Multispectral photoacoustic imaging of tumours in mice injected with an enzyme-activatable photoacoustic probe

Takeshi Hirasawa1, Ryu J Iwatate2, Mako Kamiya2, Shinpei Okawa1, Yasuteru Urano2 and Miya Ishihara1

1 Department of Medical Engineering, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama, 359-8513, Japan
2 Laboratory of Chemical Biology & Molecular Imaging, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan

E-mail: hirasawa@ndmc.ac.jp.

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Abstract
Photoacoustic (PA) imaging offers depth-resolved images of optical absorbers with the spatial resolution of ultrasound imaging. To enhance tumour contrast, tumour-specific probes are used as contrast agents. We synthesised a colourless PA probe that is activated in the presence of γ-glutamyltranspeptidase, a cancer-associated enzyme, to show its original colour and fluorescence. We have acquired high specificity fluorescence images of small tumours, using a fluorescent probe based on similar enzymatic reactions. Here, we developed a PA imaging technique to detect the PA probe. In PA imaging, depending on the concentration and excitation wavelength of the probe, the intensities of the probe signals may be lower than those of the background signals produced by intrinsic optical absorbers such as haemoglobin. For probe imaging in the presence of strong background signals, multispectral photoacoustic (MS-PA) imaging was evaluated. In MS-PA imaging, the spectral fitting method, which distinguishes the probe signals from background signals using reference spectra, has been widely used. To compensate for the decrease of fluence due to optical attenuation in biological tissue, we used a simplified compensation method that calculates fluence inside biological tissues by the Monte-Carlo model using published data on optical properties of biological tissues. The validity of the method was confirmed using tissue-mimicking phantoms. Finally, MS-PA imaging of a mouse subcutaneous tumour injected with the activatable probe was demonstrated. In conclusion, our MS-PA imaging technique afforded successful detection of the activated probe in the tumour, and time-increase of PA signals were successfully observed.

Keywords: multispectral photoacoustic imaging, multispectral optoacoustic imaging, spectral unmixing, spectral fitting, spectroscopy, activatable probe

(Some figures may appear in colour only in the online journal)

1. Introduction

Photoacoustic (PA) imaging offers depth-resolved imaging of optical absorbers in biological tissues at a spatial resolution of ultrasound (US) imaging [1–3]. In PA imaging, we detected PA signals, which are ultrasounds produced from optical absorbers that have absorbed excitation lights. Different
intrinsic optical absorbers such as haemoglobin, melanin, and lipids show different absorption spectra in the wavelength range from visible to near-infrared. Therefore, by selecting the appropriate excitation wavelength based on the absorption spectra, PA imaging can be used to generate high-contrast images [4, 5]. Thus, PA imaging can be used in medical applications such as imaging of the tumour vasculature [6, 7], diagnosis of melanoma infiltration depth [8] and detection of lipid-rich arterial plaques [9–11] to differentiate abnormal tissues from normal tissues based on intrinsic optical absorber distributions.

To image abnormal tissues that show little differences from normal tissues in their distribution of intrinsic optical absorbers, PA imaging using probes as contrast agents that are specific to the abnormal tissues has been examined [12–21]. In particular, small organic molecule-based probes that emit both PA signals and fluorescence have been developed for fluorescence imaging applications. Urano et al synthesised a colourless and enzyme-activatable fluorescent probe that is activated in the presence of γ-glutamyltranspeptidase (GGT) [22]. Whereas conventional non-activatable probes emit fluorescence regardless of their location, the activatable probe does not emit fluorescence in the absence of GGT. Because GGT is overexpressed in various cancer cells, the probe enables highly specific imaging of tumours. Urano et al successfully detected small tumours in peritoneal implant model mice using fluorescence imaging [22]. Furthermore, various medical applications such as intraoperative detection of breast lesions [23] and endoscopic detection of colon cancer [24] have been studied. To expand the range of applications of the probe, the goal of this study was to image the enzyme-activatable probe using PA imaging.

Because intrinsic optical absorbers such as haemoglobin produce strong background signals, to image the probe clearly, it is necessary to distinguish probe signals from background signals. For this purpose, multispectral photo-acoustic (MS-PA) imaging has been widely examined [25–30]. Using this method, PA images are acquired at multiple excitation wavelengths, and then PA spectra are calculated as the spectral dependence of PA image intensities at each image pixel. Spectral unmixing is performed to distinguish the probe from the background based on the PA spectra. The spectral fitting method (SFM) is a widely used spectral unmixing method [25, 27, 28]. In this method, to calculate the relative concentrations of the probe and background optical absorbers, reference spectra for the probe and the background optical absorbers are fitted to the PA spectra at each image pixel. However, the PA spectra deviate from the absorption spectra of the optical absorbers because of wavelength-dependent attenuation of the excitation light fluence in biological tissue. This phenomenon, called ‘spectral colouring’, causes errors in SFM [31]. To compensate for the effect of spectral colouring, fluence compensation methods have been proposed [28, 32, 33]. However, the methods have some disadvantages: long computational times due to iterative processing and limited accuracies because of assumptions to simplify the radiation transfer equation.

