Quantitative evaluation of intrinsic optical signals in brain tissues using diffuse reflectance spectroscopy

Dissertation Submitted for the degree of
Doctor of Engineering
March 2015

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Chapter 1.

Introductory Remarks

1.1 General Review for Tissue Optics

In the recent years, research on measurement methods using diffuse reflectance spectroscopy [1, 2] and optical coherence tomography (OCT) [3-6] in the living tissue has been developing. OCT is an observation apparatus capable of obtaining a tomographic image of the tissue with an accuracy of several ten microns. This is using the interference phenomenon of light. On the other way, diffuse reflectance spectroscopy, the internal light has repeatedly scattering and absorption by the scattering materials and absorption materials of inside biological tissues, and a diffused light which is measured at the incident surface side. The diffused reflectance light intended the information of the amount of the absorber and the scatterer of the biological tissue. In particular, it is believed that the diffuse reflection light of the living tissue has a variety of information in the near infrared region from the visible light region, such as the chromophores in living tissue. For example, if the amount of hemoglobin which is a major absorber of the biological tissue is increased, the absorption is increased and the diffuse reflectance of light is reduced. Like these, it is possible to be non-invasively and quantitatively evaluate the physiologic condition change, such as changes in blood flow in the biological tissue by measuring the diffusely reflected light.

The light incident on the tissue, the light propagating to the tissue to proceed while changing the strength and direction by absorption and multiple scattering.
Diffusely reflected light, reflects the absorption and scattering properties of biological tissue, the diffusely reflected light is used for diagnosis of the tissue since it is capable of providing a biochemical and morphological information. If the light propagates in uniform biological tissue, the intensity of light decreases exponentially with absorption and multiple scattering. The characteristic of absorption and scattering greatly depends on the wavelength of the light. According to the Lambert-Beer law [7], absorption in biological tissue is a physical property in the absorption of the medium. The major cause of absorption in the brain tissue on visible light is blood.

Scattering phenomenon in the living tissue is very complex. Because, it has been seen microscopically, not only the sum of scattering by a single-particle stacking but also the dispersion by the difference between cell membrane and refractive index with the liquid matrix and the difference of the refractive index with the organelle and the liquid matrix liquid in the cell. That is, since scattering is caused by the structural heterogeneity of the cell, the wavelength dependence of the scattering is not large, usually monotonically decreases with increasing wavelength. Biological tissue, mainly in order to elucidate the scattering phenomena against tissues such as the brain rather than observing the scattering phenomena in each cell, and it is important to understand the macroscopic scattering phenomenon caused by sum of the micro scattering phenomena. In generally, macroscopic scattering phenomena are represented by the scattering phase function $p(\theta)$ [8]. It represents the intensity distribution of the direction of the light scattering and the scattering coefficient. Scattering in the living tissue is a strong forward scattering. Parameter that represents the direction of the scattering is anisotropy parameters [9], it used the average cosine of the phase function. Anisotropy factor takes a value from -1 to +1, pure forward scattering at +1, pure backward scattering at, whereas -1 the isotropic scattering at 0. Living tissue, usually exhibit a
strong forward scattering, and takes 0.9 or more. However, repeating forward scattering in the tissue, the scattering can be similar isotropic scattering in macroscopic. Where, the scattering coefficient named as the reduced scattering coefficient.

When the light propagate in the medium, if it does not have waviness, the optical power $I$ is based on a transportation equation to expression of preservation of the light energy. Light transport equation [10] that represents the storage of light energy.

$$\frac{1}{c} \frac{\partial I}{\partial t} + \hat{s} \cdot \nabla I + (\mu_s + \mu_a)I = \mu_s \int_0^{4\pi} p(\hat{s}', \hat{s}) I(r, \hat{s}', t) d\Omega(\hat{s}') + q \quad (1.1)$$

In this equation, where $I$ is the intensity of light, $r$ means position in the media, $s$ means the angle of direction and $c$ means the speed of light. The $\mu_a$ and $\mu_s$ are the absorption coefficient and scattering coefficient respectively. The function $p(\hat{s}', \hat{s})$ is the scattering phase function, representing scattering contribution from the direction $\hat{s}'$to $\hat{s}$, and $d\Omega$ is the solid angle. Transport equation can be solved in principle given the appropriate boundary conditions if the scattering coefficient, absorption coefficient and $p(\theta, \phi)$ of optical characteristic values are known in advance. In order to perform quantitative measurements of the scattering can be considered the following methods.

i. Measurement method by integrating sphere

Integrating sphere is spheres whose inner surface processed to scatter in all directions, the light incident on the specimen is transmitted in terms of the reflected or the opposite side from the incident surface after being scattered inside the sample. The reflected and transmitted light is oriented in all directions. So the integrating sphere can be used to measure the transmittance and reflectance by collecting them all. Reflectance $R$ and transmittance $T$ obtained amount of the absorption coefficient and scattering coefficient of the material sample. When determining the scattering coefficient and absorption coefficient, it is assumed the scattering coefficient and absorption coefficient
in fast, and performs calculation of the scattering including absorption by using the estimated value, then obtain the reflectance $R$ and transmittance $T$. If obtained reflectance $R$ and transmittance $T$ do not match the measured values, it re-estimating the new reflectance $R$ and transmittance $T$ again by absorption coefficient and scattering coefficient repeatedly. If the result of reflectance $R$ and transmittance $T$ match the measured value, the absorption coefficient and estimated scattering coefficient are become the solution. This series of calculation technique is called inverse problem solution. Calculating forward problem is performed according to the mathematical model to faithfully reproduce the scattering and absorption phenomenon. An example of the statistical method, Monte Carlo simulation is used for the calculation technique.

Monte Carlo simulation [11, 12] treats light as a particle, it is a technique which slide into one by one track the path of light progresses while repeating absorption and scattering in the medium to meet the optical characteristic value statistical media. By using a large number of photons, to reveal the propagation behavior by taking the statistical average of the path. Photons tracking in Monte Carlo simulation, photons with energy collide with the scatterers, change the direction of the scattering angle and the azimuth angle of travel, and the advance step size, $s$ until the collision to the next scatterer. Of these, the scattering angle, the azimuth angle, the step size are represented using a uniform pseudo-random number.

ii. Time-resolved measurement method

Measured by the integrating sphere is a continuous light, the response time of the photo detector is not needed so quickly. Time-resolved measurement method [13, 14] is the technique that the ultra-short picosecond order pulse light enters to the semi-infinite medium, diffuse reflected light appear on the surface with response to scattering in the medium. And, this method is estimating the equivalent scattering
coefficient and absorption coefficient of the medium from time-resolved waveform obtained by measuring the temporal change in the light detector. Irradiated ultra short-pulsed light in a uniform semi-infinite medium is detected at a point of the distance \( \rho \). The result depends on the absorption coefficient and scattering coefficient of the medium. This waveform is possible to predict by Monte Carlo simulations, it can be also predicted using the light transport equation.

### iii. Spatially resolved measurement method

Incident the continuous light on bio tissue and to measure the reflected light intensity \( R_1 \) and \( R_2 \) at two points \( \rho_i \) and \( \rho_2 \) indifferent from the incident point [15-21]. By integrating the light diffusion equation time, the reflected light intensity at distance \( \rho \) is expressed by the following equation. The reflected light intensity at distance \( \rho \) is obtained by the following equation.

\[
R_i(\rho) = \frac{1}{4\pi} \left[ z_0 \left( \mu_{\text{eff}} + \frac{1}{\rho_+} \right) \frac{\exp(-\mu_{\text{eff}}\rho_+)}{\rho_+} - (z_0 + 2z_b) \left( \mu_{\text{eff}} + \frac{1}{\rho_-} \right) \frac{\exp(-\mu_{\text{eff}}\rho_-)}{\rho_-} \right]
\]

\[
\mu_{\text{eff}} = \sqrt{3\mu_a\mu_s'}, \quad \rho_+ = \rho_i^2 + z_0^2, \quad \rho_- = \rho_i^2 + (z_0 + 2z_b)^2, \quad z_0 = \frac{1}{\mu_s}, \quad z_b = 2AD
\]

### iv. Frequency resolved measurement method

Spectroscopy using a light modulated by a sine wave instead of the pulse light is used in time-resolved spectroscopy [22-27]. Relationship with the response to the signals incident light modulated in the living tissue is related by the Fourier transform. So, examining the intensity variation and phase delay with respect to a change in the modulation frequency of the transmitted light in the living tissue, to determine the scattering coefficient and absorption coefficient.
1.2 Motivation

In the recent years, to quantify biological absorbance materials which absorb the visible light in the brain is needed a technique for imaging brain tissue oxygen saturation or cerebral blood flow state. It is important vital signs during surgery. In the measurement of in vivo cerebral blood flow, the evaluation of cerebral blood flow has been performed already using a laser Doppler method or a laser speckle method [28, 29]. In addition, these techniques can be obtaining a non-invasive and quantitative patient's condition, so these techniques are required in the medical field such as brain surgery and emergency medicine. On the other hand, the technique that measuring the absorption and scattering properties of the brain tissue have been studied. For example, an imaging method for the tissue oxygen saturation and the blood flow state of the brain tissue on the basis of intrinsic optical signal (IOSs) [30]. Usually, the spectral image obtained by multispectral imaging and hyperspectral imaging. For example, multispectral imaging obtained by narrow-band interference filters [31], liquid crystal variable filter and acoustic-optical filter [32]. And hyperspectral imaging obtained by diffraction grating and interferometer. In these conventional multispectral imaging methods have problems such as high cost and low time resolution of the imaging system when they obtained the multispectral imaging. Therefore, there is a need for a technology capable of obtaining a spectral image using a simple imaging system, low cost and with high time resolution. By using technique of low cost and with high time resolution, it is possible to know the change of optical characteristic values in the brain more simply in brain surgery and emergency medical field.
1.3 Synopsis of Contents

The purpose of this dissertation is to study the method for evaluating the hemoglobin and tissue conditions in the brain tissue using the diffuse reflectance spectra. The dissertation may be divided into two main parts. The first part is the measurement for the brain tissue without blood flow using rat brain slices (in vitro measurement). The second part is the spectroscopic analysis of the hemoglobin and tissue conditions in the exposed rat brain tissue (in vivo measurement). Chapter 2 describes the fundamental theories and principles treated through this dissertation. The Lambert-Beer Law, Multiple Regression Analysis [33] and the Monte Carlo method are mentioned as the quantitative description of the light propagation model in the tissue, respectively.

