Multispectral imaging of absorption and scattering properties of in vivo exposed rat brain using a digital red-green-blue camera

Keiichiro Yoshida
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1 Introduction

Evaluating the optical properties of brain tissue is important for the application of light in clinical diagnosis, surgery, and therapeutic procedures for brain diseases. The optical properties of biological tissue have been used to evaluate spatial and/or temporal changes of neuronal activity and tissue viability in the brain as intrinsic optical signals (IOSs). IOSs in the brain are believed to be caused primarily by the following processes: hemodynamic-related changes in absorption and scattering properties, changes in absorption due to redox states of cytochromes in mitochondria, changes in scattering generated by cell swelling or shrinkage caused by water movement between intracellular and extracellular compartments, and changes in scattering and absorption caused by chromophore contents and cell deformations. Light in the visible to near-infrared spectral range is sensitive to the absorption and scattering properties of biological tissue. The absorption and scattering properties of in vitro tissue slices can be estimated from the measured diffuse reflectance and transmittance of tissue slice based on several light transport models, such as the Kubelka-Munk theory, the diffusion approximation to the transport equation, and the Monte Carlo method and the adding-doubling method. Numerous spectroscopic methods have been investigated for in vivo determination of the scattering and absorption properties in living tissues, including time-resolved measurements, a frequency-domain method, and spatially resolved measurements with continuous wave (CW) light. Diffuse reflectance spectroscopy (DRS) based on the measurement of CW light can be simply achieved with an incandescent white light source, inexpensive optical components, and a spectrometer. DRS is one of the most promising methods for evaluating the absorption and scattering properties of in vivo brain tissue. Several approaches using a Monte Carlo simulation-based lookup table have been investigated for determining the absorption and scattering properties of biological tissue. Several imaging methods based on DRS have been used to investigate cortical hemodynamics based on changes in the absorption properties of brain tissue. In order to achieve rapid multispectral imaging, the use of an acousto-optical tunable filter and the combination of a lenslet array with narrow-band filters have been proposed. On the other hand, the reconstruction of multispectral images from a red green blue (RGB) image acquired by a digital RGB camera is promising as a method of rapid and cost-effective multispectral imaging. Several reconstruction techniques for multispectral images, such...
as the pseudo-inverse method, finite-dimensional modeling, the nonlinear estimation method, and the Wiener estimation method (WEM) have been investigated. Among these reconstruction techniques, the WEM is one of the most promising methods for practical applications because of its simplicity, cost-effectiveness, accuracy, time efficiency, and the possibility of high-resolution image acquisition.

In the present study, we investigate a simple spectral imaging of reduced scattering coefficients \( \mu_s(\lambda) \) and the absorption coefficients \( \mu_a(\lambda) \) of \textit{in vivo} exposed brain tissues in the range of wavelengths from visible to near-infrared based on DRS using a multispectral imaging system. Multispectral diffuse reflectance images of \textit{in vivo} exposed brain are estimated from an RGB image captured by a digital RGB camera. The Monte Carlo simulation-based multiple regression analysis for the estimated absorbance spectra at nine wavelengths (500, 520, 540, 560, 570, 580, 600, 730, and 760 nm) is then used to specify the concentrations of oxygenated and deoxygenated hemoglobin as the absorption parameters, and the coefficient \( a \) and the exponent \( b \) of the reduced scattering coefficient spectrum approximated by a power law function as the scattering parameters. The absorption coefficient spectra and the reduced scattering coefficient spectra are finally reconstructed from the hemoglobin concentrations and the scattering parameters, respectively. In order to confirm the feasibility of a method by which to evaluate the absorption and scattering properties of the cerebral cortex, we performed \textit{in vivo} experiments using exposed rat brain while changing the fraction of inspired oxygen (FiO2).

2 Principle

2.1 Estimation of Spectral Diffuse Reflectance Images by Wiener Estimation

The response of a digital color camera in spatial coordinates \((x, y)\) with the \(i\)th \((i = 1, 2, 3)\) color channel, or red, green, and blue can be calculated as

\[
e_i(x, y) = \int u_i(\lambda)E(\lambda)S(\lambda)r(x, y; \lambda)d\lambda,
\]

where \(\lambda\) is the wavelength, \(u_i(\lambda)\) is the transmittance spectrum of the \(i\)th filter, \(E(\lambda)\) is the spectrum of the illuminant, \(S(\lambda)\) is the sensitivity of the camera, and \(r(x, y; \lambda)\) is the reflectance spectrum in the spatial coordinates \((x, y)\). For convenience, Eq. (1) is expressed in discrete vector notation as

\[
v = Fr,
\]

where \(v\) is a vector having a three-element column and \(r\) is a vector having a \(k\)-element column, which corresponds to the reflectance spectrum of a pixel of an image. Moreover, \(F\) is a \(3 \times k\) matrix and is expressed as

\[
F = UES,
\]

where \(U = [u_1, u_2, u_3]^T\). Column vector \(u_i\) denotes the transmittance spectrum of the \(i\)th filter, and \([\cdot]^T\) represents the transpose of a vector. Also, \(E\) and \(S\) are \(k \times k\) diagonal matrices representing the spectrum of the illuminant and the sensitivity of the camera, respectively. The Wiener estimation of \(r\) is given by

\[
\tilde{r} = Wv,
\]

where \(W\) is the Wiener estimation matrix. The purpose of \(W\) is to minimize the minimum square error between the original and estimated reflectance spectra. In this case, the minimum square error is expressed as

\[
e = \langle (r - \tilde{r})^T(r - \tilde{r}) \rangle.
\]

From Eqs. (1) and (3), the minimum square error is rewritten as

\[
e = \langle (r - \tilde{r})^T(r - \tilde{r}) \rangle = \langle r^T(r - \tilde{r})^T \rangle = \langle r^Tv + v^TRv \rangle = 0.
\]