In this study, the feasibility of MS-PA imaging of tumours using the activatable PA probe was demonstrated. First, to image the activated PA probe in the presence of strong background signals, a MS-PA imaging technique was developed. To distinguish the PA probe from the background signals, the SFM was performed on the PA images acquired at multiple excitation wavelengths. To compensate for the spectral colouring effect due to wavelength-dependent attenuation of excitation light fluence, fluence in the biological tissue was calculated using Monte-Carlo model; then, fluence compensation was performed using the calculated fluence. To evaluate the validity of the MS-PA imaging technique, experiments using a tissue-mimicking phantom were performed. Next, the PA signal generation efficiency of the PA probe that was synthesised to have low fluorescence quantum yields was evaluated. The effects of fluorescence quantum yields on PA signal generation efficiencies were demonstrated by comparing the intensities of PA signals produced from aqueous solutions of an activated fluorescent probe and the activated PA probe. To validate the specificity of the PA probe for cancer cells with GGT overexpression, time-course PA measurements of cancer cells with or without GGT overexpression were performed. Finally, in vivo imaging of a subcutaneous tumour of a mouse injected with the probe was performed. Time-course MS-PA imaging showed that the probe signal intensity increased over time, reflecting probe activation. Additionally, 3D MS-PA imaging was performed to confirm that the probe was detected at the position of the tumour.

2. Materials and methods

2.1. Activatable PA probe

Figure 1 shows the synthesis of gGlu-HMDiMeR, the activatable PA probe. HMDiMeR was synthesised as previously.
described [34]. HMDiMeR (93.1 mg, 0.270 mmol, 1.0 equiv), tert-butyldimethylchlorosilane (124.3 mg, 0.825 mmol, 3.1 equiv), and imidazole (202.2 mg, 2.97 mmol, 11.0 equiv) were added to dry dimethylformamide (DMF; 10 ml). The mixture was stirred overnight at room temperature under an Ar atmosphere. The reaction was quenched with water, and the mixture was extracted using CH₂Cl₂ three times. The organic layer was dried over Na₂SO₄ and evaporated to dryness. The residue was stirred for 3.5 h. The solvent was evaporated, and the residue was dissolved in 5 ml of trifluoroacetic acid, and the solution was stirred for 3.5 h. The solvent was evaporated, and the residue was purified by preparative high-performance liquid chromatography to obtain a red powder (92.8 mg, 0.196 mmol, yield: 72.4%).

The colourless PA probe is activated in the presence of GGT, an enzyme associated with cancer, to show its original fluorescence. In a previous study, an activatable fluorescent probe based on a similar enzymatic reaction, gGlu-HMRG, was successfully used for tumour detection [22]. The PA probe was designed to have low fluorescence quantum yields to enhance PA signal generation efficiency. The fluorescence quantum yields of the activated PA probe and activated fluorescent probe were 0.044 and 0.81, respectively. The absorption spectrum, measured using a spectrophotometer (U-3900, Hitachi High-Tech Science Corp., Tokyo, Japan) and the fluorescence spectrum, measured using a multi-mode plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) of 100 μM activated PA probe are shown in figure 2.

2.2. PA measurement system

A tuneable optical parametric oscillator (OPO; Versascan MBI-FE, Spectra-Physics, CA, USA) pumped by third harmonic generation of a pulsed Nd:YAG laser (Quanta-Ray Pro-190-THDA-FE, Spectra-Physics) were used as an excitation light source. The excitation light pulses had a pulse width and a pulse repetition frequency of 6–8 ns and 10 Hz, respectively. The energy of each light pulse was monitored using a beam sampler (BSF-A; Thorlabs, Newton, NJ, USA) and an energy meter (PE25-C; Ophir Optics, Jerusalem, Israel). The light pulses were coupled to an optical fibre with core diameter of 400 μm (M40L02, Thorlabs). The optical fibre was guided through the centre of an unfocused ring-shaped acoustic sensor with outer and inner diameters of 4.0 and 1.4 mm, respectively. The sensor composed of a 20 μm thick P(VDF-TrFE) film (KF piezo-film; Kureha Corp., Tokyo, Japan) had a −6 dB bandwidth ranging from 3.0–19.5 MHz. A low-noise field effect transistor amplifier (SA220F5; NF Electronic Instruments, Kanagawa, Japan) was used to amplify the PA signals detected by the sensor. Amplified PA signals were recorded by a 10 bit resolution PXI digitizer (M9210A; Agilent Technologies, Santa Clara, CA, USA) operated at a sampling frequency of 2 GHz. The sensor was connected to a three axes stepping motor stage to perform well scanning in a 96-well cell culture plate.

2.3. MS-PA imaging system

The MS-PA imaging system is shown in figure 3. The light pulses generated from the OPO described in section 2.2 were coupled to an optical fibre bundle (FBF-19-220-400-SUS02-3-SUS0; Photonics Science Technology, Hokkaido, Japan) consisting of 19 optical fibres with a core diameter and numerical aperture of 400 μm and 0.22, respectively. The output ends of 19 optical fibres circularly surrounded an acoustic sensor. The optical fibres were tilted by 25° towards the acoustic detection axis of the sensor and directed to the acoustic focus of the sensor at 25 mm away from the sensor detection surface. The sensor composed of 20 μm thick P(VDF-TrFE) film had a −6 dB bandwidth from 3.0–13.1 MHz. The sensor had a concave detection surface with a diameter of 12 mm and curvature radius of 25 mm. PA signals were recorded using the amplifier and the digitizer described in section 2.2. The digitizer was operated at a sampling frequency of 100 MHz.