Chapter 3 deals with the in vitro study on optical properties of rat cerebral cortical slices. In this chapter, rat brain slices with an artificial cerebrospinal fluid perfusion experimental system was performed simultaneous measurement of the transmitted light and reflected light. This method is not affected by oxygenation and deoxygenation of hemoglobin and blood flow, which can be measured with maintaining the viability of the tissue [34, 35]. In the next, we used the inverse Monte Carlo method [36, 37] and estimated the absorption coefficient and scattering coefficient from the measurement results of transmittance $T$ and reflectance $R$. Experiments was carried out in a state of oxygen and glucose deprivation. I observed the change of optical properties of rat brain slice during low viability.

Chapters 4-6 are devoted to the spectroscopic studies on the exposed rat brain tissue in in vivo state.

In Chapter 4, in vivo multispectral imaging of scattering and absorption properties of exposed rat brain. To estimate the concentrations of hemoglobin with the
oxygen saturation in the exposed rat brain tissue, obtained images of the brain surface, using an interference filter. The possibility of this method is experimental investigated for absorbance spectra and scattering spectra with typical optical parameters of the rat brain tissue. This experiment can obtain a spectral image of a rat brain surface quantitatively, and performs measurement of the optical parameters associated with the physiological conditions change.

Chapter 5-6 deals with the method of evaluating by using the RGB camera. In the Chapter 5, a method for estimating the hemoglobin concentration, absorption coefficient, scattering coefficient and oxygen saturation of the exposed rat brain tissue were proposed. In the first, we conducted the quantitatively multispectral imaging method of exposed rat brain surface using the RGB camera. The spectral analysis of the spectral reflectance, it was estimated multispectral reflectance image by applying the Wiener estimation [38-40] for RGB images. Next, I performed a multiple regression analysis based on the results of light propagation, Monte Carlo simulation to multispectral reflectance images estimated by the Wiener estimation method for each pixel in the image. Hemoglobin content and tissue oxygen saturation in the brain tissue, the scattering parameters are obtained. Finally, I obtained the images of the absorption coefficient spectra and scattering coefficient spectra. By using this analytical method, the oxygenated hemoglobin concentration, deoxygenated hemoglobin concentration, total hemoglobin concentration, tissue oxygen saturation and the scattering parameters are estimated from the multispectral reflectance images. And, scattering coefficient spectrum and absorption coefficient spectrum are estimated. From the above results, possibility that spatial distribution of light scattering properties and absorption properties of hemoglobin at low cost and high time resolution was shown from RGB
images of the exposed rat brain tissue.

In the Chapter 6, the measurement was performed on brain dysfunction using Chapter 5 techniques. In this chapter, I performed the Cortical Spreading Depression (CSD). CSD is the depolarization wave that propagates on the cerebral cortex. And it said that the cause of dysfunction, nerve damage, migraine, ischemia and stroke. Propagating of CSD occurs that the blood flow increase and reduced blood flow after passing through. In addition, negative changes in the extracellular local field potential (LFP) occurs at the same time. In this study, I discussed relevance of changes in cerebral blood flow and the propagation of the CSD.

In the Chapter 7, summarizes the results of in vitro and in vivo, and I considered the change of optical properties in the physiological condition change.
Chapter 2.

Fundamental Theories and Principles

2.1 Optical parameters

A number of methods have been proposed to explain the phenomenon of light propagation in the rat brain tissue with a certain thickness directly. One is to solve the radiative transport equation by diffusion approximation derived from the storage of light energy in the continuum. The other is to solve the approximate expression. In such a situation, to determine the optical properties of a rat brain tissue the absorption coefficient $\mu_a$, the scattering coefficient $\mu_s$ and the scattering phase function $p(\theta)$ are needed.

Light scattering by the living tissue is known to be a strong forward scattering. However, the light advanced by scattering could lose direction with a certain thickness of the living tissue by repeated scattering. Eventually light scattering can be approximated the macroscopic isotropic scattering instead of forward scattering, depicted in Fig.2.1. While, the reduced scattering coefficient $\mu'_s$ can be expressed by

![Diagram showing change from forward to isotropic scattering approximation](Image)

Fig.2.1 Change of forward scattering to isotropic scattering approximation.
Anisotropy scattering parameter $g$ takes a value in the range from -1 to +1, isotropy scattering in the 0, +1 which represent the full forward scattering, and -1 represent the full backward scattering. In addition, the refractive index $n$ is a parameter important to determine the optical properties of the skin tissue. Normally, the refractive index $n$ often takes a value of 1.4 [41] to a living body.

![Diagram of scattering light](image)

**Fig. 2.2** Scattering of light by a single particle.

Fig. 2.2 illustrates the scattering directions of light by a single particle. Anisotropy parameter $g$ is a parameter representing the scattering direction of light, and the scattering phase function $p(\theta)$ is obtained the zenith angle of distribution for $\theta$ cosine of the average. Typically, anisotropy parameter $g$ takes a value from 0.8 to 0.95.
[42-44] because of the strong forward scattering in the living tissue in the visible and near-infrared wavelength region. Absorption coefficient $\mu_a$ and scattering coefficient $\mu_s$ are proportional to the rate of light intensity by absorption and scattering when light is transmitted by flat samples with the small thickness, Fig.2.3.

**Fig.2.3** Absorption and scattering of the light.

Fig.2.3 represents the spectroscopy of the uniform clear solution to measure the absorbance $OD$ (Optical Density) = $\ln (I_0/I)$. The light $I_0$ enters the solution of thickness $d$. The absorbance of the solution based on equation (2.3) which represents Beer-Lambert law (see section 2.2) and the concentration $c$ [mol/l]. The Lambert-Beer law is proportional to the optical path length. The $\varepsilon$ [mm$^{-1}$ (mol/l)$^{-1}$] means the molar extinction coefficient of the solute, which depends on the wavelength. In addition, $\mu_a=\varepsilon c$ [mm$^{-1}$] is the absorption coefficient of the solution. Also, $I_0$ is the incident light intensity and $I$ is the transmitted light intensity.

\[
\begin{align*}
OD &= \ln \left( \frac{I_0}{I} \right) = A \\
I &= I_0 \exp(-A) \\
A &= \mu_a d = C \varepsilon d
\end{align*}
\]

(2.3)
However, equitation (2.3) is not well pleased for incident light scattering of rat brain tissue, because of its scattering in all directions. Hence, the equation (2.3) can be modified into equation (2.4), where $\bar{l}$ is the optical path length.

\[
\begin{aligned}
I &= I_0 \exp(-S) \\
S &= \mu_s \bar{l}
\end{aligned}
\]  \hspace{1cm} (2.4)

In this equation, $S$ represents the attenuation due to scattering, which is related the thickness $d$, concentration $c$, therefore $S$ cannot be expressed as a simple equation. That is why it is difficult to quantitatively measure the scattering system. Moreover, it is described below how to measure the scattering quantitatively.

![Integrating sphere](image)

**Fig.2.4 Integrating sphere.**

There is one method of using an integrating sphere to measure the scattering. An integrating sphere with the inner surface processed to scatter light in all directions. Providing a number of windows as the installation location of the sample, shown in Fig.2.4. The light which incident on the sample is exited from the incident surface (reflected) or the opposite side (transmitted) after being scattered inside the sample. The reflected light and the transmitted light are oriented in all directions, the integrating
sphere can collect them all and measure the rate of reflectance $R$ and transmittance $T$. The measuring parameters reduced scattering coefficient $\mu'_s$ and the absorption coefficient $\mu_a$ of physical state can be obtained, from the measurement results of the sample reflectance $R$ and transmittance $T$. It is possible to obtain the absorption coefficient $\mu_a$, if the molar extinction coefficient $\varepsilon$ is known, where concentration $c$ is derived from $\mu_a = \varepsilon c$ quantitatively.

2.2 Beer-Lambert Law

In the case, the light propagating in the living tissue, the light intense reduced exponentially by multiple scattering and absorption. In generally, Beer-lambert law [7] is used for the law about the formulation of light absorption in the tissue. If the material which is absorbance the light contains in the tissue. When placing the molar concentration of the material is $c$, the attenuation of the light intensity $dl_0$ when the light passed the layer including the material is proportional to the layer thickness $dx$, concentration and the light intensity $I_0$. Thus it is represented by the following equation.

$$dl_0 = -kcl_0dx$$  \hspace{1cm} (2.5)

Where $k$ is the constant of proportionality. By modifying the above equation, it becomes the following equation (2.6).

$$dlnI_0 = -kcdx$$  \hspace{1cm} (2.6)

This equation can be fitted each layers, so this equation can also used when the sample is in layers. Therefore, when the incident light intensity is $I_0$, and to determining the light intensity $I$ out from the samples which thickness is $l$, the sum of the changes in each layers, equation (2.7).

$$\int_{lnI_0}^{lnI} dlnI_0 = -k \int_0^l c \, dx$$  \hspace{1cm} (2.7)

If concentration is uniform, $c$ is independent of $x$. And an expression of following
Beer-Lambert obtained integrating this equation (2.7).

\[ I = I_0 \exp(-kcl) \] (2.8)

Therefore, the light intensity will be attenuated exponentially for the thickness and concentration of the sample. Beer-Lambert law is often expressed by the following equation (2.9).

\[ I = I_0 10^{-\varepsilon cl} \] (2.9)

\[ (k = \varepsilon \ln10) \]

In this way, the above equation (2.9) can be written as follow equation (2.10).

\[ \log \frac{I}{I_0} = -\varepsilon cl \] (2.10)

Where \( \varepsilon \) is molar extinction coefficient of the sample, it is dependent on the frequency of the incident light. The unit of molar extinction coefficient is 1/(concentration × length), it represent \( M^{-1}cm^{-1} \).