From Eq. (1), the matrix \(W\) is derived as

\[
W = \langle rv^T \rangle(vv^T)^{-1} = \langle rv^T \rangle(F(rt)^TF)^{-1},
\]

where \(\langle \cdot \rangle\) is an ensemble-averaging operator. The derivation of matrix \(W\) requires the autocorrelation matrix \(r^Tr\). In the present study, we determine \(r^Tr\) based on 720 different reflectance spectra obtained from \textit{in vivo} rat brain under various physiological conditions. To test the accuracy in spectral reconstruction, the estimated spectrum at 27 different wavelengths by WEM was compared with the measured spectrum by spectrometer using a goodness-of-fit coefficient (GFC). The GFC is based on the inequality of Schwartz and it is described as

\[
GFC = \frac{\sum_j r_{\text{est}}(\lambda_j)r_{\text{mes}}(\lambda_j)}{\sqrt{\sum_j [r_{\text{mes}}(\lambda_j)]^2} \sqrt{\sum_j [r_{\text{est}}(\lambda_j)]^2}},
\]

where \(r_{\text{meas}}(\lambda_j)\) is the measured original spectral data at the wavelength \(\lambda_j\) and \(r_{\text{est}}(\lambda_j)\) is the estimated spectral data at the wavelength \(\lambda_j\). Hernández-Andrés et al. suggested that colorimetrically accurate \(r_{\text{meas}}(\lambda_j)\) requires a GFC > 0.995; a “good” spectral fit requires a GFC ≥ 0.999, and GFC ≥ 0.9999 is necessary for an “excellent” spectral fit.

2.2 Estimation of the Absorption Coefficient and the Reduced Scattering Coefficient

Figure 1 shows a flow diagram of the method used to estimate the absorption coefficient \(\mu_a(\lambda)\) and the reduced scattering coefficient \(\mu_s(\lambda)\). The absorbance spectrum \(A(\lambda)\) is defined as

\[
A(\lambda) = -\log_{10} r(\lambda),
\]

where \(r(\lambda)\) is the diffuse reflectance spectrum normalized by the incident light spectrum. Since attenuation due to light scattering can be treated as a pseudochromophore, the absorbance spectrum \(A(\lambda)\) can be approximated as the sum of attenuations due to absorption and scattering in the brain as

\[
A(\lambda) = A(\text{absorption}) + A(\text{scattering}).
\]
\[ A(\lambda) = C_{\text{HBO}} l(\lambda, C_{\text{HBO}}, C_{\text{HR}}, \mu'), \varepsilon_{\text{HBO}}(\lambda) + C_{\text{HR}} l(\lambda, C_{\text{HBO}}, C_{\text{HR}}, \mu'), \varepsilon_{\text{HR}}(\lambda) + D(\lambda, \mu'). \]  

where \( C \) is the concentration, \( l \) is the mean path length, \( \varepsilon(\lambda) \) is the extinction coefficient, and \( D(\lambda, \mu') \) indicates attenuation due to light scattering in the tissue. The subscripts HBO and HBR denote oxygenated hemoglobin and deoxygenated hemoglobin, respectively. The absorption coefficient of the cortical tissue was assumed to depend only on the concentrations of HBO and HBR as

\[ \mu_{\text{a}}(\lambda) = C_{\text{HBO}} \varepsilon_{\text{HBO}}(\lambda) + C_{\text{HR}} \varepsilon_{\text{HR}}(\lambda). \]  

The total hemoglobin concentration \( C_{\text{HBT}} \) is defined as the sum of \( C_{\text{HBO}} \) and \( C_{\text{HR}} \) as follows:

\[ C_{\text{HBT}} = C_{\text{HBO}} + C_{\text{HR}}. \]  

The tissue oxygen saturation is determined as

\[ \text{StO}_2[\%] = 100 \times \frac{C_{\text{HBO}}}{C_{\text{HBT}}}. \]  

The reduced scattering coefficient of the brain tissue was assumed to take the following form as a power law function:

\[ \mu'_{\text{s}}(\lambda) = a\lambda^{-b}, \]  

where the coefficient \( a \) and the exponent \( b \) represent the scattering amplitude and the scattering power, respectively.
represents the bias component of $A(\lambda)$. Thus, $a_0$ describes the degree of contribution of the attenuation due to light scattering in the brain to the absorbance spectrum $A(\lambda)$ and so is related to the coefficient $a$ and the exponent $b$ in Eq. (10). At the same time, $a_0$ is also affected by the absorption coefficient of the brain, since $A(\lambda)$ is generally a function of the tissue absorption coefficient and the reduced scattering coefficient.

In order to investigate the relationship between the regression coefficients and the values of $C_{\text{HbO}}$, $C_{\text{HbR}}$, $a$, and $b$, we performed MCS for the diffuse reflectance from the rat cortical tissue through the skull at $\lambda = 500, 520, 540, 560, 570, 580, 600, 730,$ and 760 nm using various values of $C_{\text{HbO}}$, $C_{\text{HbR}}$, $a$, and $b$. We used the MCS source code developed by Wang et al.\textsuperscript{5} in which the Henyey-Greenstein phase function is applied to the sampling of the scattering angle of photons. The simulation model consisted of a single layer representing cortical tissue. In a single simulation of diffuse reflectance at each wavelength, 5,000,000 photons were randomly launched. The absorption coefficients of oxygenated hemoglobin $\mu_{a,\text{HbO}}(\lambda)$ and deoxygenated hemoglobin $\mu_{a,\text{HbR}}(\lambda)$ were obtained from the values of $\varepsilon_{\text{HbO}}(\lambda)$ and $\varepsilon_{\text{HbR}}(\lambda)$ in the literature where the hemoglobin concentration of blood with a 44% hematocrit is 2326 $\mu$M of hemoglobin. For the reduced scattering coefficients, the values of $a$ were 60,258, 80,344, 100,430, 120,516, and 180,774 in the simulation, which were derived by multiplying the typical value of $a$ by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively. The values of $b$ were 1.2442, 1.31332, 1.3824, 1.45156, and 1.52068, which were derived by multiplying the typical value of $b$ by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively. The reduced scattering coefficients $\mu_s(\lambda)$ of the cortical tissue were obtained from Eq. (15). The sum of the absorption coefficients of oxyhemoglobin and deoxyhemoglobin, $\mu_{a,\text{HbO}}(\lambda)$ and $\mu_{a,\text{HbR}}(\lambda)$, represents the absorption coefficient of total hemoglobin $\mu_{a,\text{HT}}(\lambda)$. The values for $C_{\text{HbT}} = 4.652, 23.26,$ and 116.3 $\mu$M were used as input to the cortical tissue in the MCS. Tissue oxygen saturation was assumed to be StO$_2 = 60\%$ for all combinations. For all simulations, the refractive index of the cortical tissue $n_c$ was fixed at 1.4. The thickness of the cortical tissue in each case was set to 5.0 mm.