Raster scanning of the sensor was performed using a pair of stepping motor stages (SGSP20-200; SIGMAKOKI, Tokyo, Japan). By replacing the field effect transistor amplifier with a pulsar receiver (5900PR; Olympus, Tokyo, Japan), the system could measure ultrasound echo signals.

2.4. Fluence compensation

PA signal intensities were proportional to the product of the absorbance of samples and excitation light fluences at the samples. Because the excitation lights attenuate during
propagation in biological tissues with wavelength-dependent optical properties, the excitation light fluence strongly depends on both excitation wavelengths and depth of the biological tissue surface. To calculate the values proportional to the absorbance of the samples, we applied a simplified fluence compensation method [28]. In the method, the fluence of excitation light in biological tissues was calculated as a function of distance from skin surface. To calculate the fluence based on Monte Carlo model, the MCML software developed by Wang was used [35]. For phantom experiments, absorbances of both skin and soft tissue phantoms were measured by a spectrophotometer. Scattering coefficients and anisotropies were calculated based on an equation shown in the literature [36]. For in vivo measurements, because spectroscopic data of optical properties of mice tissues were limited, optical properties of rat skin [37] and rat muscle [38] at 500, 550, and 600 nm were obtained from the literature. The absorbances of skin and soft tissues were interpolated by cubic interpolation. Scattering coefficients were interpolated by power-law fitting. Diameter of incident excitation light on the tissue surface was 10 mm. Lateroabdominal skin thickness of 0.5 mm was measured from ultrasound images. The skin surface was identified from ultrasound images. To justify the image pixel sizes of ultrasound images and PA images, both images were interpolated and the distance between the skin surface and each image pixels were calculated [28]. Fluences at each image pixels were calculated from the relation of fluence and the distance from the skin surface. PA images acquired at multiple excitation wavelengths were divided by the calculated fluences.

2.5. Spectral fitting method

The SFM was performed to distinguish probe signals from background signals by fitting the PA spectra at each image pixel to reference spectra of the probes and backgrounds. PA spectra were obtained as wavelength dependence of the PA image intensities at each image pixel. Because oxy-haemoglobin (HbO2), deoxy-haemoglobin (HHb), and melanosome are dominant optical absorbers in biological tissues in the visible to near-infrared wavelength range, the absorption spectra of those optical absorbers [39, 40] were used as reference background spectra. The reference background spectra are shown in figure 4. The reference background spectra were standardized to have a Euclidean norm of 1 before performing SFM.

The relative concentrations of the probe and background optical absorbers were calculated using the following least square equation:

$$\min \left| \sum_{j=1}^{M} c_j(\mathbf{r}) \varepsilon_j(\lambda) - S(\mathbf{r}, \lambda) \right|^2,$$

where $S(\mathbf{r}, \lambda)$ is the intensity of the PA image at position $\mathbf{r}$, the excitation wavelengths $\lambda$, $\varepsilon_j(\lambda)$ are the reference spectra of the probe and background optical absorbers, and $c_j(\mathbf{r})$ are the relative concentrations of the probe and background optical absorbers [41].

Equation (1) was solved using the lsqnonneg function in MATLAB2014b, which is widely used to solve least square problems while constraining solutions to non-negative values. Use of this function prevented the concentrations of the probes and background absorbers from showing negative values, which have no physical meaning [28].
To evaluate the performance of our MS-PA imaging system, we performed an experiment using a tissue-mimicking phantom. Optical properties of the skin differ from that of other soft tissues [42]; thus, to validate the performance of the system (imaging of the PA probe accumulated in tumours, including subcutaneous tumours), a phantom consisting of a skin layer, a soft tissue layer and a probe layer was used. The skin layer and soft tissue layer were made of 20 wt% gelatine (G2500; Sigma-Aldrich Corp., St. Louis, MO) in Tris-buffered saline. India ink (S0216630; Rotring, Hamburg, Germany) and a haemoglobin solution were added as optical absorbers. Intralipid was added as an optical scatterer. The concentrations of the optical absorbers and the optical scatterer are shown in Table 1. The concentration of the intralipid was calculated based on an equation shown in the literature [36]. Because scattering coefficients of intralipid solutions decrease with increasing concentrations of gelatine [43], the concentration of the intralipid in the phantom was adjusted to have the same transmittance as that of an aqueous solution of intralipid. The thicknesses of skin and soft tissue layers are also shown in Table 1. The probe layer was made of a 1.0 mm thick clear silicone rubber sheet (SKSC-6000-01, SK Co., Ltd, Tokyo, Japan). The sheet had holes with diameters of 3 mm and the target samples (dissolved in 10% gelatine) filled the holes. The activated PA probes (10, 32, 100 μM) and the haemoglobin solution (527 μM, which is equivalent to 10% haematocrit blood) [44] were used as the samples. The haemoglobin solution was prepared from 10 ml of blood obtained from a Japanese white rabbit (National Defense Medical College Committee for Animal Use approval number 13091). Then, 2000 units of heparin were added to the blood sample. To separate red blood cells (RBCs) from other constituents, the blood was centrifuged. The plasma and buffy coat in the blood were removed with a laboratory pipette and then PBS was added to obtain 10 ml of a RBC suspension. The process was repeated three times to purify the RBCs suspension. The RBCs suspension was centrifuged again and then 2 ml of centrifuged RBCs were added to 8 ml of distilled water to haemolyse RBCs and to dilute the suspension to 20% in haematocrit. To obtain 1.05 mM of the haemoglobin solution, the haemolysed RBC suspension was centrifuged, and then the precipitated membranes were removed. All centrifuge processes were conducted in a EX-126 centrifuge (TOMY-SEIKO, Tokyo, Japan) operated in a centrifugal force of 2000 G, duration time of 15 min, and sample temperature of 4°C.

