it uses polarization modulation for the alternating excitation wavelength. However, there are many multi-spectral applications where the number of wavelengths used is only two and thus is very advantageous. For example, it can be used as a real-time functional PAM system measuring oxygen saturation by selecting 545 and 558 nm as alternating excitation wavelengths and by controlling the intensity of the pump light using two 5 m long Raman fibers [24]. In addition, when the wavelength range is changed, the applicable range is also further expanded. When this method is used with a 1064 nm pump light, it is possible to acquire a multi-spectral PA image by selecting two wavelength lengths from various 1097, 1150, and 1215 nm, which are various Raman Stokes peaks of 1064 nm [29]. For example, using 1064 and 1215 nm, it is possible to acquire photoacoustic signals of nanoparticles and lipids simultaneously.

Compared to our previous study of selecting wavelength between 532 nm and 1064 nm [6], the current proposed approach is more flexible and can be applied to the real-time multi-spectral OR-PAM imaging of various contrast agents with different absorption peaks in the visible region (i.e., green-to-red region), such as Prussian blue, methylene blue, and gold nanoparticles. In addition, the proposed laser source has the potential to be used for monitoring drug delivery or photothermal therapy. This is possible because the exogenous contrast agents used as targets can be easily separated from the background blood vessels in real-time.

4. Conclusion

In this study, we demonstrated a real-time multispectral OR-PAM imaging system based on a novel optimized multi-wavelength switchable SRS fiber laser as a source. Since this proposed laser can selectively alternate between 545 nm and 655 nm wavelength at high speed, signals from the exogenous contrast agents can be easily differentiated from blood vessels in real-time. Optimizing the two excitation wavelengths in this laser system is very easy and is achieved simply by adjusting the length of the Raman fibers. The combined excitation pulse lights (545 nm and 655 nm) can be switched at a frequency of 150 kHz, and the pulse energy of more than 200 nJ could be achieved using this laser source. Further work is to alternate wavelengths at 600 kHz PRR corresponding to the image display speed over 1 fps. Such a system with a higher rate would be useful to measure dynamic changes in biomolecules with real-time molecular OR-PAM images and for pre-clinical study.

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Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pacs.2020.100204.

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