Figures 2(a) and 2(b) show the values of $\alpha_{\text{HbO}}$ and $\alpha_{\text{HbR}}$ versus the volume concentrations of oxyhemoglobin $C_{\text{HbO}}$ and deoxyhemoglobin $C_{\text{HbR}}$, respectively, for various values of $a$, as obtained from the MCS. Figures 2(c) and 2(d) show the values of $\alpha_{\text{HbO}}$ and $\alpha_{\text{HbR}}$ versus the volume concentrations of oxyhemoglobin $C_{\text{HbO}}$ and deoxyhemoglobin $C_{\text{HbR}}$, respectively, for various values of $b$, as obtained from the MCS. In both Figs. 2(a) and 2(c), the value of $\alpha_{\text{HbO}}$ increases with the increase of $C_{\text{HbO}}$. Moreover, the value of $\alpha_{\text{HbO}}$ changes with the increase

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**Fig. 2** Regression coefficients versus concentrations of oxygenated hemoglobin $C_{\text{HbO}}$ and deoxygenated hemoglobin $C_{\text{HbR}}$, coefficient $a$, and exponent $b$, obtained from the Monte Carlo simulations.

(a) Regression coefficient $\alpha_{\text{HbO}}$ versus concentration $C_{\text{HbO}}$ for various values of $a$. (b) Regression coefficient $\alpha_{\text{HbR}}$ versus concentration $C_{\text{HbR}}$ for various values of $a$. (c) Regression coefficient $\alpha_{\text{HbO}}$ versus concentration $C_{\text{HbO}}$ for various values of $b$. (d) Regression coefficient $\alpha_{\text{HbR}}$ versus concentration $C_{\text{HbR}}$ for various values of $b$. (e) Regression coefficient $\alpha_0$ versus coefficient $a$ for various values of $C_{\text{HbO}}$ and $C_{\text{HbR}}$. (f) Regression coefficient $\alpha_0$ versus exponent $b$ for various values of $C_{\text{HbO}}$ and $C_{\text{HbR}}$. 

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*Yoshida et al.: Multispectral imaging of absorption and scattering properties of in vivo exposed rat brain.*
in the values of \(a\) and \(b\). The same tendency can be seen for \(a_{\text{HbR}}\), as shown in Figs. 2(b) and 2(d). Figures 2(e) and 2(f) show the values of \(a_0\) versus the values of \(a\) and \(b\), respectively, for various values of \(C_{\text{HbO}}\) and \(C_{\text{HbR}}\). The value of \(a_0\) decreases with the increase in the value of \(a\). Moreover, the value of \(a_0\) increases with the increases in \(C_{\text{HbO}}\) and \(C_{\text{HbR}}\). On the other hand, the value of \(a_0\) increases with the increase in the value of \(b\). Therefore, the regression coefficients \(a_{\text{HbO}}, a_{\text{HbR}}, a_0\) are related to the volume concentration of oxyhemoglobin \(C_{\text{HbO}}\), that of deoxyhemoglobin \(C_{\text{HbR}}\), the coefficient \(a\), and the exponent \(b\), respectively. However, \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\) are not determined by a unique regression coefficient when using only MRA1.

Thus, we conducted further multiple regression analyses to estimate the values of \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\) based on the combination of regression coefficients \(a_{\text{HbO}}, a_{\text{HbR}}, a_0\) that were obtained from MRA1. In this analysis, \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\) were regarded as response variables, and the three regression coefficients \(a_{\text{HbO}}, a_{\text{HbR}}, a_0\) in Eq. (17) were regarded as predictor variables to determine the regression equations for \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\). The regression equations for \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\) are written as

\[
C_{\text{HbO}} = \beta_{\text{HbO}} \cdot a_1,
\]

\[
C_{\text{HbR}} = \beta_{\text{HbR}} \cdot a_1,
\]

\[
b = \beta_b \cdot a_1,
\]

\[
\alpha_1 = [1, a_{\text{HbO}}, a_{\text{HbR}}, a_0]^T,
\]

\[
\beta_{\text{HbO}} = [\beta_{\text{HbO},0}, \beta_{\text{HbO},1}, \beta_{\text{HbO},2}, \beta_{\text{HbO},3}],
\]

\[
\beta_{\text{HbR}} = [\beta_{\text{HbR},0}, \beta_{\text{HbR},1}, \beta_{\text{HbR},2}, \beta_{\text{HbR},3}],
\]

\[
\beta_b = [\beta_{b,0}, \beta_{b,1}, \beta_{b,2}, \beta_{b,3}].
\]

The symbol \([\cdot]^T\) represents the transposition of a vector. We refer to this analysis as MRA2. On the other hand, in the preliminary investigation, we found that adding \(b\) to the predictor variables can improve the accuracy of the estimation of \(a\). Therefore, \(b, a_{\text{HbO}}, a_{\text{HbR}}, a_0\) are regarded as the predictor variables, and the given values of \(a\) are regarded as the response variables for determining the regression equation for \(a\), which is written as

\[
a = \beta_a \cdot \alpha_2,
\]

where

\[
\alpha_2 = [1, a_{\text{HbO}}, a_{\text{HbR}}, a_0, b]^T,
\]

\[
\beta_a = [\beta_{a,0}, \beta_{a,1}, \beta_{a,2}, \beta_{a,3}, \beta_{a,k}].
\]

We refer to this analysis as MRA3. The coefficients \(\beta_{a,0}, \beta_{a,1}, \beta_{a,2}, \beta_{a,3}, \beta_{a,k}\) are unknown and must be determined before estimating \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\).