2.3 Monte Carlo Model of light propagating

Monte Carlo simulation (MCS) method [11,12] describes the local rules of photon propagation, as probability distribution that explains the step size of photon movement between sites of photon-tissue interaction, and the angle of deflection in a photon’s trajectory when a scattering event occurs. Movement of the photon is determined by a random number in accordance with the scattering coefficient and phase function. Monte Carlo simulation can be performed by using the absorption coefficient, scattering coefficient, anisotropy factor, refractive index and geometrical thickness.

2.3.1 Initialization of the photon

A simple variance reduction technique which implicit the photon capture is used to improve the efficiency of the MCS model. This technique allows one to
equivalently propagate many photons as a photon packet along a particular pathway. Usually, one photon leads to the course, while the other photons might be absorbed or scattered by each path. If the photon packet was exited the tissue, when its weight is below the threshold weight, the weight is set to 1. The size of this photon packet has been called weights ($w$).

Fig. 2.5 Initialization value of the photon.

### 2.3.2 Propagation distance of the photon

The successive moving distance $\Delta s$ represents the distance between two interactions, or scattering in the tissue. The certain moving distance $\Delta s$ should be small compared to the average mean free path length of photons in tissue. The mean free path length is the inverse of the total attenuation coefficient.

$$\Delta s \ll \frac{1}{\mu_t} = \frac{1}{\mu_s + \mu_a} \quad (2.11)$$
In this equation, \( \mu_t, \mu_a, \) and \( \mu_s \) reflects the entire damping coefficient, absorption coefficient and scattering coefficient respectively. If the moving distance \( \Delta s \) is too small, photons does not act on the tissue and each other. Which turns the Monte Carlo method ineffective. Conversely, if the moving distance \( \Delta s \) is too large, then the distance propagated by the photon becomes poor approximately of the actual distance.

According to Lambert-Beer Law, the moving distance for each photon movement \( \Delta s \), is more likely for a photon to travel a short distance than a long distance and the probability is proportional to \( e^{-\mu t \Delta s} \). The function of the random variable \( \xi \) is uniformly distributed between 0 to 1. Variable with this distribution, which yields a random expressed in equation (2.12).

\[
\Delta s = -\frac{\ln \xi}{\mu_t} \tag{2.12}
\]

Moving distance \( \Delta s \) can be calculated from the above mentioned equation (2.12), which represents the previous distance to move the photons before the photons interaction with the tissue through absorption or scattering.
2.3.3 Photon movement

Once the moving distance $\Delta s$ is determined, the photon is moved in the tissue. While, the photon location is described by the Cartesian coordinates $(x, y, z)$. The propagating direction of a photon can be described by the directional cosines $(\mu_x, \mu_y, \mu_z)$. Required formula for photon propagation is simple, where variable angle representing the direction of the photons does not change as long as the direction of the photons unchanged. Direction cosine is identified by the direction of the photon considering the cosine of the angle between. Each axes $(x, y, z)$ axis. These axis specified to each axis which corresponds to each of $(\mu_x, \mu_y, \mu_z)$ respectively. If the photon that places the coordinates $(x, y, z)$ moves the distance $\Delta s$ to the direction of $(\mu_x, \mu_y, \mu_z)$, the new coordinates $(x', y', z')$ is given by equation (2.13). Where the arrows indicate quantity substitutions. The variables on the left-hand side have the new updated values, and the variables on the right-hand side have the old values.

$$
\begin{align*}
    x' &= x + \mu_x \Delta s \\
    y' &= y + \mu_y \Delta s \\
    z' &= z + \mu_z \Delta s
\end{align*}
$$

(2.13)

2.3.4 Absorption of photons

Fig.2.7 shows the change of the weights of the photon in the tissue. The indirect capture technology gives a weight to each of the photons immediately after the photon enters the tissue. The packet of photons is divided into two parts, the photon propagating the distance $\Delta s$ in each of the path. One part is intended to be absorbed, the other part is scattered. Percentage of absorbed packets is given by equation (2.14).

$$
\text{fraction absorbed} = \frac{\mu_a}{\mu_a + \mu_s} = 1 - \frac{\mu_a}{\mu_a + \mu_s} = 1 - a
$$

(2.14)
In this equation, \( a \) denotes the albedo of single particles. Therefore, the weight of the new photon \( (w') \) is given by \( w' = aw \). This equation represents the percentage of packets that are scattered on this route.

\[
w_i = \frac{\mu_s}{\mu_a + \mu_s} \mu_a w_i^{i-1}
\]

Fig.2.7 Change of photon weight.

### 2.3.5 Scattering of photons

Fig.2.8 shows the change in the scattering direction of the photons within the tissue. When a photon is scattered, the normalized phase function express the probability density function about the azimuth angle \( \phi \) and direction cosine \( \theta \). If the phase function has no dependencies on the azimuth angle \( \phi \), then the azimuth angle \( \phi \) distributed from 0 to \( 2\pi \). And, it is generated by multiplying \( 2\pi \) and the pseudo-random number \( \xi \) which is uniformly distributed between 0 and 1 (i.e. \( \varphi = 2\pi \xi \)). Traveling direction of the angle \( \theta \) on isotropic scattering \( (g=0) \) is given by equation (2.15). In this equation, anisotropy scattering parameter \( g \) is a parameter representing the scattering direction of light, and it takes the cosine average of the scattering phase function \( p(\theta) \).
\[
\cos \theta = 2\xi - 1 \quad (2.15)
\]

Scattering in the tissue characterized by Henyey-Greenstein [11]. Henyey-Greenstein phase function generated by equation (2.16).

\[
\cos \theta = \frac{1}{2g}\left\{1 + g^2 - \left[\frac{1+g^2}{1-2g+2g^2}\right]^2\right\} \quad (2.16)
\]

If scattering is isotropic \((g = 0)\), then equation (2.15) is used.

![Figure 2.8 Scattering of photons.](image)

If the photon move to the angle \((\theta, \varphi)\) from the direction \((\mu_x, \mu_y, \mu_z)\), the new scattered direction \((\mu'_x, \mu'_y, \mu'_z)\) specified by equation (2.17).

\[
\begin{align*}
\mu'_x &= \frac{\sin \theta}{\sqrt{1 - \mu_z^2}} \left(\mu_x \mu_z \cos \varphi - \mu_y \cos \theta\right) \\
\mu'_y &= \frac{\sin \theta}{\sqrt{1 - \mu_z^2}} \left(\mu_y \mu_z \cos \varphi - \mu_x \cos \theta\right) \\
\mu'_z &= -\sin \theta \cos \varphi \sqrt{1 - \mu_z^2} + \mu_z \cos \theta
\end{align*}
\quad (2.17)
\]

If the angle is close enough to vertical (for example, \(|\mu_e| > 0.99999\)), the following formulas should be used to obtain the direction of a new photon (equation (2.18)).
\[
\begin{align*}
\mu'_{x} &= \sin \theta \cos \varphi \\
\mu'_{y} &= \sin \theta \cos \theta \\
\mu'_{z} &= \frac{\mu_{z}}{|\mu_{z}|} \cos \varphi
\end{align*}
\] (2.18)

2.3.6. Internal reflection of photons

When photons propagate across the boundaries of the different refractive index region, the possibility of internal reflection occurs. Possibility of photons internal reflection are determined the Fresnel reflection coefficient \( R (\theta) \).

\[
R (\theta) = \frac{1}{2} \left[ \frac{\sin^2 (\theta_i - \theta_t)}{\sin^2 (\theta_i + \theta_t)} + \frac{\tan^2 (\theta_i - \theta_t)}{\tan^2 (\theta_i + \theta_t)} \right]
\] (2.19)

Fig.2.9 (a) Internal reflection. (b) Diffused reflectance and transmittance.

In the equation (2.19), \( \theta_i = \cos^{-1} \mu_e \) is the angle of incidence on the boundary line, whereas the transmission angle \( \theta_t \) is given by Snell's law.

\[
n_i \sin \theta_i = n_t \sin \theta_t
\] (2.20)
In the equation (2.20), \( n_i \) and \( n_t \) are the refractive index of the incident medium and transmitted medium, respectively. The random number \( \xi \) which is uniformly distributed between 0 to 1 is used to determine whether photons are transmitted or reflected. At that time, if \( \xi < R(\theta) \), the photon is reflected internally. Otherwise, photons will be out of the tissue, the event is recorded as transmitted light (when out of the bottom surface) or diffuse reflected light (when out of the surface of photons). If the photon is reflected internally, the position and direction of the photon is adjusted accordingly.