The experiment was performed using the MS-PA imaging system described in section 2.3. A container with the bottom partly replaced with an 11 μm thick clear film filled with water at 15°C was placed on the phantom. The clear film on the bottom of the container contacted the phantom through distilled water. The detection surface of the acoustic sensor combined with the optical fibres was immersed in the water. The wavelengths of the excitation light pulses that irradiated from the optical fibres were changed from 500 to 600 nm in 5 nm steps. The pulse energies of the excitation light pulses at the output of the optical fibres were varied within a range of 0.2 to 9.0 mJ/pulses to acquire PA images with high signal-to-noise ratio (SNR) while preventing saturation of PA images at the depth of the probe layer. Three-dimensional MS-PA imaging was performed by scanning the sensor in both the x and y directions with a scan area of 20 × 6 mm and scan step of 0.4 mm.

2.7. Comparison of fluorescent probe and photoacoustic probe

The PA measurement system described in section 2.2 was used to measure PA signals produced from aqueous solutions of the activated fluorescent probe and the activated PA probe with various absorbance values. The absorbances of the aqueous solutions measured using a spectrophotometer (U-3900; Hitachi High-Tech Science Corp., Tokyo, Japan) were 0.3–4.2 cm⁻¹. The sensor was immersed in a small water tank filled with degassed water. The distance between the detection surface of the sensor and bottom of the water tank was 10 mm. The bottom of the water tank was replaced with an 80 μm thick cover glass (Micro Cover Glass No. 00; Matsunami Glass Inc., Tokyo, Japan). The aqueous probe solutions (100 μl) were dropped onto the glass slide. The cover glass on the bottom of the water tank contacted the aqueous solution and was fixed 1 mm away from the glass slide surface. To measure PA signals, excitation lights with pulse energies of 100 μJ/pulses were used to irradiate the aqueous solutions. PA signals were averaged over 20 light pulses. The excitation wavelengths were set to the absorption maximum wavelengths for the fluorescent probe and PA probe. The absorption maximum wavelengths of the fluorescent probe and PA probe were 500 and 530 nm, respectively.

The maximum values of the PA signals were measured after removing the offset. The offset was originated from pyroelectric signals generated by back-reflected excitation.

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**Table 1.** Thickness, constitution, absorbance and reduced scattering coefficients \(\mu'_s\) of phantom layers.

| Layer     | Thickness (mm) | Haemoglobin (μM) | India ink (vol%) | Intralipid 20% (vol%) | Gelatine (wt%) | Absorbance @ 530 nm (cm⁻¹) | \(\mu'_s\) @ 530 nm (cm⁻¹) |
|-----------|----------------|------------------|------------------|-----------------------|---------------|-----------------------------|-----------------------------|
| Skin      | 0.5            | 1.35             | 0.0023           | 28.1                  | 10            | 0.204                       | 34.2                        |
| Soft tissue | 0.0–5.5     | 15.0             | 0                | 6.37                  | 10            | 0.660                       | 7.76                        |
| Probe     | 1.0            | 0                | 0                | 0                     | 0             | 0                           | 0                           |
| Soft tissue | 6.0         | 15.0             | 0                | 6.37                  | 10            | 0.660                       | 7.76                        |
lights absorbed by the acoustic sensor. Signal intensity per energy was calculated by dividing the maximum values of PA signals by the pulse energy of the excitation light. PA signal generation efficiencies were calculated from the linear fit of the dependence of the signal intensity per energy on absorbance.

2.8. PA measurement of the probe in cancer cells

We used human lung cancer cell lines in this study, including A549 (DS Pharma Biomedical Co. Ltd, Osaka, Japan) and H226 (American Type Culture Collection, Manassas, VA, USA). GGT is highly expressed in A549 cells, whereas its expression is low in H226 cells \[45\]. A549 cells were grown in Dulbecco’s modified Eagle’s medium containing D-glucose, L-glutamine, and sodium pyruvate (DMEM, 11885–084; Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (SH3091003; Life Technologies, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (15240-062; Life Technologies, Carlsbad, CA, USA) in 5% CO\(_2\). H226 cells were grown in RPMI Medium 1640 containing D-glucose and L-glutamine (11875–093; Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (SH3091003; Life Technologies, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (15240-062; Life Technologies, Carlsbad, CA, USA) in 5% CO\(_2\).

Next, 2 × 10\(^4\) cells from each cell line were seeded into 4 wells of 96-well dishes. The cells were incubated for 42 h and then washed with phenol red-free medium: DMEM containing D-glucose (11054–020; Life Technologies, Carlsbad, CA, USA) and 2 mM of L-glutamine (KBDSM202, DS Pharma Biomedical Ltd, Osaka, Japan) was used for A549 cells, and RPMI Medium 1640 containing D-glucose and L-glutamine was used for H226 cells. After removing the medium from each well, 400 \(\mu\)l of the activatable probe (1 \(\mu\)M, containing 0.1% v/v DMSO as a co-solvent) in phenol red-free medium was added.