We used MCS as the foundation to determine reliable values of \(\mu_{\text{HbO},0}, \mu_{\text{HbR},0}, \mu_{\text{HbR},j},\) and \(\mu_{a,k}\). The simulation model used here consisted of a single layer of cortical tissue, in which \(\mu_a(\lambda)\) and \(\mu_s(\lambda)\) are homogeneously distributed. The absorption coefficients \(\mu_a(\lambda)\) converted from the concentrations \(C_{\text{HbO}}\) and \(C_{\text{HbR}}\) and the reduced scattering coefficient \(\mu_s(\lambda)\) deduced by the coefficient \(a\) and the exponent \(b\) were provided as inputs to the simulation, whereas the diffuse reflectance spectrum \(r(\lambda)\) was derived as output. The input values of \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\) and the output reflectance spectra are useful as the data set in statistically determining the values of \(\mu_{\text{HbO},0}, \mu_{\text{HbR},0}, \mu_{\text{HbR},j},\) and \(\mu_{a,k}\) for determining the absolute values of \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\). The five different values of 60,258, 80,344, 100,430, 120,516, and 180,774 were calculated by multiplying the typical value of \(a\) by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively, whereas the five values of 1.2442, 1.31332, 1.3824, 1.45156, and 1.52068 were calculated by multiplying the typical value of \(b\) by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively. The reduced scattering coefficients \(\mu_s(\lambda)\) of the cortical tissue with the 25 different values were derived using Eq. (1). The sum of the absorption coefficients of oxyhemoglobin and deoxyhemoglobin \(\mu_a(\lambda) + \mu_s(\lambda) = \mu_a(\lambda)\) for \(C_{\text{HbR}} = 4.652, 23.26,\) and 116.3 \(\mu M\) was used as input to the cortical tissue in the simulation. Tissue oxygen saturation \(\text{StO}_2\) was determined by \(\mu_a(\lambda) / \mu_a(\lambda)\), and values of 0%, 20%, 40%, 60%, 80%, and 100% were used for the simulation. The above values were also used for the refractive index of the cortical layer. In total, 450 diffuse-reflectance spectra at \(\lambda = 500, 520, 540, 560, 570, 580, 600, 700,\) and 760 nm were simulated under the various combinations of \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\). The MRA1 analysis for each simulated spectrum based on Eq. (17) generated the 450 sets of vector \(\alpha_1\) and concentrations \(C_{\text{HbO}}\) and \(C_{\text{HbR}}\) and exponent \(b,\) and the 450 sets of vector \(\alpha_2\) and coefficient \(a\). The coefficient vectors \(\beta_{\text{HbO}}, \beta_{\text{HbR}},\) and \(\beta_b\) were statistically determined by performing MRA2, whereas the coefficient vector \(\beta_{\text{HbO}}\) was determined statistically by performing MRA3. Once \(\beta_{\text{HbO}}, \beta_{\text{HbR}},\) and \(\beta_b\) were obtained, \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\) were calculated from \(\alpha_{\text{HbO}}, \alpha_{\text{HbR}}, a_0,\) which were derived from MRA1 for the measured reflectance spectrum, without the MCS, as shown in Fig. 2(b). Therefore, the spectrum of the reduced scattering coefficient \(\mu_s(\lambda)\) and that of the absorption coefficient \(\mu_a(\lambda)\) were reconstructed by Eqs. (2) and (3), respectively, from the measured reflectance spectrum.

3 Experiments

3.1 Imaging System

Figure 4(a) shows a schematic diagram of the experimental system used in the present study. A white-light emitting diode (LED) (LA-HDF158A, Hayashi Watch Works Co., Ltd., Tokyo, Japan) illuminated the surface of the exposed cortex via a light guide and a ring-shaped illuminator with a polarizer. The light source covered a range from 400 to 780 nm. Diffusely reflected light was received by a 24-bit RGB CCD camera (DFK-31BF03.H, Imaging Source LLC, Charlotte, NC, USA) without an IR cut filter via an analyzer and a camera lens to acquire an RGB image of 640 × 480 pixels. The primary polarization plate (ring-shaped polarizer) and the secondary polarization plate (analyzer) were placed in a crossed Nicol alignment in order to reduce specular reflection from the sample surface. A standard white diffuser was used to regulate the white balance of
the camera. In order to evaluate the accuracy of the WEM, the reflectance spectra of cortex were simultaneously measured by a fiber-coupled spectrometer (USB4000-XRS-ES, Ocean Optics Inc., Dunedin, Florida, USA) for an integration time of 130 ms as reference data. Before the measurements of RGB images and reflectance spectra, the area measured using the spectrometer was confirmed by projecting light from a halogen lamp (HL-2000, Ocean Optics Inc.) onto the surface of the cortex via one lead of a bifurcated fiber, a lens, and a beam splitter. The RGB image of the sample, including the spot of light illuminated by the halogen lamp, was stored in the PC, and the size and coordinates of the spot were then specified as the area measured by the spectrometer. After the halogen lamp was turned off, the measurements of RGB images and reflectance spectra were performed simultaneously. Using the WEM, reflectance images ranging from 500 to 760 nm at intervals of 10 nm were reconstructed from an RGB image acquired at an exposure time of 65 ms. This means that the spectral reflectance images at 27 wavelengths can be obtained with a temporal resolution of 15 fps. The field of view of the system was 9.31 × 6.98 mm² with 1024 × 768 pixels. The lateral resolution of the images was estimated to be 9.1 μm. The multispectral reflectance images with 400 × 400 pixels at nine wavelengths (500, 520, 540, 560, 570, 580, 600, 730, and 760 nm) were then used to estimate the images of μₐ(λ), μₛ(λ), and μₛ′(λ) according to the process described in Fig. 1.

3.2 Phantom Experiments

To confirm the validity of the proposed method, we performed experiments using tissue-like optical phantoms. We prepared agar solution by diluting agarose powder (FastGene AG01; NIPPON Genetics EUROPE GmbH, Duren, NRW, Germany) with saline at a weight ratio of 1.0%. To simulate the scattering condition, a mixture of polystyrene latex beads solution with a 0.1-μm mean particle size (LB1-15L; Sigma-Aldrich Japan K. K., Tokyo, Japan) and that with a 1.1-μm mean particle size (LB11-15L; Sigma-Aldrich Japan K. K.) was added to the agarose solution. The resultant solution was used as the base material. The volume concentration of the polystyrene solutions ranged from 2.75% to 11.0%. An optical phantom layer was made by adding a small amount of fully oxygenated hemoglobin extracted from horse blood to the base material. All phantoms were hardened in molds having the required thickness and size by being cooled at approximately 5.5°C for 30 min. The thickness of each phantom was 0.5 cm, while the area of each phantom was 2.6 × 4.5 cm². In the preliminary experiments, we attempted to measure the phantoms with hemoglobin deoxygenated by Na₂S₂O₄ solution. However, the measured diffuse reflectance spectra had a tendency to exhibit unexpected fluctuation and it was difficult to maintain the stable condition of the deoxygenated spectra during the experiments. To avoid the potential uncertainty of the estimation of the total hemoglobin with the condition of O₂ = 0%, the phantoms with hemoglobin deoxygenated by Na₂S₂O₄ solution were excluded from the measurements in this study. We made 22 optical phantoms with different combinations of C_HBO₂, C_HBR, a, and b.