About the geometric conditions of the slab, the thickness \( t \) in the z-direction, \( x \) direction and \( y \) direction are infinite, the position of the photons \((x'', y'', z'')\) reflected internally is obtained by only coordinate changes of the photon in the z-component.

\[
(x'', y'', z'') = f(x) = \begin{cases} 
(x, y, -z), & \text{if } z < 0 \\
(x, y, 2t - z), & \text{if } z > t 
\end{cases} \tag{2.21}
\]

New photon direction \((\mu'_x, \mu'_y, \mu'_z)\) is expressed by equation (2.22).

\[
\left(\mu'_x, \mu'_y, \mu'_z\right) = \left(\mu_x, \mu_y, -\mu_z\right) \tag{2.22}
\]

In this case, \( \mu_x \) and \( \mu_y \) do not change.
2.3.7 Terminate of the photon

The weight of photon never reaches zero, and the photon which propagated inside the tissue with very small weights bears very little information. If the photon weight has been sufficiently decreased from the threshold value after many steps of interaction, then further propagation of the photon yields little information unless the interest is in the very late stage of photon propagation. A technique called Russian roulette is used to terminate the photon packet when the weight of photons falls below a certain minimum value. Roulette technology gives the photon packet one chance in $m$ of surviving with a weight of $mw$. If the photon packet does not survive the Roulette, the photon weight is reduced to zeros and the phonon is terminated. Therefore, without continuing the propagation until the weight of the photon reaches zero, the photon is erased in an impartial manner. And, the weight of photons reduced below a certain
minimum value, while photons propagation are terminated by roulette technology, the new photons with a new weight begin to move.

![Diagram of photon termination](image)

Fig.2.12 Terminate of the photon.

### 2.3.8 Monte Carlo simulation

Based on the above conditions, Monte Carlo simulation is performed on the tissue model which incorporates the optical parameters required to calculate, the reflectance $R$ and transmittance $T$. This simulation is included both mismatched boundary conditions and anisotropic scattering, therefore, it is to enhance the realism of the model. Furthermore, the Monte Carlo method in comparison with the deterministic approach of using the diffusion approximation, it can be used to accurately predict the light propagation in tissue. Thus, the tissue model handled by Monte Carlo method can be simulated on a computer where the light propagates in a plurality of layers of tissue by giving the thickness of the layers in each layer $d$, the anisotropy parameter $g$, the
refractive index $n$, the scattering coefficient $\mu_s$ and the absorption coefficient $\mu_a$. If required coefficients are highly accurate the results can be obtained as correct, and it is possible to estimate the spectral reflectance of the tissue at different concentration values. On the other hand, in order to track the movement of light photons one by one in a group, it takes very long calculation time. To improve this drawback, various algorithms have been proposed.

![Tissue model for Monte Carlo simulation.](image)

**Fig.2.13 Tissue model for Monte Carlo simulation.**

### 2.4 Inverse Monte Carlo method

The Inverse Monte Carlo method [36,37] which helps to estimate the absorption coefficient and scattering coefficient from the known reflectance $R$ and transmittance $T$. Equivalent scattering coefficient $\mu'_s$ and the absorption coefficient $\mu'_a$ can be obtained by repeating the procedure, like as Fig.2.14. Firstly, scattering coefficient $\mu'_s$ and the absorption coefficient $\mu'_a$ are assumed, and then calculation of the scattering including absorption performed by using the assumed value, to determine the
reflectance $R$ and transmittance $T$ (calculated forward problem). If the obtained reflectance $R$ and the transmittance $T$ is not consistent with the measured value, re-assumed the scattering coefficient $\mu'_s$ and the absorption coefficient $\mu_a$ and calculated the new transmittance $T$ and reflectance $R$ again. This calculation technique is called inverse problem solution.

Calculating forward problem can be calculated according to the mathematical model which faithfully represents the scattering phenomenon. Several techniques have been established as a mathematical model. In this study, I used a Monte Carlo method for statistical technique. Monte Carlo method is considered the light as a photon which has an energy, it can change the direction collides with the scatter, and also tracking process during lose of energy by absorption. I want to refer to Section 2.3 for details about Monte Carlo method. The reflectance $R$ and transmittance $T$ can be obtained by performing the calculation for the large number of photons (100,000~10,000,000 packets). If changing the wavelength of the incident light is changed the scattering coefficient $\mu'_s$ and the absorption coefficient $\mu_a$, I can obtain the spectrum value.
Furthermore, it is necessary to reduce the thickness of the sample (optical thickness) to measure the transmittance $T$. Therefore in many cases, isotropic scattering approximation does not hold. It needs the scattering phase function $p(\theta)$ in the calculation. Phase function $p(\theta)$ is commonly assumed the anisotropy parameter $g$ as approximately 0.9 in the case of skin tissue.

### 2.5 Multiple regression analysis

The multiple regression analysis [33] is one of the multivariate analysis. This technique obtain unknown variables from known variables. If chosen appropriate variables, it is easy to calculate and make a predictive expression with a few errors.

#### 2.5.1 Regression relationship

When I already know the way to predict the unknown variables $Y$ from the known variables $X$, it is possible to determine the unknown variables $Y$ by the equation (2.23).

$$ Y = f(X) $$  \hspace{1cm} (2.23)

In the equation (2.23), substituting the value of the variable $X$ to the function $f(X)$ of equation (2.23), it is possible to determine the value of the unknown variables $Y$. Where variable $X$ is predictor variables, and variable $Y$ is response variables. However, due to unexpected error factors, equation (2.23) is not established well. After termination of such an error factor, it can be expressed as equation (2.24) by using variable $D$ in error factor.

$$ Y = f(X) + D $$  \hspace{1cm} (2.24)

In the equation (2.24), variable $D$ can changes at random, which may called a residual error variable. Therefore, even if a value of predictor variables $X$ are given, response
variables $Y$ are not decided constantly, and variables $Y$ will have a probabilistic change by variable $D$. Hence, the relationship containing the stochastic variations is called a regression relationship.

$$\hat{Y} = f(X)$$ (2.25)

The equation (2.25) is a regression equation $Y$ by $X$, where $\hat{Y}$ is a predicted value of $Y$ by $X$. For example, I want to know the information about $(Y)$ from available information $(X_1)$ and $(X_2)$, the following equation to the predicted value $\hat{Y}$ holds.

$$\hat{Y} = a_0 + a_1 X_1 + a_2 X_2$$ (2.26)

Where the factor $a_0$, $a_1$ and $a_2$ are valuables.

### 2.5.2 Multiple regression analysis

In many case, the regression equation is prompted to the best fit the data when the event is obtained concerning the phenomenon. The unknown variables $a_0$, $a_1$ and $a_2$ are determined only after a certain phenomenon happened. Regarding the $N$ number of individual $(i=1,2,3,...,N)$, criteria for adaptation of the predicted value and data are set in the situation that reduced as much as possible the error $e_i$ between the observation data $y_i$ of response variables $Y$ and predicted value $\hat{y}_i$ from predictor variables $X$ of observed data by the equation (2.25). The criteria is used the least square criterion which is sum of the squares of the difference in minimum. Thus, the method of obtaining the regression equation from the data means the least squares method. See in the equation (2.27).

$$Q = \sum_{i=1}^{N} (y_i - \hat{y}_i)^2 = \sum_{i=1}^{N} e_i^2$$ (2.27)

The method of obtaining the regression equation from the data by the least squares method, it called the regression analysis. If the N-number of predictor variables $X_1$, $X_2$, $X_3$... , the equation (2.28) is used.
\(X_2, \ldots, X_N\) are present, linear regression equation can be represented by the equation (2.28). This analysis technique called multiple regression analysis.

\[
\hat{Y} = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + \cdots + a_NX_N \tag{2.28}
\]

Where \(a_0\) is a constant term and coefficient according to each explanatory variables are partial regression coefficients. In addition, an index representing the goodness of the true of linear regression equation, correlation coefficients between the predicted value \(\hat{Y}\) and the response variable \(Y\) is used. This correlation coefficients are called a multiple correlation coefficient. If the goodness of the true predictive value of the response variable \(Y\) is considered to be insufficient, it is necessary to devise such further to add or convert the explanatory variables.
Chapter 3.

In vitro study on optical properties of rat cerebral cortical slices

3.1 Introduction

Changes in optical properties of the brain have been used to evaluate spatial and/or temporal changes of neuronal activity and physiological conditions in the brain as intrinsic optical signals (IOSs). It is believed that IOSs in the brain occur mainly by the following three processes: changes in absorption and scattering originating from hemodynamics, changes in absorption due to redox states of cytochromes in mitochondria, and changes in scattering generated by cell swelling or shrinkage caused by water movement between intracellular and extracellular compartments [30].

IOSs also have potential for real-time and continuous monitoring of tissue viability of the brain in clinical situations. Yamashita et al. [45] reported that reduced scattering coefficient of intact brain tissue decreases significantly during hypoxic condition, which are obtained by time-resolved diffuse reflectance measurements. Kawauchi et al. [1] investigated the tissue viability of in vivo bloodless rat brain based on IOSs related to redox changes in cytochrome c oxidase (CcO) in mitochondria and to light scattering, which are derived from steady-state diffuse reflectance measurements at specific wavelengths. Sato et al. [4] reported that the signal intensity in depth profiles of in vivo exposed rat brain measured by optical coherence tomography (OCT) is
significantly changed before cardiac arrest. Because these techniques rely on changes in IOSs related to alterations in scattering and absorption of the tissue itself, it is important to evaluate the degree to which changes in tissue viability affect the optical properties of brain tissue. Optical properties of the brain tissue reported so far, however, have all been derived from post-mortem tissue kept at room temperature [46-48], in which tissue viability is certainly lost. The results may necessarily give neither an accurate representation of in vivo optical properties nor an estimation of changes in optical properties due to this loss of tissue viability.