The PA measurement system described in section 2.2 was used to measure PA signals produced in the cells. The signals were measured by contact of the detection surface of the sensor with the medium. The signals were measured each minute for 60 min, beginning at 1.5 min after probe application. The distance between the detection surface of the sensor and the bottom of the well was 10 mm. Wavelength and pulse energy of the excitation light pulses were 530 nm and 100 \(\mu\)l/pulses, respectively. PA signals were averaged over 20 light pulses. During the experiment, the temperature of the cells and medium were maintained at 37°C using a plate heater (MATS-55AXK-DG-AN; Tokai Hit, Shizuoka, Japan). The maximum values of the PA signals were measured after removing the offset due to the pyroelectric signal described in sections 2.4. The maximum values of PA signals were divided by the pulse energy of the excitation light.

The fluorescence images were acquired by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan) using a filter cube (49003, Ex 490–510 nm, DM 515 nm, Em 520–550 nm, M SQUARE Co., Ltd, Tokyo, Japan). Transmitted light phase contrast images were also acquired. The images were acquired before probe application and after PA measurement.

2.9. MS-PA imaging of subcutaneous tumours in mice

All animal procedures were performed according to a protocol approved by the National Defense Medical College Committee for Animal Use (approval number 14109). Male nude mice (BALB/c-nu/nu) 6 weeks of age were purchased from Nihon SLC Co. Ltd (Shizuoka, Japan). Subcutaneous tumours were initiated in the mice by subcutaneously injecting 5 × 10\(^6\) A549 cells in 100 \(\mu\)l of DMEM containing D-glucose, L-glutamine, and sodium pyruvate supplemented with 10% foetal bovine serum and 1% antibiotic-antimycotic. After tumours had grown to 3–6 mm in diameter, the following experiments were performed.

The experiment was performed using the MS-PA imaging system described in section 2.3. A tumour-bearing mouse was initially anesthetized with 4% isoflurane (Forane, Abbott, IL, USA), and the experiments were performed with 1.5% isoflurane using an anesthesia apparatus (Shinano Manufacturing, Tokyo, Japan). The mouse was placed on a heater (MATS-55AXK-DG-AN; Tokai Hit, Shizuoka, Japan) with a surface temperature of 38°C. Then, a container with the bottom partly replaced with an 11 \(\mu\)m thick clear film filled with water at 36–38°C was placed on the mouse. The clear film on the bottom of the container contacted the mouse through a low-viscosity clear ultrasound coupling gel (Towa gel; Towatech, Tokyo, Japan). The detection surface of the acoustic sensor combined with the optical fibres was immersed in the water. The wavelengths of the excitation light pulses irradiated from the optical fibres were changed from 500 to 600 nm in 5 nm steps. The pulse energies of the excitation light pulses at the output of the optical fibres ranged from 0.3 to 0.5 mJ/pulses because the energies of the light pulses generated from the OPO depended on the wavelength.

Three-dimensional MS-PA images and fluorescence images were obtained before probe injection to measure background signals for each imaging method. Next, the subcutaneous tumour of the mouse was injected intratumorally with 20 nmol of the activatable PA probe in 40 \(\mu\)l of PBS containing 5.0% DMSO as a co-solvent. Immediately after probe injection, a fluorescence image was acquired and then time-course MS-PA imaging was performed. In time-course MS-PA imaging, cross-sectional MS-PA images were acquired every 5 min for 60 min to determine increases in probe signal intensity over time in the tumour. After the experiments, the mouse was sacrificed and 3D MS-PA imaging and fluorescence imaging were performed.

Three-dimensional MS-PA imaging was performed by scanning the sensor in both the \(x\) and \(y\) directions with a scan area of 20 × 10 mm and a scan step of 0.4 mm. The signal acquisition time was approximately 60 min. In time-course MS-PA imaging, cross-sectional MS-PA imaging was performed by scanning the sensor in the \(x\) direction with a scan width of 20 mm and scan step of 0.4 mm. The signal acquisition time was approximately 3 min. PA signals were wavelet-filtered and then band-pass filtered (zero-phase filter,
should not be significant. Changes in PA signal waveforms among wavelength steps were validated if all of the following conditions are met: (a) PA signals acquired at previous wavelength steps. The method was valid if all of the following conditions are met; (b) changes in PA signal waveforms among wavelength steps should not be significant; (c) a PA signal with enough SNR should be commonly detected in all wavelengths; (d) the motion should happen along the acoustic detection axis. The method was valid in our experiment because the wavelength step of 5 nm was sufficient; (e) strong PA signals from mouse skin were detected at all excitation wavelengths, and the mouse was tightly held by a holder, except for the top surface which contacts the bottom of the water tank via an acoustic coupling gel. Ultrasound echo images were also acquired by transmitting an electrical pulse with a pulse energy of 4 μJ/pulses to the sensor using a pulser receiver.