As preparatory measurements, we first determined the absorption coefficient μₐ and reduced scattering coefficients μₛ(λ) of each phantom at 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm, because they are necessary for the MCS to deduce the empirical formula for C_HBO₂, C_HBR, a, and b. The spectra of the absorption coefficient μₐ(λ) and reduced scattering coefficients μₛ(λ) are also required to calculate the given values for C_HBO₂, C_HBR, a, and b. For this purpose, we measured the diffuse reflectance and total transmittance spectra of each phantom individually. A 150-W halogen-lamp light source (LA-150SAE; Hayashi Watch Works Co., Ltd.) illuminated the phantom via a light guide (LGCl-5L1000; Hayashi Watch Works Co., Ltd.) and lens with a spot diameter of

![Fig. 3](image-url)
0.2 cm. The diameter and focal length of the lens are 5.0 and 10 cm, respectively. The thickness of each phantom in the preparatory measurements was 0.1 cm, while the area of each phantom was 2.6 × 4.5 cm². The phantom was placed between two glass slides having a thickness of 0.1 cm and fixed at the sample holder of an integrating sphere (RT-060-SF, Labsphere Incorporated). The detected area of the phantom was circular with a diameter of 2.2 cm. Light diffusely reflected from the detected area was received at the input face of an optical fiber probe having a diameter of 400 μm which was placed at the detector port of the sphere. The fiber transmits the received light into a multichannel spectrometer (USB2000, Ocean Optics Inc.), which measured reflectance or transmittance spectra in the visible to near-infrared wavelength region under the control of a personal computer.

To determine μ_s(λ) and μ'_s(λ) from the measured diffuse reflectance and total transmittance spectra, we utilized the inverse Monte Carlo method (IMC). In the IMC, the MCS of the reflectance and transmittance spectra were iterated for different values of μ_s(λ) and μ'_s(λ) until the difference between the simulated and measured spectral values decreased below a predetermined threshold. The values used in the last step of the iteration were adopted as the final results. This process was carried out at 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm, and wavelength-dependent properties of μ_s(λ) and μ'_s(λ) were obtained for each phantom. In these calculations, the refractive index was assumed to be 1.33 for all phantoms in the whole wavelength range. The concentrations of oxygenated hemoglobin C_{HBO} and deoxygenated hemoglobin C_{HBR} in each phantom were calculated from Eq. (4) with the estimated absorption coefficient μ_s(λ) and the known values of ε_{HBO}(λ) and ε_{HBR}(λ). The concentration of total hemoglobin C_{HT} and the tissue oxygen saturation SO_{2T} in each phantom was calculated from Eqs. (1) and (3), respectively. The coefficient a and the exponent b for each phantom were calculated from the estimated μ'_s(λ) based on Eq. (2). Those values of μ_s, C_{HBO}, C_{HBR}, C_{HT}, SO_{2T}, μ'_s, a, and b obtained by IMC are used as the given values to evaluate the validity of the proposed model experimentally.

We also need to have the conversion vectors for the phantoms used in this study. We generated diffuse reflectance spectra at 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm using the MCS with the conditions of the phantoms. For this simulation, the values of μ_s and μ'_s at 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm were set to be the same as those estimated by IMC. Performing MRA1, MRA2, and MRA3 as described in Sec. 3.1.1, we derived the conversion vectors and the corresponding empirical formula for estimating the volume concentrations of oxygenated hemoglobin C_{HBO}, that of deoxygenated hemoglobin C_{HBR}, the coefficient a, and the exponent b.

### 3.3 Animal Experiments

Animal care and experimental procedures were approved by the Animal Research Committee of Tokyo University of Agriculture and Technology. Intraperitoneal anesthesia was implemented with α-chloralose (50 mg/kg) and urethane (600 mg/kg) in five adult male Wistar rats (134 to 426 g). Anesthesia was maintained at a depth such that the rat had no response to toe pinch. The rat head was placed in a stereotaxic frame. A longitudinal incision of ~20 mm in length was made along the head midline. The skull bone overlying the parietal cortex was removed with a high-speed drill to form an ellipsoidal cranial window, as shown in Fig. 1A. In order to confirm the change in reflectance spectra of exposed rat brain, we performed the measurements during normoxia (t = 0 to 5 min), hyperoxia (t = 5 to 10 min), and anoxia (t = 10 to 30 min) by varying FiO_2. Hyperoxia (FiO_2 = 95%) was induced by 95%O_2–5%CO_2 gas inhalation, for which a breath mask was used under spontaneous respiration, whereas anoxia (FiO_2 = 0%) was induced by 95%N_2–5%CO_2 gas inhalation. In order to identify the respiration arrest (RA), the respiration of the rat was confirmed by observing the periodical movement of the lateral region of the abdomen during the experiments.

In order to evaluate the magnitude of signal S induced by hyperoxia and anoxia, we calculated the change in the signal based on the time series data. The signal at the onset of measurement was selected as a control S_c, which was subtracted from each of the subsequent signals S in the series. Each subtracted value, which demonstrated the change in the signal, S − S_c, over time, was normalized by dividing by S_c. The change in the signal is expressed as ΔS = (S − S_c)/S_c × 100. The above calculation was applied to the time series of C_{HBO}, C_{HBR}, C_{HT}, SO_{2T}, a, b, and μ'_s(λ). The time of RA can differ from sample to sample. Therefore, we divided the time course of the estimated value during anoxia into two periods: anoxia 1 and anoxia 2. Anoxia 1 is the period between the onset of anoxia and RA, whereas anoxia 2 is the period between RA and the end of the measurement. The time averages of the estimated values over the periods of normoxia, hyperoxia, anoxia 1, and anoxia 2 were then calculated and averaged over all five samples.

### 3.4 Statistical Considerations

A region of interest (ROI) of 40 × 40 pixels was placed in an image for each resultant image. An unpaired Student’s t-test was used for statistical analysis when comparing the in vivo results of the normoxic condition with those for the hyperoxic condition or with those for the anoxic conditions. The normality of the averaged value over the seven samples for each condition was tested using a Shapiro-Wilk test before the Student’s t-test. A P value of <0.05 was considered to be statistically significant.