In contrast, in vitro brain slices perfused with artificial cerebrospinal fluid (aCSF) enable evaluation of changes in the optical properties of living brain tissue, while maintaining tissue viability and without obstructing hemodynamics. IOSs related to scattering properties of brain slices under various physiological conditions have been investigated based on changes in reflectance and transmittance [49-53]. Varying osmolarity, elevated potassium concentration, hypoxia, and electrical stimulation lead to changes in reflectance and transmittance of the slices, to regulate degrees [49]. To simulate the loss of tissue viability and neuronal damage, ischemia like condition induced by oxygen/glucose deprivation (OGD) of aCSF has been applied to in vitro brain slices [10,11,21,22]. Tao et al. [54] have investigated back scattered light intensity from layer III-V of somatosensory cortex region in rat brain slices during OGD by use of a photon counting fiber optic system. Mean percent change in the reflected light intensity of brain tissue slice at the wavelength of 500 nm significantly decreased under OGD condition. Andrew et al. [55] have reported that light transmittance change of cell body region in hippocampal slice during OGD remains increase, whereas that of dendrite region increases initially and then decreases irreversibly. Histological and
electrophysiological features attributed the changes in IOSs related to the light scattering during OGD to acute neuronal damage due to alteration of cellular or subcellular structure and loss of tissue viability, respectively [34]. However, to the best of our knowledge, no report has investigated reduced scattering coefficients $\mu_s'(\lambda)$ and absorption coefficients $\mu_a(\lambda)$ of living, healthy brain tissue slices and their changes due to loss of tissue viability.

In Chapter 3, I present the simultaneous measurement of the diffuse reflectance spectrum and transmittance spectrum for *in vitro* healthy rat brain slices perfused with aCSF at 35°C. The spectra of $\mu_s'(\lambda)$ and $\mu_a(\lambda)$ for the brain slices are estimated based on the Inverse Monte Carlo method. Changes in $\mu_s'(\lambda)$ and $\mu_a(\lambda)$ of the brain slices are also demonstrated to simulate loss of viability in living brain tissue during OGD of aCSF.

### 3.2 Materials and methods

#### 3.2.1 Brain slice preparation

All experiments were performed under a research protocol approved by the Animal Research Committee of Tokyo University of Agriculture and Technology. Seven male Wistar rats, 28-35 days old, were housed in a controlled environment (24°C, 12-h light/dark cycle) with food and water *ad libitum*. Each rat was decapitated using a guillotine, and the brain was quickly removed and placed in cold, oxygenated (95% O$_2$-5% CO$_2$) aCSF. Using a vibratome, coronal slices were cut at 400 $\mu$m and placed in the cold aCSF. Figure 3.1 shows a photograph of a rat cerebral cortical slice. Twelve cerebral cortical slices in total were prepared and each slice was then hemisected along the midline and equilibrated in the aCSF at room temperature for at least 1 h prior to
data acquisition. The aCSF was prepared by dissolving in double-distilled water (in mM) 120 NaCl, 3.3 KCl, 26 NaHCO₃, 1.3 MgSO₄×7H₂O, 1.2 NaH₂PO₄, 11 D-glucose, and 1.8 CaCl₂ (pH 7.3-7.4). Oxygen/glucose deprivation, which simulates ischemia in vitro, was induced by reducing aCSF glucose from 11 to 0 mM and gassing the aCSF with 95% N₂-5% CO₂. NaCl was added to the aCSF to balance osmolarity.

![Region of interest](image)

Fig.3.1 Typical photograph of a rat cerebral cortical slice perfused by artificial cerebrospinal fluid (aCSF).

### 3.2.2 Experimental setup

Figure 3.2 schematically shows an experimental setup used in this study. Each brain slice was placed individually in a recording chamber (RC-27NE, Warner Instruments LLC, Hamden, CT, USA) with a glass cover slip as the base and supported
using a slice tissue anchor. The brain slice was submerged in oxygenated aCSF flowing at a rate of 3 ml/min. A slide glass was placed directly above the slice so that the level of fluid above the slice remained constant. The temperature of the flowing aCSF was raised by 2\(^{\circ}\)C every 5 min to 35\(^{\circ}\)C in all experiments. A halogen lamp light (HL-2000, Ocean Optics Inc., Dunedin, FL, USA) which covers the visible-to-near-infrared wavelength range, was used to illuminate the sample perpendicularly via an optical fiber with a core diameter of 400 \(\mu m\) and an achromatic lens. The illuminating area of the sample was circular, with a diameter of 400 \(\mu m\), which corresponds to layers III-V of the primary somatosensory cortex region (gray matter), as shown in Figure 3.1. Diffusely reflected light from the illuminated area was collected by an achromatic lens and coupled to a detection fiber with a core diameter of 400 \(\mu m\). The detection angle was 45\(^{\circ}\), to exclude the specular component of the reflected signal. Diffusely transmitted light from the brain slice entered into the integrating sphere (SPH-1-4,
SphereOptics LLC, Concord, NH, USA) via the sampling port (diameter, 6.35 mm) and was received at the input face of a detection fiber (diameter, 400 μm) placed at the detector port of the sphere. Detection fibers for reflectance and transmittance delivered the received light into two different multichannel spectrometers (USB4000, Ocean Optics Inc.) under the control of a personal computer. A white silicone chip (thickness, 400 μm, of which the reflectance spectrum was calibrated by the Spectralon white standard, was prepared as a reference object. The diffuse reflectance spectrum \( R(\lambda) \) of the brain slice was calculated based on measurement of the reflected intensities of the white silicone chip. The transmittance spectrum \( T(\lambda) \) of the brain slice was calculated based on measurement of transmitted intensity through the recording chamber without the brain slice. Measurements of \( R(\lambda) \) and \( T(\lambda) \) were obtained simultaneously in the wavelength range from 500 to 900 nm at 30-s intervals for 45 min. The inflow of aCSF under the OGD condition into the recording chamber started at 2.5 min after initiation of the measurements.

### 3.2.3 Estimation of scattering and absorption coefficients

To calculate \( \mu' (\lambda) \) and \( \mu_a (\lambda) \) from measured reflectance and transmittance spectra, I employed the Inverse Monte Carlo method (IMC), using the Monte Carlo simulation code developed by Wang et al., [11] in which the Henyey-Greenstein phase function is applied to sampling of the scattering angle of photons. In the present study, the source code was partly modified for iterative calculation. In these calculations, the geometry of the optical setup and radiation losses were taken into account. The refractive index \( n(\lambda) \) and the anisotropy factor \( g(\lambda) \) were assumed to be 1.4 and 0,
respectively, in the whole wavelength range, and the layer thickness was set as 400 \( \mu \)m. The Monte Carlo calculation of diffuse reflectance and transmittance spectra was iterated for varying values of \( \mu_s'(\lambda) \) and \( \mu_o(\lambda) \) until the difference between the simulated and measured spectral values decreased below a predetermined threshold. The values of \( \mu_s'(\lambda) \) and \( \mu_o(\lambda) \) used in the last iteration step were adopted as the final results. This process was carried out at intervals of 5 nm from 500 to 900 nm, and the spectra of \( \mu_s'(\lambda) \) and \( \mu_o(\lambda) \) were obtained at each time point. To evaluate the magnitude of signal \( S \) induced by OGD, I calculated the change of the signal based on the time series data. The signal immediately before OGD onset 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 signal \( S - S_c \) over time, was normalized by dividing by \( S_c \). The change of the signal is expressed as \( \Delta S/S_c = (S - S_c)/S_c \). The above calculation was applied to the time series of \( \mu_s'(\lambda) \) and \( \mu_o(\lambda) \).

### 3.3 Results and discussion

The typical diffuse reflectance spectra of (a) \( R(\lambda) \) and (b) transmittance spectra \( T(\lambda) \), obtained from a rat cerebral cortical slice under the normal (immediately before OGD onset) and OGD conditions (at 12.5 min after OGD onset) are shown in Figure 3.3. For each condition, \( R(\lambda) \) and \( T(\lambda) \) show a decrease and increase, respectively, with the wavelength. The magnitudes of the increase of \( T(\lambda) \) and the decrease of \( R(\lambda) \) were greater for the OGD condition. These trends of change in \( R(\lambda) \) and \( T(\lambda) \) are similar to those reported previously regarding cerebral cortical slices during OGD [34,54]. Small peaks at 520, 550, and 605 nm for both of \( R(\lambda) \) and \( T(\lambda) \) are attributable to absorption of
bands of cytochrome $b+c$, cytochrome $c$, and heme $aa_3$ in CcO, respectively, \[56, 57\]; these peaks clearly differentiate the normal and OGD conditions.
The typical estimated values of (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s'(\lambda)$ of rat cerebral cortical slice for the normal and OGD conditions are shown in Figure 3.4. It is noted that the peak at 550 nm becomes more remarkable and that two shoulders appear at around 520 and 605 nm in the spectra of $\mu_a(\lambda)$ obtained from the OGD slices. Those changes in $\mu_a(\lambda)$ at 520, 550, and 605 nm indicate reductions in $\beta$-bands of cytochrome $b + c$, cytochrome $c$, and heme $aa_3$ in CcO, respectively, [56,57] in the OGD condition. Decrease in broad absorption centered at 830 nm is due to the reduction of copper (CuA) in CcO [56,57]. The reduced scattering coefficients $\mu_s'(\lambda)$ noted for both conditions have a broad scattering spectrum, exhibiting larger magnitude at shorter wavelengths. Table.3.1 shows the mean estimated values of absorption coefficient $\mu_a(\lambda)$ and reduced scattering coefficient $\mu_s'(\lambda)$ obtained from twelve rat cerebral cortical slices for the normal (immediately before OGD onset) and OGD conditions (at 12.5 min after OGD onset). It should be noted that scattering for normal healthy tissue is significantly larger compared to that for the OGD condition. The spectral feature in $\mu_s'(\lambda)$ for the OGD condition is comparable with that previously obtained for gray matter in post-mortem brain slices [46,48].
Table 3.1 Reduced scattering coefficients $\mu_s$ (cm$^{-1}$) and absorption coefficients $\mu_a$ (cm$^{-1}$) for twelve rat cerebral cortical slices for the normal and OGD conditions.