Fluorescence images were acquired using a digital camera (D90, Nikon, Tokyo, Japan), Xenon lump (MAX302; Asahi Spectra, Tokyo, Japan), laboratory-made dark box, excitation band-pass filter with a transmission wavelength range of 490-500 nm (YFPHQ; Nikon, Tokyo, Japan), and emission long-pass filter with a cut-off wavelength of 550 nm (LV0550; Asahi Spectra, Tokyo, Japan). The digital camera was operated at an International Organization for Standardization (ISO) sensitivity of 1600, shutter speed of 100 ms, focal length of 105 mm, and F-number of 16. White light photographs were also taken using the digital camera at a shutter speed of 0.5 ms. The 8 bit green channel images were extracted from the 24 bit colour fluorescence images and then displayed in a jet colormap in MATLAB2014b (Mathworks, Natick, MA, USA). The fluorescence images were thresholded and then merged onto the white light images.

Figure 5. C-scan images of 3D MS-PA images of haemoglobin (a) and (b) and 100 μM of probe samples (c) and (d) placed below the skin phantom and 2 mm thick soft tissue phantom (a) and (c) PA images acquired at 530 nm. (b) and (d) Probe images calculated by performing simplified fluence compensation and SFM.

3. Results and discussion

3.1. Experimental validation of the MS-PA imaging system using a tissue-mimicking phantom

To evaluate the performance of the MS-PA imaging system in PA probe detection, experiments using tissue-mimicking phantoms were performed. Figure 5 shows MS-PA images of haemoglobin and 100 μM PA probe samples placed below the 0.5 mm thick skin phantom and 2 mm thick soft tissue phantom. The C-scan images shown in figure 5 were maximum amplitude projection of the 3D MS-PA images at the depth of the sample detection with a slice thickness of 1.0 mm. To evaluate variance in PA signal intensities, three samples were placed in 6 mm spacing. In figure 5(a), because the absorbance of the haemoglobin sample was larger than that of the PA probe, a strong signal produced from haemoglobin samples was detected. Probe images calculated by performing simplified fluence compensation and SFM are shown in figures 5(b) and (d). In the probe images, the signals produced from the probe samples (figure 5(d)) were emphasized, whereas the signals produced from the haemoglobin samples (figure 5(b)), by contrast, were suppressed. From the result, the SFM with simplified fluence compensation successfully distinguished the probe signals from background signals produced by haemoglobin.

To validate the effectiveness of the simplified fluence compensation in cancellation of the spectral colouring effect, the PA spectra before and after fluence compensation were compared. PA spectra were calculated by subtracting PA signals produced from the bottom of the soft tissue phantom from the PA signals observed at the position of the probe phantom. Figure 6 shows PA spectra of a 100 μM PA probe sample and haemoglobin sample placed below the 0.5 mm thick skin phantom and 5.5 mm thick soft tissue phantom before and after fluence compensation. Before performing
fluence compensation, the PA spectra of the probe sample and haemoglobin sample deviated from their absorption spectra due to the spectral colouring effect caused by wavelength-dependence optical attenuation in the skin phantom and soft tissue phantom. After performing fluence compensation, the peak of the PA spectra appeared at the peak wavelength of its absorption spectrum. In figure 7, determination coefficients of the absorption spectrum of the PA probe and PA spectra before and after fluence compensation were compared. As a result, an increase in skin-tissue phantom thickness led to a gradual decrease of the determination coefficients of absorption spectrum of the PA probe and PA spectra of the PA probe. This was because an increase of soft tissue phantom thickness increased the deviation of the PA spectra from the absorption spectra due to the spectral colouring effect. After performing fluence compensation, the decrease in the determination coefficient was prevented. Thus, the simplified fluence compensation technique successfully cancelled the spectral colouring effects.

To validate the effectiveness of the SFM in discrimination of the PA probe signals from background signals produced from haemoglobin, relative PA signal intensities of the PA probe samples and the haemoglobin samples before and after SFM were compared in figure 8. The PA signals produced from haemoglobin samples were $2.6 \pm 0.2$ times stronger than that produced from the 100 $\mu$M PA probe at an

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Figure 6. Spectral characteristics of PA signals produced from the PA probe sample and haemoglobin sample placed below the 0.5 mm thick skin phantom and 5.5 mm thick haemoglobin phantom before and after fluence compensation.

Figure 7. Determination coefficients of absorption spectrum of the PA probe and PA spectra measured through 0.5 mm thick skin phantom and various thicknesses of soft tissue phantoms (a) before and (b) after fluence compensation.
excitation wavelength of 530 nm. After performing SFM, the background signals produced from the haemoglobin samples were suppressed. The PA signals produced from the haemoglobin samples were 0.058 ± 0.072 times stronger than the intensities produced from the 100 μM PA probe. This result clearly demonstrated the effectiveness of SFM in discrimination of the PA probe signals from background signals produced from haemoglobin.

In figure 8, 100 μM of the probe was imaged through the 0.5 mm thick skin phantom and the 5.5 mm thick soft tissue phantom. Similarly, 32 μM of the probe was imaged through the 0.5 mm thick skin phantom and the 4.0 mm thick soft tissue phantom. The maximum imaging depths at each concentration were limited by a decrease in the intensity of the PA signals due to strong optical attenuation.

In the case of the 10 μM probe, the probe below the 0.5 mm thick skin phantom was not imaged. This result was not consistent to the results of other probe concentrations. Because the absorbance of the probe was lower than that of the skin phantom, the signal produced from the probe was concealed by the signal produced from skin phantom. Thus, absorbance of background tissue also limits the minimum detectable concentration of the PA probe.