### 4 Results and Discussion

Figure 8 shows the comparisons between the estimated and given values for (a) oxygenated hemoglobin C_{HBO}, (b) deoxygenated hemoglobin C_{HBR}, (c) total hemoglobin C_{HT}, (d) tissue oxygen saturation SO_{2T}, (e) coefficient a, and (f) exponent b, obtained from the phantom experiments. In Figs. 4H, 4I, 4C, 4E, and 4T, the estimated values are well correlated with the given values. Correlation coefficients between the estimated and given values are 0.91 (P < 0.0001), 0.95 (P < 0.0001), 0.84 (P < 0.0001) and 0.46 (P = 0.029) for C_{HBO}, C_{HBR}, a, and b, respectively. In Figs. 4B and 4D, all estimated values of C_{HBO} and SO_{2T} are close to 0 μM and 100%, respectively, which is consistent with the fact that the hemoglobin solution used in the phantoms was fully oxygenated hemoglobin, as described above. These results indicate the validity of the proposed model for estimating the volume concentrations C_{HBO} and C_{HBR}, the coefficient a, and the exponent b. Figure 9 shows the comparisons between the estimated and given values for (a) absorption coefficient μ_s and (b) reduced scattering coefficient μ'_s. Reasonable results were obtained for both μ_s and μ'_s. Correlation coefficients between the estimated and given values are 0.97 (P < 0.0001) and 0.78 (P < 0.0001) for μ_s and μ'_s, respectively. These results demonstrate the validity of the
The proposed method for estimating absorption and reduced scattering coefficients from the diffuse reflectance spectrum.

Figure 6 shows the typical spectral reflectance images at 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm estimated from the RGB image of in vivo rat brain under normoxia by the WEM. Spectral reflectance images of the cerebral cortex were successfully reconstructed from the RGB image using the proposed method. The distribution of blood vessels in the cortical tissue can be clearly recognized in the estimated reflectance images at 500, 520, 540, 560, 570, and 580 nm. The images at 600, 730, and 760 nm have low contrast between the parenchyma region and the blood vessel region, which indicates the lower absorption coefficient of hemoglobin at lower wavelengths.

Figure 4 Comparisons between the estimated and given values for (a) oxygenated hemoglobin $C_{\text{HBO}}$, (b) deoxygenated hemoglobin $C_{\text{HBRI}}$, (c) total hemoglobin $C_{\text{HBT}}$, (d) tissue oxygen saturation $StO_2$, (e) coefficient $a$, and (f) exponent $b$, obtained from the phantom experiments.

Figure 5 Comparisons between the estimated and given values for (a) absorption coefficient $\mu_a$ and (b) reduced scattering coefficient $\mu_s'$, obtained from the phantom experiments.
Figure 7 shows the reflectance spectra estimated using the WEM and the reflectance spectra measured using the spectrometer for (a) normoxia, (b) hyperoxia, and (c) anoxia immediately after RA. The estimated reflectance spectra for a blood vessel and that for parenchyma in each graph of Fig. 7 is the average values over the ROI_b and ROI_p, respectively. In this case, both ROI_b and ROI_p were selected to be the same as the measured area by the spectrometer. The reflectance spectra estimated using the WEM are comparable to the spectra measured using the spectrometer for the different respiration conditions. The estimated spectra under hyperoxia and anoxia are dominated by the spectral characteristics of oxygenated hemoglobin and deoxygenated hemoglobin, respectively. The values of GFC obtained from five samples summarized in Table 1 in the revised manuscript indicate the accurate spectral reconstruction by WEM.

Figure 8 shows the typical estimated images of exposed rat brain under normoxia obtained using the proposed method for (a) oxygenated hemoglobin $C_{HbO}$, (b) deoxygenated hemoglobin $C_{HbR}$, (c) total hemoglobin $C_{HbT}$, (d) tissue oxygen saturation $StO_2$, (e) coefficient $a$, and (f) exponent $b$. In Figs. 8(a)–8(c), the values of $C_{HbO}$, $C_{HbR}$, and $C_{HbT}$, respectively, in the blood vessel region are higher than those in the parenchyma region, which indicates the difference in blood volume between

![Figure 6](image1.png)

**Fig. 6** Typical estimated multispectral images $r(\lambda)$ of the exposed rat brain under normoxia.

![Figure 7](image2.png)

**Fig. 7** Comparison of the estimated reflectance spectra averaged over the ROIs in Fig. 6 (white squares) and the reference spectra measured by spectrometer for (a) normoxia, (b) hyperoxia, and (c) anoxia.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Region</th>
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<th>$\pm$ SD</th>
<th>Max</th>
<th>Min</th>
<th>Number of spectra</th>
<th>Accuracy</th>
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<td>0.9999</td>
<td>0.9935</td>
<td>361</td>
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</table>
the blood vessels and the parenchyma. The distribution of StO2 in the blood vessel region in Fig. 8(d) is probably due to the difference between artery and vein. The average values over the entire region were calculated to be $C_{\text{HBO}} = 150.08 \pm 50.61 \ \mu M$, $C_{\text{HBR}} = 40.11 \pm 12.12 \ \mu M$, $C_{\text{HTT}} = 190.19 \pm 52.05 \ \mu M$, StO2 = 75.52 ± 7.62%, $a = 134.458 \pm 3.634$, and $b = 1.33 \pm 0.02$, whereas those over the ROI on parenchyma (white square in each image of Fig. 8) were calculated to be $C_{\text{HBO}} = 41.16 \pm 3.13 \ \mu M$, $C_{\text{HBR}} = 42.34 \pm 5.51 \ \mu M$, $C_{\text{HTT}} = 190.19 \pm 52.05 \ \mu M$, StO2 = 75.52 ± 7.62%, $a = 134.458 \pm 3.634$, and $b = 1.33 \pm 0.02$, whereas those over the ROI on parenchyma (white square in each image of Fig. 8) were calculated to be $C_{\text{HBO}} = 41.16 \pm 3.13 \ \mu M$, $C_{\text{HBR}} = 42.34 \pm 5.51 \ \mu M$, $C_{\text{HTT}} = 190.19 \pm 52.05 \ \mu M$, StO2 = 75.52 ± 7.62%, $a = 134.458 \pm 3.634$, and $b = 1.33 \pm 0.02$.