<table>
<thead>
<tr>
<th>$\lambda$(nm)</th>
<th>$\mu_s$(cm$^{-1}$)</th>
<th>$\mu_a$(cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>OGD</td>
</tr>
<tr>
<td>500</td>
<td>43.7 ± 9.2</td>
<td>29.4 ± 7.6</td>
</tr>
<tr>
<td>510</td>
<td>42.6 ± 8.7</td>
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<tr>
<td>520</td>
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<td>27.5 ± 6.9</td>
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<td>38.0 ± 7.1</td>
<td>25.2 ± 6.0</td>
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<tr>
<td>560</td>
<td>36.7 ± 7.1</td>
<td>24.4 ± 5.9</td>
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<td>10.6 ± 1.3</td>
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*Values are means ± SD.*
Fig. 3.5 Typical time courses of the changes in the absorption coefficients $\Delta \mu_a/\mu_{a,c}$ and the scattering coefficients $\Delta \mu_s/\mu_{s,c}$ of a rat cerebral cortical slices (a) at four specific wavelengths of 520, 550, 605, and 830 nm for 45 min. (b) Enlarged view of a part enclosed by dashed square in (a) for 605 and 830 nm.

Typical time courses of the changes in the absorption coefficients $\Delta \mu_a/\mu_{a,c}$ and the reduced scattering coefficients $\Delta \mu_s/\mu_{s,c}$ at four specific wavelengths, 520, 550, 605, and 830 nm, are shown in Figure 3.5 (a). The values of $\Delta \mu_a/\mu_{a,c}$ at 520, 550, and 605 nm showed a gradual increase. The decrease of $\Delta \mu_a/\mu_{a,c}$ at 830 nm is probably due to
reduction of CuA in CcO. The value of $\Delta \mu_a/\mu_{a,c}$ at each wavelength reached a maximum amplitude at almost 12.5 min after onset of OGD. Enlarged view of a part enclosed by dashed square in Figure 3.5 (a) for 605 and 830 nm are shown in Figure 3.5 (b). It should be noted that the change in $\Delta \mu_a/\mu_{a,c}$ at 605 nm was triphasic, with a rapid phase (Phase I, 2.5-3.0 min), a slow phase (Phase II, 3.0-5.5 min), and a second rapid phase (Phase III, 5.5-8.0 min), whereas decrease in $\Delta \mu_a/\mu_{a,c}$ at 830 nm was monophasic. The phasic change in $\Delta \mu_a/\mu_{a,c}$ at 605 nm and that at 830 nm correspond to the redox behavior of heme $aa_3$ and that CuA observed in in vivo rat perfused brain under the ischemia-like condition, respectively [57]. Changes in the reduced scattering coefficient $\Delta \mu_s/\mu_{s,c}$ for each wavelength were significantly decreased by OGD, reaching a maximum amplitude at almost 12.5 min after OGD onset. After reaching a plateau, $\Delta \mu_s/\mu_{s,c}$ began to increase slightly again until the end of the measurement. The mean maximum amplitudes of $\Delta \mu_a/\mu_{a,c}$ at 520, 550, 605, and 830 nm were 0.33±0.14, 0.30±0.12, 0.30±0.14, and −0.04±0.16, respectively, whereas these of $\Delta \mu_s/\mu_{s,c}$ at 520, 550, 605, and 830 nm were −0.37±0.08, −0.38±0.08, −0.38±0.08, and −0.39±0.08 ($n=12$).

Mitochondrial respiration is inhibited during the ischemia-like condition by the rapid drop in O$_2$ tension, which causes depletion of the adenosine triphosphate (ATP). The initial rapid phase of change in absorbance at 605 nm during ischemia-like condition is independent of the energy state, i.e., ATP level, while the slow phase and second rapid phase depend on the reduction of heme $aa_3$ accompanied by a decrease in the energy change of the adenylate pool [58]. Hence, the slow phase (Phase II) and second rapid phase (Phase III) in $\Delta \mu_a/\mu_{a,c}$ at 605 nm shown in Fig.3.5 (b) probably
reflect the reduction of heme $aa_3$ due to the decrease of ATP. On the other hand, the
degree of the redox state of CuA depends on the tissue oxygen concentration and it
closely correlates with cerebral ATP level [57,59]. Therefore, the monophasic change in
$\Delta \mu_\sigma/\mu_{a,c}$ at 830 nm is indicative of the decreases of oxygen concentration and cerebral
ATP level during OGD. It should also be noted that the values of $\Delta \mu_\sigma'/\mu_{b,c}$ at each
wavelength begin to decrease significantly, after onset of the Phase II ($t = 3$ min) in
$\Delta \mu_\sigma/\mu_{a,c}$ at 605 nm. In other words, the light scattering decreases with the reduction of
heme $aa_3$ or decrease of ATP. Reduced ATP production due to the inhibition of
mitochondrial respiration leads to failure of the $Na^+/K^+$ATPase pump [60]. In such a
case, extracellular $Na^+$, $Cl^-$, and $Ca^{2+}$ rush in, with water following osmotically, causing
cell swelling that shrinks the extracellular space [34,53]. Thus, the significant decreases
in $\mu_\sigma' (\lambda)$ shown in Figures 3.4 and 3.5 are most likely caused by cell swelling due to
failure of the $Na^+/K^+$ATPase pump. The minor increases in $\mu_\sigma' (\lambda)$ after $t=15$ min is
probably caused by the dendritic beading effect, which develops even as the tissue
continues to swell, and is indicative of neuronal damage [34]. 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
transmitted light intensity [61-63].

I assumed refractive index $n$ is 1.4 and the slice thickness is 400 $\mu$m in
the IMC for both normal and OGD conditions. However, cell swelling will
cause the tissue thickness to increase slightly. In such a case, the tissue
thickness assumed in the IMC will be thinner than the actual thickness under
OGD condition. If the values of $\mu_\sigma', \mu_\sigma$ and $n$ are constant, the diffuse
reflectance and transmittance are decreased and increased, respectively, as the
tissue thickness decreases. Therefore, the IMC for OGD condition may overestimate the values of $\mu_e'$ to compensate these differences in diffuse reflectance and transmittance. Cell swelling will also cause the tissue refractive index to match the surrounding aCSF. The incident light from surrounding aCSF to tissue will be increased as the refractive index of tissue approaches that of surrounding aCSF. In this case, both of diffuse reflectance and transmittance will somewhat increase if the values of $\mu_e'$, $\mu_a$ and tissue thickness are unchanged. As a consequence, the IMC for OGD condition may overestimate the values of $\mu_a$ to make up for these differences in diffuse reflectance and transmittance. The results for OGD condition may include these two effects due to cell swelling. This needs further investigation in the future works.

3.4 Conclusions

In summary, I presented reduced scattering coefficients $\mu_e'(\lambda)$ and absorption coefficients $\mu_a(\lambda)$ of rat cerebral cortical tissue slices perfused with aCSF. The magnitudes of differences in $\mu_e'(\lambda)$ and $\mu_a(\lambda)$ between the normal and ischemia-like conditions were demonstrated. The spectral data of $\mu_a(\lambda)$ and $\mu_e'(\lambda)$ obtained from normal slices perfused with aCSF with glucose at 35°C shown in Table.3.1 provide a more accurate representation of in vivo optical properties than the data obtained from post mortem brain tissue that have been reported to date. The mean maximum amplitudes of changes in the absorption coefficient $\Delta\mu_a/\mu_{a,c}$ at 520, 550, 605, and 830 nm were 0.33±0.14, 0.30±0.12, 0.30±0.14, and −0.04±0.16, respectively, whereas these
in the reduced scattering coefficient $\Delta\mu_s'/\mu_{s,c}'$ at 520, 550, 605, and 830 nm were $-0.37\pm0.08$, $-0.38\pm0.08$, $-0.38\pm0.08$, and $-0.39\pm0.08$. The variations in $\mu_s'(\lambda)$ during OGD imply cell deformation, whereas changes of $\mu_e(\lambda)$ indicate reductions of heme $aa_3$ and CuA in CcO and cytochrome $c$. The results presented in this paper may be useful for evaluating IOSs related to loss of tissue viability in the \textit{in vivo} intact brain.
Chapter 4.

In vivo multispectral imaging of scattering and absorption properties of exposed rat brain

4.1 Introduction

Determining the optical properties of brain tissue is important for application of light in clinical diagnosis, surgery, and therapeutic procedures for brain disorders. Changes in the optical properties of the brain have been used to deduce spatial and/or temporal changes of neuronal activity and tissue viability in the brain as intrinsic optical signals (IOSs). It is believed that IOSs in the brain are mainly caused by the following three processes: hemodynamic-related changes in absorption and scattering, changes in absorption due to redox states of cytochromes in mitochondria, changes in scattering generated by cell swelling or shrinkage due to the movement of water between intracellular and extracellular compartments [30], and changes in scattering and absorption caused by chromophore content and cell deformation. 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 slices [64] based on the several light transport models such as the Kubelka–Munk theory [65], the diffusion approximation to the transport equation [66], the Monte Carlo method [11], and the adding-doubling method [67]. Numerous spectroscopic methods have been studied for in vivo determination of the scattering and absorption properties in living
tissues, including time-resolved measurements [13], a frequency-domain method [22], and spatially resolved measurements with continuous wave (CW) light [15-21].