Another factor that limits the minimum detectable concentration of the PA probe was the dynamic range of the measurement system, which was limited by SNR. The noise of our measurement system, which was mainly caused by electromagnetic interference with a pulsed laser and stepping motor stages, had intensities of 10 mV. The saturation level of the digital oscilloscope was 1000 mV. Thus, the dynamic range of the system was approximately 40 dB. The haemoglobin sample with a concentration equivalent to 10% haematocrit produced 3.74 ± 0.85 times a stronger signal compared to the 100 μM PA probe at their peak wavelengths. Because whole blood generally contains 40% haematocrit, whole blood produces four times a stronger PA signal compared to that produced from the haemoglobin sample. Thus, whole blood produces 15.0 ± 3.41 times stronger signals than the 100 μM PA probe. To prevent saturation of the measurement system, the maximum intensity of the PA signal produced from whole blood should be limited to 1000 mV by adjusting the excitation light intensity. In this case, the PA signal produced from the 15 μM PA probe becomes noise; thus, this concentration was used as the lower detection limit of the PA probe (in the presence of whole blood as background optical absorber).

To improve sensitivity, we are currently developing an activatable probe with a longer excitation wavelength because the lower detection limit of PA probes was limited by several factors associated with the large absorbance of haemoglobin. While the absorbance of haemoglobin in the blood was more than 100 cm⁻¹ in the wavelength range of 500–600 nm, the absorbance was less than 20 cm⁻¹ in the wavelength range of 610–1000 nm [39]. The decrease in absorbance directly decreases the intensity of background signals, enabling probe detection at lower concentrations. In addition, a decrease in absorbance extends the penetration depth of the excitation light, enabling probe detection deep inside biological tissues. As result of simulation using the Monte-Carlo model (using optical properties cited from literature) [38], the fluence at 9.4 mm from the soft tissue surface at 650 nm was equivalent to the fluence at 5.5 mm from the soft tissue surface at 530 nm. Thus, by extending the excitation wavelength to 650 nm, the 100 μM PA probe at 9.4 mm from the soft tissue surface can now be imaged.

3.2. Comparison of fluorescent probe and PA probe

PA signal intensities measured in aqueous solutions of the PA probe and fluorescent probe with various absorbance values are compared in figure 9(a). The intensities of PA signals measured from both probes linearly increased with increasing absorbance. PA signal generation efficiencies, calculated as the slopes of the curves shown in figure 9(a), were compared in figure 9(b). The aqueous solutions of the PA probe produced PA signals 7.0 times more efficient than the aqueous solution of the fluorescent probe. Differences in PA signal generation efficiencies were related to fluorescence quantum yields of the probes. Because the fluorescent probe showed a large fluorescence quantum yield of 0.81, 81% of the absorbed optical energies were converted to fluorescence and only 19% of the energies remained. In the PA probe with a
fluorescence quantum yield of 0.044, 95.6% of the energies remained. Thus, in the PA probe, the fraction of the remaining optical energies was 5.0 times larger than that of the fluorescent probes, a result consistent with PA signal generation efficiency. The differences in PA signal generation efficiencies and the fraction of remained energies may be related to the presence of optical energies converted to neither fluorescence nor PA signals. In addition, the effect of ground state depopulation, which decreases PA generation efficiencies of optical absorber with high fluorescence quantum yields, may also be a cause for the differences [46]. The PA probe designed to have a low fluorescence quantum yield produced PA signals efficiently, and thus the probe was effective for PA imaging.

3.3. PA measurement of the probe in cancer cells

The time course of PA signal intensities measured in A549 and H226 cells determined using the activatable PA probe are shown in figure 10(a). Whereas the intensities of PA signals produced from A549 cells increased over time, the intensities remained constant in H226 cells. The time-dependent increase in PA signal intensities was caused by activation of the probe. Because the probe is activated by GGT, the probe was activated only in A549 cells, where GGT is overexpressed. Fluorescence images of the cells acquired after the PA measurement are shown in figure 10(b). Although the fluorescence quantum yield of the probe was low, it was possible to detect fluorescence from the probes. Fluorescence from the probe was detected in A549 cells, consistent with the results of PA measurement. We demonstrated that the activatable probe could be used for imaging in cancer cells where GGT is overexpressed to produce both PA signals and fluorescence.

3.4. In vivo MS-PA imaging of subcutaneous tumours using the PA probe

An initial result of in vivo MS-PA imaging of subcutaneous tumours using the activatable PA probe are presented in this section. Fluorescence images acquired from a mouse before probe injection, immediately after intratumour injection of the probe, and after performing MS-PA imaging are shown in figures 11(a), (b) and (c), respectively. In figure 11(b), although the probe was located in the tumour, fluorescence
was not detected because the probe had not been activated. However, after performing MS-PA imaging, a strong fluorescence signal was detected at the position of the tumour, as shown in figure 11(c). Thus, the probe was activated in the tumour during MS-PA imaging.

Three-dimensional MS-PA imaging was performed before probe injection and 60 min after probe injection. XY maximum amplitude projections of MS-PA images acquired at two representative wavelengths are shown in figure 12. The signal acquisition time required to acquire each image was approximately 60 min. Because haemoglobin showed large absorbance at both 530 and 570 nm, blood vessels are clearly shown in the images. Although 530 nm was the absorption maximum wavelength of the probe, no noticeable signal was observed in the tumour. It was difficult to find the probes from PA images acquired at a single wavelength because of the strong background signals produced by haemoglobin.