Figure 9 shows the reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_s(\lambda)$ and (b) the reduced scattering coefficient spectrum $\mu'_s(\lambda)$ under normoxia. The average values of $\mu_s(\lambda)$ and $\mu'_s(\lambda)$ over the ROIs were calculated to be $\mu_s(\lambda) = 48.29 \pm 4.19\%$, $a = 136.811 \pm 2298$, and $b = 1.32 \pm 0.01$.

Figure 10 shows the reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_s(\lambda)$ and (b) the reduced scattering coefficient spectrum $\mu'_s(\lambda)$ under normoxia. The average values of $\mu_s(\lambda)$ and $\mu'_s(\lambda)$ over the ROIs were calculated to be $\mu_s(\lambda) = 48.29 \pm 4.19\%$, $a = 136.811 \pm 2298$, and $b = 1.32 \pm 0.01$. 

Figure 11 shows the reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_s(\lambda)$ and (b) the reduced scattering coefficient spectrum $\mu'_s(\lambda)$ under normoxia. The average values of $\mu_s(\lambda)$ and $\mu'_s(\lambda)$ over the ROIs were calculated to be $\mu_s(\lambda) = 48.29 \pm 4.19\%$, $a = 136.811 \pm 2298$, and $b = 1.32 \pm 0.01$. 

\[ \text{StO2, C, total hemoglobin CHTT, (d) tissue oxygen saturation StO2, (e) coefficient a, and (f) exponent b.} \]

Fig. 8 Estimated images of exposed rat brain obtained using the proposed method under normoxia for (a) oxygenated hemoglobin $C_{\text{HBO}}$, (b) deoxygenated hemoglobin $C_{\text{HBR}}$, (c) total hemoglobin $C_{\text{HTT}}$, (d) tissue oxygen saturation StO2, (e) coefficient a, and (f) exponent b.

Fig. 9 Typical estimated images of exposed rat brain obtained using the proposed method under normoxia for (a) absorption coefficient $\mu_s(\lambda)$ and (b) reduced scattering coefficient $\mu'_s(\lambda)$.

Fig. 10 Estimated spectra averaged over the ROI (white square in Fig. 9) for (a) absorption coefficient $\mu_s(\lambda)$ and (b) reduced scattering coefficient $\mu'_s(\lambda)$. The error bars mean the standard deviations for all pixels in the ROI.
[white squares in Figs. 9(a) and 9(b)] are also shown in Fig. 10. The error bars mean the standard deviations for all pixels in the ROI. The wavelength dependence of $\mu_a(\lambda)$ is dominated by the spectral characteristics of hemoglobin. The reduced scattering coefficients $\mu'_s(\lambda)$ have a broad scattering spectrum, exhibiting a larger magnitude at shorter wavelengths. The spectral features of $\mu'_s$ correspond to the typical spectrum of brain tissue published in the literature.

Figure 11 shows the comparison between the measured reflectance spectrum and the predicted reflectance spectrum for the $\mu_a$ and $\mu'_s$ shown in Fig. 10. The predicted reflectance spectrum is comparable to the measured spectrum. The value of GFC in this case was 0.9974, which shows a good agreement between the measured reflectance spectrum and the predicted reflectance spectrum.

Figure 12 shows the typical in vivo results for $C_{HBO}$, $C_{HBR}$, $C_{HBT}$, $StO_2$, $a$, and $b$ while varying FiO$_2$. Figure 13 shows the time courses of change in $C_{HBO}$, $C_{HBR}$, $C_{HBT}$, and $StO_2$ averaged over the area for the ROI in the parenchyma, as shown in Fig. 12 while varying FiO$_2$. The values of $C_{HBO}$ and $C_{HBR}$ were increased and decreased, respectively, during hyperoxia, which caused the increase in $StO_2$. After the onset of anoxia, the values of $C_{HBO}$ and $C_{HBR}$ decreased and increased, respectively. Consequently, the value of $StO_2$ was dramatically decreased. The value of $C_{HBT}$ begins to increase before RA and reaches a maximum amplitude approximately 1 min after...
RA, which is indicative of an increase in blood flow for compensating hypoxia. The period between the onset of anoxia and respiratory arrest averaged over all five samples was $499 \pm 207\ s$. Immediately after RA, the values of both $C_{HBO}$ and $C_{HBT}$ decreased rapidly. The time courses of $C_{HBO}$, $C_{HBR}$, $C_{HBT}$, and $StO_2$ while varying $FiO_2$ are consistent with the well-known hemodynamic responses to the change in the fraction of inspired oxygen. Minor fluctuations in both $C_{HBO}$ and $C_{HBT}$ approximately 2.5 min after RA are probably caused by the changes in blood volume due to vasoconstriction and vasodilatation.

Figure 13 shows the time courses of $\Delta C_{HBO}$, $\Delta C_{HBR}$, $\Delta C_{HBT}$, and $\Delta StO_2$ averaged over the ROI in the parenchyma region shown in Fig. 8 while varying $FiO_2$.

Figure 14 shows the time averages over the period of normoxia, hyperoxia, anoxia 1 (before RA), and anoxia 2 (after RA) after respiratory arrest for (a) $\Delta C_{HBO}$, (b) $\Delta C_{HBR}$, (c) $\Delta C_{HBT}$, and (d) $\Delta StO_2$ averaged over the ROIs for all five samples. The trends of $C_{HBO}$, $C_{HBR}$, $C_{HBT}$, and $StO_2$ shown in Figs. 2 and 3 were apparent in all five samples. The average values of $StO_2$ during normoxia over all five samples were $47.49 \pm 14.42\%$, which is lower than the value of around 60% reported in the literature.4 This may be due to the experimental conditions of the present study, such as the type of anesthetic used, the anesthetic depth, or uncontrolled body temperature.

Figure 15 shows the time courses of coefficient $a$, exponent $b$, $\mu'_s(500)$, and $\mu'_s(760)$ averaged over the area corresponding to the ROIs in the parenchyma, as shown in Fig. 8 while varying $FiO_2$. During the period from the onset of anoxia until RA, coefficient $a$ and exponent $b$ decrease slightly and increase slightly, respectively. The changes in the scattering parameters decrease the reduced scattering coefficient $\mu'_s$. On the other hand, the values of $\mu'_s(500)$ and $\mu'_s(760)$ increased remarkably after RA.