Diffuse reflectance spectroscopy (DRS) with CW light can be simply achieved with a white light source, inexpensive optical components, and a spectrometer. DRS is one of the most promising techniques for evaluating the optical properties of in vivo brain tissue. Several imaging techniques based on DRS have been used to investigate cortical hemodynamics based on changes in the absorption properties of brain tissue [68-70]. In observations of the brain surface using multispectral imaging based on CW light, the scattering properties of brain tissue have often been evaluated using the diffuse reflectance in the near-infrared wavelength region. Although the absorption of light by hemoglobin in the near-infrared wavelength region is much smaller than that in the visible wavelength region, hemodynamic-related changes in the absorption properties of brain tissue can lead to incorrect estimation of the scattering properties. On the other hand, several methods with CW light have been used to estimate the scattering and absorption properties of brain tissue separately [68,71,72]. The reduced scattering coefficients $\mu'_s$ of brain tissue in the visible to near-infrared wavelength region have a broad scattering spectrum, exhibiting larger magnitude at shorter wavelengths. Therefore, the spectrum of the reduced scattering coefficient $\mu'_s(\lambda)$ was assumed to be in the following form of the power law function [73,74]:

$$\mu'_s(\lambda) = a\lambda^{-b},$$  \hspace{1cm} (4.1)

It has been reported that the scattering amplitude $a$ and the scattering power $b$ are related to the scatterer density and the scatterer size, respectively [73,74]. Several approaches to evaluate the scattering properties of brain tissue with diffuse reflectance spectra focus on the change in the scattering amplitude $a$ under the assumption that the
scattering power \( b \) is constant \([68,71,73,74]\). However, morphological alterations of tissues due to pathophysiological changes and loss of tissue viability in living brain tissue can affect the size and density of biological scatters \([34,55,60-63]\). Therefore, it is important to estimate both the scattering amplitude \( a \) and the scattering power \( b \) quantitatively to evaluate the scattering properties of brain tissue.

In Chapter 4, I proposed a simple spectral imaging of reduced scattering coefficients \( \mu'_e(\lambda) \) and the absorption coefficients \( \mu_a(\lambda) \) of \textit{in vivo} exposed brain tissues in the range from visible to near-infrared wavelengths based on DRS using a multispectral imaging system. Multispectral diffuse reflectance images of \textit{in vivo} exposed brain are acquired at nine wavelengths (500, 520, 540, 560, 570, 580, 600, 730, and 760 nm) by a 16-bit cooled charge-coupled device (CCD) camera with a motorized filter wheel system. The Monte Carlo simulation (MCS)-based multiple regression analysis for the absorbance spectra is then used to specify the concentrations of oxygenated and deoxygenated hemoglobin, scattering amplitude, and scattering power. The absorption coefficient spectrum and the reduced scattering coefficient spectrum are finally reconstructed from the hemoglobin concentrations and the scattering parameters, respectively. Experiments with optical phantoms validated the proposed method. To confirm the possibility of the method to evaluate changes in absorption and scattering properties of the cerebral cortex, I performed \textit{in vivo} experiments with exposed rat brain during normoxia, hyperoxia, and anoxia.
4.2 Materials and Methods

4.2.1 The proposed algorithm for estimating absorption and scattering spectra

Figure 4.1 shows a flow diagram of the proposed method. The absorbance spectrum \( A(\lambda) \) is defined as

\[
A(\lambda) = -\log_{10} r(\lambda),
\]  
(4.2)

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

\[
A(\lambda) = C_{\text{HbO}} I(\lambda, C_{\text{HbO}}, C_{\text{HbR}}, \mu'_t) e_{\text{HbO}}(\lambda) + C_{\text{HbR}} I(\lambda, C_{\text{HbO}}, C_{\text{HbR}}, \mu'_t) e_{\text{HbR}}(\lambda) + D(\lambda, \mu'),
\]  
(4.3)

where \( C \) is the concentration, \( l \) is the mean path length, \( \alpha(\lambda) \) is the extinction coefficient, and \( D(\lambda, \mu') \) indicates attenuation due to light scattering in the tissue. Subscripts \( \text{HbO} \) and \( \text{HbR} \) denote oxygenated hemoglobin and deoxygenated hemoglobin, respectively. The absorption coefficient of the cortical tissue was assumed to depend only on the concentrations of \( \text{HbO} \) and \( \text{HbR} \) \( (C_{\text{HbO}}, C_{\text{HbR}}) \) as

\[
\mu_a(\lambda) = C_E(\lambda) = C_{\text{HbO}} e_{\text{HbO}}(\lambda) + C_{\text{HbR}} e_{\text{HbR}}(\lambda).
\]  
(4.4)

Total hemoglobin concentration \( C_{\text{HbT}} \) is defined as the sum of \( C_{\text{HbO}} \) and \( C_{\text{HbR}} \):

\[
C_{\text{HbT}} = C_{\text{HbO}} + C_{\text{HbR}}.
\]  
(4.5)

Tissue oxygen saturation is determined as

\[
\text{StO}_2 = 100 \times \frac{C_{\text{HbO}}}{C_{\text{HbT}}},
\]  
(4.6)
The reduced scattering coefficient of the brain tissue was assumed to be in the form of Eq. (4.1). Using $A(\lambda)$ as the response variable and $\alpha(\lambda)$ as the predictor variables, the multiple regression analysis based on the modified Lambert–Beer law (MRA1) can be applied to Eq. (4.3) as

$$A(\lambda) = \alpha_{HbO} \varepsilon_{HbO}(\lambda) + \alpha_{Hbr} \varepsilon_{Hbr}(\lambda) + \alpha_0,$$

(4.7)
where $\alpha_{HbO}$, $\alpha_{HbR}$, and $\alpha_0$ are the regression coefficients. The regression coefficients $\alpha_{HbO}$ and $\alpha_{HbR}$ describe the degree of contribution of each extinction coefficient to $A(\lambda)$ and are closely related to the concentrations $C_{HbO}$ and $C_{HbR}$, respectively. The regression coefficient $\alpha_0$ is expressed as

$$\alpha_0 = \bar{A} - \bar{\varepsilon}_{HbO}\alpha_{HbO} - \bar{\varepsilon}_{HbR}\alpha_{HbR},$$

(4.8)

where $\bar{A}$, $\bar{\varepsilon}_{HbO}$, and $\bar{\varepsilon}_{HbR}$ are the averages of $A(\lambda)$, $\varepsilon_{HbO}(\lambda)$, and $\varepsilon_{HbR}(\lambda)$ over the wavelength range, respectively. $\alpha_0$ represents the bias component of $A(\lambda)$. Thus, $\alpha_0$ describes the degree of contribution of the attenuation due to light scattering in the brain to the absorbance spectrum $A(\lambda)$ and hence it is related to the coefficient $a$ and the exponent $b$ in Eq. (4.1). At the same time, $\alpha_0$ is also affected by the absorption coefficient of the brain, since $A(\lambda)$ is generally a function of the tissue absorption coefficient and reduced scattering coefficient.

To investigate the relationship between the regression coefficients and the values of $C_{HbO}$, $C_{HbR}$, $a$, and $b$, I performed MCS for the diffuse reflectance from the rat cortical tissue through the skull at $\lambda = 500, 520, 540, 560, 570, 580, 600, 730, \text{ and } 760$ nm under the various values of $C_{HbO}$, $C_{HbR}$, $a$, and $b$. I used the MCS source code developed by Wang et al., [11] in which the Henyey–Greenstein phase function is applied to 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 launched into the medium with the scattering and absorption properties. Photons were propagated into the medium under the scattering and absorption. Then, some portion of the scattered light comes back from the medium, and finally, is emitted from the surface of the medium. The absorption
coefficients of oxyhemoglobin $\mu_{a,HbO}(\lambda)$ and deoxyhemoglobin $\mu_{a,HbR}(\lambda)$ were obtained from the values of $\varepsilon_{HbO}(\lambda)$, and $\varepsilon_{HbR}(\lambda)$ in the literature [64], where the hemoglobin concentration of blood, having a 44% hematocrit with 150 g/L of hemoglobin, was converted to 100% volume concentration (100 vol.% of hemoglobin. For the reduced scattering coefficients, the values of scattering amplitude $a$ were 60,258, 80,344, 100,430, 120,516, and 180,774 in the simulation, which were derived by multiplying the typical value [75] of scattering amplitude $a$ by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively. The values of scattering power $b$ were 1.2442, 1.31332, 1.3824, 1.45156, and 1.52068, which were derived by multiplying the typical value [75] of scattering power $b$ by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively. The reduced scattering coefficients $\mu_r(\lambda)$ of the cortical tissue were obtained from Eq. (4.1). The sum of the absorption coefficients of oxyhemoglobin and deoxyhemoglobin, $\mu_{a,HbO}(\lambda)$ and $\mu_{a,HbR}(\lambda)$, represents the absorption coefficient of total hemoglobin $\mu_{a,HbT}(\lambda)$; the values for $C_{HbT} = 0.2$, 1.0, and 5.0 vol.% 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 thicknesses of the cortical tissue were set to 5.0 mm.