To suppress the background signals produced by haemoglobin, fluence compensation and SFM were performed. In the probe images acquired after probe injection...
(figure 13(b)), the strong background signals (figures 12(c) and (d)) were removed to then detect the probe at the tumour position. Thus, the probe could be imaged by suppressing the background signal produced by haemoglobin.

The time course of signal intensities at the tumour and background are compared in figure 14. Based on the result, an increase in probe signals at the tumour position was clearly observed. Thus, probe activation was confirmed in vivo by MS-PA imaging. Because the temporal change in probe signal intensities differed from that of background signals, it may be possible to distinguish the probe signals using time course information without using spectral information [25]. In addition, because probe activation is closely related to GGT expression levels in the tumour, the rate of the time-increase may be useful for understanding tumour characteristics [47]. To monitor the dynamics of the activation of the probe in higher temporal resolution, we are currently developing an array of acoustic sensors based on the MS-PA imaging system that can image a single wavelength cross-sectional PA image at 10 fps. Another group have already demonstrated the time-course measurement of PA images using real time PA imaging instruments in hemodynamics monitoring [48] and contrast agent perfusion monitoring [49].

In the in vivo experiment, the simplified fluence compensation was performed using the optical properties of the skin and soft tissues of mice established in the literature. By calculating the fluence inside biological tissues as a function of distance from skin surface before performing experiment, the fluence compensation can be performed during the experiment in real time.

The calculation model with cylindrically symmetric structure having optically homogeneous layers was accurate in the phantom experiment. However, the model did not match perfectly with the in vivo experiment because of heterogeneity of the mouse tissues. Although solution of three-dimensional radiative transfer equation in accurate heterogeneous tissue model may offer accurate fluence inside heterogeneous biological tissue [31], the method is impractical because of large computational cost for modelling the accurate heterogeneous tissue and solving the equation. To reduce computational cost, diffusion approximation has been discussed [31], however the approximation was invalid in the in vivo experiment because the tumour injected by the activatable PA probe located at depth corresponds to the ballistic regime rather than the diffusion regime. In that circumstance, use of Monte Carlo method with simple geometry was relatively useful and efficient for fluence compensation. The optical properties of Monte Carlo model that consist of optically uniform two layers might not precisely agree with the optical properties of actual mouse tissues because of heterogeneity of biological tissues. In addition, the optical properties of mouse tissues may involve individual differences and may be varied by physiological conditions. To compensate the fluence more accurately, accurate optical properties should be prepared based on actual measurement [50] or estimation by iterative algorithm [32]. By use of those measurement and estimation of the optical properties, the fluence compensation will be improved. However, the simplified fluence compensation functioned well and the probe was successfully distinguished from the background signals by our MS-PA imaging technique. In the in vivo experiment, the probe located just below the skin with a thickness of 0.5 mm, which corresponds to the ballistic regime. Monte Carlo method can simulate the ballistic photon migration. However, because the spectral colouring effect was small, the result of MS-PA imaging was possibly less sensitive to the error of the fluence compensation. In the imaging of the probe that locates deep inside tissue, the result of MS-PA imaging becomes sensitive to the error of fluence compensation. Thus, improvement of the simplified fluence compensation may be needed.

Although systematic injection such as venous injection was more reliable for various medical applications, we directly injected the activatable PA probe into the tumour. Currently, due to limited sensitivity of the MS-PA imaging, it was difficult to accumulate the activatable probe in concentration sufficiently imaged by MS-PA imaging. To image the tumours with the aid of the activatable probe injected by systematic method, decrease of the minimum detectable concentration by extending the excitation wavelength of the PA probe or development of an efficient method to deliver the PA probe to a tumour while maintaining the activation efficiency were required.

In this study, the feasibility of tumour imaging using MS-PA with an activatable PA probe was demonstrated. MS-PA imaging enables depth-resolved imaging of subcutaneous tumours selectively stained with the activatable PA probe. The use of activatable probes is particularly useful for detecting small tumour and micrometastasis, making it possible to obtain depth-resolved images that are difficult to obtain by fluorescence imaging.

4. Conclusion

In this study, the feasibility of a MS-PA imaging technique of tumours using an activatable PA probe was demonstrated. To
image the PA probe in the presence of a strong background signal produced from haemoglobin, a MS-PA imaging technique using SFM and simplified fluence compensation was developed. Experiments using tissue-mimicking phantoms revealed that the MS-PA imaging technique made it possible to image the PA probe with a concentration of 100 μM through a 0.5 mm thick skin phantom and a 5.5 mm thick soft tissue phantom in the presence of haemoglobin as background. The activatable PA probe designed to have a low fluorescence quantum yield produced PA signals more efficiently than fluorescent probes with high fluorescence quantum yields. Tumour selectivity of the probe was confirmed through in vitro experiments. Probe activation in A549 cells that overexpress GGT was successfully detected using both PA measurement and fluorescence imaging. Finally, an initial result of in vivo imaging to demonstrate that MS-PA imaging can be used to image probes injected into a tumour was presented. Although the background signals were much stronger than probe signals, by performing fluence compensation and SFM, the background signals were suppressed and clear probe images were obtained.

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