Figure 16 shows the time average over the period of normoxia, hyperoxia, anoxia 1, and anoxia 2 for (a) coefficient $a$, (b) exponent $b$ ($c\ \mu'_s(500)$), and (d) $\mu'_s(760)$ averaged over the ROIs for all five samples. The same tendencies of $a$, $b$, $\mu'_s(500)$, and $\mu'_s(760)$ shown in Figs. 2 and 3 were applicable to the average values over all five samples. Changes in the reduced scattering coefficients after the onset of $N_2$-inhalation imply morphological changes in brain tissue. Mitochondrial respiration is inhibited during the ischemia-like condition by the
rapid drop in O₂ tension, which causes depletion of adenosine triphosphate (ATP). Reduction of ATP production due to the inhibition of mitochondrial respiration leads to failure of the Na⁺/K⁺-ATPase pump. Thus, the slight decreases in \( \mu ' \) in Figs. 12, 13, and 14 are most likely caused by cell swelling due to failure of the Na⁺/K⁺ ATPase pump. The remarkable increases in \( \Delta \mu ' \) after RA are probably caused by the dendritic beading effect, which is indicative of neuronal damage. The necklace-like structure of a large amount of dendritic processes is highly efficient at scattering light, such that bead formation over several minutes reduces the transmitted light intensity.

Since the method relying on diffusing reflection integrates all information along the depth direction, the method does not have depth resolution. The depth and diameter of blood vessels usually differ among samples and may change due to the age of the rat. Their correct estimation is essential for precisely estimating the concentrations of oxygenated hemoglobin and deoxygenated hemoglobin for the blood vessel regions. In the present study, we assumed the scattering spectrum of the in vivo brain tissue as a power law function. This assumption may be applicable to the parenchyma region with a low volume concentration of blood. However, the diffuse reflectance spectrum from the blood vessel region with a higher volume concentration of blood can be strongly influenced by the scattering properties of the red blood cells (RBCs). It has been reported that RBCs behave as strong scatterers of light, and the reduced scattering coefficient spectrum has a similar wavelength dependence to the absorption spectrum of hemoglobin. Using the empirical formulas obtained from the MCS with an inhomogeneous layer model in which the reduced scattering coefficient spectrum is considered may enable more accurate estimations of the reduced scattering coefficients for the blood vessel regions.

It has been reported that the use of the Lambert-Beer approximation in the analysis of data for ischemia condition in which the values of \( C_{HBO} \) and \( C_{BR} \) are dramatically changed provides serious errors. The most serious problem is a large overestimation of the reduction in \( C_{HBO} \), leading to calculated values of \( C_{HBO} \) of less than zero. For this problem, they used...
a nonlinear minimization method to improve the accuracy of the estimations for $C_{\text{HBO}}$ and $C_{\text{HBR}}$. We also analyzed the absorbance spectrum based on the Lambert-Beer law for estimating $C_{\text{HBO}}$ and $C_{\text{HBR}}$. In this case, the absorbance spectrum was linearly related to $C_{\text{HBO}}$ and $C_{\text{HBR}}$. In addition, we used a linear regression model to specify the relationship between the sets of regression coefficients $a_{\text{HBO}}, a_{\text{HBR}},$ and $a_0$ obtained from MRA1 and the values of $C_{\text{HBO}}, C_{\text{HBR}}, a,$ and $b$. Therefore, the results obtained by the proposed method may also have some errors in $C_{\text{HBO}}$ and $C_{\text{HBR}}$ due to the linearization. Although the phantom experiments showed reasonable results for $C_{\text{HBO}}, C_{\text{HBR}}, C_{\text{HBR}}$, and $\text{SiO}_2$, the estimated values of $a$ and $b$ have relative large deviations from the given values, as shown in Fig. 8. Those deviations account for the estimation error in $\mu_s^*$ shown in Fig. 9. The use of $a_{\text{HBO}}, a_{\text{HBR}},$ and $a_0$ and their higher order terms for the vector $a_1$ and $a_2$ can extend the current linear empirical formula to the nonlinear regression models. It may be useful to improve the accuracy of the estimations for $C_{\text{HBO}}, C_{\text{HBR}}, a,$ and $b$. These issues should be investigated in the future.

5 Conclusions

In summary, a method for imaging reduced scattering coefficients $\mu_s' (\lambda)$ and the absorption coefficients $\mu_a (\lambda)$ of in vivo exposed brain tissues based on spectral reflectance images reconstructed from a single snapshot of an RGB image using the WEM was demonstrated in the present report. In vivo experiments using exposed rat brain while changing the fraction of inspired oxygen confirmed the feasibility of the proposed method for evaluating both cortical hemodynamics and changes in tissue morphologies due to loss of tissue viability in the brain.

Since the proposed method visualizes both the hemodynamic response and the morphological changes in brain tissue, it may be useful for evaluating brain function and tissue viability in neurosurgery as well as in the diagnosis of several neurological disorders, such as neurotrauma, seizure, stroke, and ischemia. We intend to extend the proposed method in order to investigate brain function in cortical spreading depression and anoxic depolarization.

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References


Keiichiro Yoshida is a PhD student in the Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Japan, where he received his MS degree in engineering in 2012.

Izumi Nishidate is an associate professor in the Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology. He received his MS and PhD degrees from Muroran Institute of Technology, Japan. His research interests include diffuse reflectance spectroscopy, light transport in biological tissues, multispectral imaging, and functional imaging of skin and brain tissues.

Tomohiro Ishizuka is a MS student in the Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Japan.

Satoko Kawachi is an assistant professor in the Division of Biomedical Information Sciences, National Defense Medical College Research Institute, Japan. She completed her MS degree at Keio University and received a PhD degree in medicine from Kyorin University, Japan. Her research interests encompass functional imaging of brain, photodynamic therapy, and diagnosing/imaging tissue viability using light scattering.

Shunichi Sato is an associate professor in the Division of Biomedical Information Sciences, National Defense Medical College Research Institute, Japan. His research interests include photomechanical waves and their application to gene/drag delivery systems, photomechanical imaging, photodynamic therapy, as well as diagnosing/imaging brain tissue viability.

Manabu Satō is a professor in the Graduate School of Science and Engineering, Yamagata University, Japan. He received his MS and PhD degrees from Tohoku University, Japan. His research focus is on optical coherence tomography, spectral imaging, and their applications to imaging of intrinsic optical signals in brain, as well as development of instruments with optical fiber for biological tissues.