Figures 4.2(a) and 4.2(b) show the values of $\alpha_{HbO}$ and $\alpha_{HbR}$ versus the volume concentrations of oxyhemoglobin $C_{HbO}$ and deoxyhemoglobin $C_{HbR}$, respectively, under the different conditions of $a$, obtained from the MCS. Figures 4.2(c) and 4.2(d) show the values of $\alpha_{HbO}$ and $\alpha_{HbR}$ versus the volume concentrations of oxyhemoglobin $C_{HbO}$ and deoxyhemoglobin $C_{HbR}$, respectively, under the different conditions of $b$, obtained from the MCS. In both Fig. 4.2(a) and Fig. 4.2(c), the value of $\alpha_{HbO}$ increases with the increase of $C_{HbO}$. Moreover, the value of $\alpha_{HbO}$ changes with the increase of the values of
$a$ and $b$. The same tendency can be seen for $\alpha_{HbR}$ shown in both Fig. 4.2(b) and Fig. 4.2(d). Figures 4.2(e) and 4.2(f) show the values of $\alpha_0$ versus the values of $a$ and those of $b$, respectively, for the different values of $C_{HbO}$ and $C_{HbR}$. The value of $\alpha_0$ decreases with the increase in the value of $a$. Moreover, the value of $\alpha_0$ increases with the increases of $C_{HbO}$ and $C_{HbR}$. On the other hand, the value of $\alpha_0$ increases with the increase of the value of $b$. Therefore, it is clear that the regression coefficients $\alpha_{HbO}$, $\alpha_{HbR}$, and $\alpha_0$ are related to the volume concentration of oxyhemoglobin $C_{HbO}$, that of deoxyhemoglobin $C_{HbR}$, the coefficient $a$, and the exponent $b$, respectively; however, $C_{HbO}$, $C_{HbR}$, $a$, and $b$ are not determined by a unique regression coefficient when using only MRA1.

Thus, I used further multiple regression analyses to estimate the values of $C_{HbO}$, $C_{HbR}$, $a$, and $b$ based on the combination of regression coefficients $\alpha_{HbO}$, $\alpha_{HbR}$, and $\alpha_0$ that were obtained from MRA1. In this analysis, $C_{HbO}$, $C_{HbR}$, and $b$ were regarded as response variables, and the three regression coefficients $\alpha_{HbO}$, $\alpha_{HbR}$, and $\alpha_0$ in Eq. (4.7) were regarded as predictor variables to determine the regression equations for $C_{HbO}$, $C_{HbR}$, and $b$. The regression equations for $C_{HbO}$, $C_{HbR}$, and $b$ are written as

$$C_{HbO} = \beta_{HbO} \cdot \alpha_1,$$

$$C_{HbR} = \beta_{HbR} \cdot \alpha_1,$$

$$b = \beta_b \cdot \alpha_1,$$

$$\alpha_1 = [1, \alpha_{HbO}, \alpha_{HbR}, \alpha_0]^T,$$

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

$$\beta_{HbR} = [\beta_{HbR,0}, \beta_{HbR,1}, \beta_{HbR,2}, \beta_{HbR,3}].$$
Fig. 4.2 Regression coefficients versus concentrations of oxygenated hemoglobin $C_{HbO}$ and deoxygenated hemoglobin $C_{HbR}$, scattering amplitude $a$, and scattering power $b$, obtained from the Monte Carlo simulations. (a) Regression coefficient $\alpha_{HbO}$ versus concentration $C_{HbO}$ under the different conditions of $a$. (b) Regression coefficient $\alpha_{HbR}$ versus concentration $C_{HbR}$ under the different conditions of $a$. (c) Regression coefficient $\alpha_{HbR}$ versus concentration $C_{HbO}$ under the different conditions of $b$. (d) Regression coefficient $\alpha_{HbR}$ versus concentration $C_{HbR}$ under the different conditions of $b$. (e) Regression coefficient $\alpha_0$ versus scattering amplitude $a$ under the different conditions of $C_{HbO}$ and $C_{HbR}$. (f) Regression coefficient $\alpha_0$ versus scattering power $b$ under the different conditions of $C_{HbO}$ and $C_{HbR}$.

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

(4.15)

The symbol $[\cdot]^T$ represents the transposition of a vector. I called this analysis MRA2. On the other hand, in the preliminary investigation, I found that adding $b$ to the predictor variables can improve the accuracy of the estimation of $a$. Therefore, $b$, $\alpha_{HbO}$, $\alpha_{HbR}$, and $\alpha_0$ are regarded as the predictor variables, and given values of $a$ are regarded as the
response variables for determining the regression equation for \(a\). The regression equation for \(a\) is written as

\[
a = \mathbf{a}_2 \cdot \mathbf{b}^T,
\]

(4.16)

\[
\mathbf{a}_2 = [1, \alpha_{\text{HbO}}, \alpha_{\text{HbR}}, \alpha_0, b]^T,
\]

(4.17)

\[
\mathbf{b} = [\beta_{a,0}, \beta_{a,1}, \beta_{a,2}, \beta_{a,3}, \beta_{a,4}].
\]

(4.18)

I called this analysis MRA3. The coefficients \(\beta_{\text{HbO},j}\), \(\beta_{\text{HbR},j}\), \(\beta_{h,j}\) \((j = 0, 1, 2, 3)\) and \(\beta_{a,k}\) \((k = 0, 1, 2, 3, 4)\) are unknown and must be determined before the estimations of \(C_{\text{HbO}}\), \(C_{\text{HbR}}\), \(b\), and \(a\).

I adopted MCS as the foundation to establish reliable values of \(\beta_{\text{HbO},j}\), \(\beta_{\text{HbR},j}\), \(\beta_{h,j}\), and \(\beta_{a,k}\). The simulation model used in this part also consisted of a single cortical tissue layer. The absorption coefficients \(\mu_a(\lambda)\) converted from the concentrations \(C_{\text{HbO}}\) and \(C_{\text{HbR}}\) and the reduced scattering coefficient \(\mu_s'(\lambda)\) specified by the coefficient \(a\) and the exponent \(b\) were provided as inputs to the simulation, while the diffuse reflectance spectrum \(r(\lambda)\) was produced as output. The input values of \(C_{\text{HbO}}, C_{\text{HbR}}, a,\) and \(b\), and the output reflectance spectra are helpful as the data set in specifying the values of \(\beta_{\text{HbO},i}\), \(\beta_{\text{HbR},i}\), \(\beta_{h,i}\), and \(\beta_{a,j}\) statistically 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 \([75]\) of \(a\) by 0.5, 0.75, 1.0, 1.25, and 1.5, respectively, whereas the five different values of 1.2442, 1.31332, 1.3824, 1.45156, and 1.52068 were calculated by multiplying the typical value \([75]\) 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. (4.1). The sum of the absorption coefficients of oxyhemoglobin and deoxyhemoglobin \(\mu_{a,\text{HbO}}(\lambda) + \mu_{a,\text{HbR}}(\lambda) =\)
\(\mu_{a,HbT}(\lambda)\) for \(C_{HbT} = 0.2, 1.0, \) and 5.0 vol.\% were used as input to the cortical tissue in the simulation. Tissue oxygen saturation \(StO_2\) was determined by \(\mu_{a,HbO}(\lambda)/\mu_{a,HbT}(\lambda)\), and values of 0\%, 20\%, 40\%, 60\%, 80\%, and 100\% were used for the simulation. For the refractive index of the cortical layer, the same values as described above were again employed. In total, 450 diffuse-reflectance spectra at \(\lambda = 500, 520, 540, 560, 570, 580, 600, 730,\) and 760 nm were simulated under the various combinations of \(C_{HbO}, C_{HbR}, a,\) and \(b.\) MRA1 for each simulated spectrum based on Eq. (4.7) generated the 450 sets of vector \(\alpha_1\) and concentrations \(C_{HbO}, C_{HbR}, b,\) and the 450 sets of vector \(\alpha_2\) and \(a.\) The coefficient vectors \(\beta_{HbO}, \beta_{HbR},\) and \(\beta_{b} \) were determined statistically by performing MRA2, while the coefficient vector \(\beta_{HbO}\) was determined statistically by performing MRA3. Once \(\beta_{HbO}, \beta_{HbR}, \beta_{b},\) and \(\beta_{b}\) were obtained, \(C_{HbO}, C_{HbR}, a,\) and \(b\) were calculated from \(\alpha_{HbO}, \alpha_{HbR},\) and \(\alpha_{b},\) which were derived from MRA1 for the measured reflectance spectrum, without the MCS. Therefore, the spectrum of reduced scattering coefficient \(\mu_s'(\lambda)\) and that of absorption coefficient \(\mu_a(\lambda)\) were reconstructed by Eqs. (4.1) and (4.4), respectively, from the measured reflectance spectrum.

### 4.2.2 Imaging system

Figure 4.3(a) shows a schematic illustration of the imaging system. A 150-W halogen-lamp light source (LA-150SAE; Hayashi Watch Works Co., Ltd, Tokyo, Japan) illuminated the surface of the sample via a light guide (LGC1-5L1000; Hayashi Watch Works Co., Ltd) and a collecting lens (SH-F16; Hayashi Watch Works Co., Ltd). The light source covers a range from the visible to near-infrared wavelengths. The angle of illumination is approximately 45\(^\circ\) with respect to the sample surface. By using nine
interference filters having the center wavelengths of 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm with 10 nm spectral full-width at half-maximum (#65088, #65093, #65096, #67766, #67767, #65646, #65102, #65115, and #67777, Edmund Optics Japan Ltd, Tokyo, Japan) mounted in a motorized filter wheel (FW-MOT-12.5; Andover Corporation, NH, USA), diffuse reflectance images of 772 × 580 pixels at the nine wavelengths were acquired by a 16-bit cooled CCD camera (BS-40, Bitran, Japan) and a video zoom lens (VZM™300i; Edmund Optics Japan Ltd, Tokyo, Japan). The

Fig. 4.3 (a) Schematic illustration of the imaging system and (b) the representation of the rat skull with a reflectance image acquired at 500 nm.
spectrum of reflected intensity from the sample was normalized by that from a spectralon white standard with 99% reflectance (SRS-99-020; Labsphere Incorporated, North Sutton, NH, USA). The normalized spectrum was treated as the diffuse reflectance spectrum. The integration time of the CCD camera for each reflectance image was <0.5 s. The access time for adjacent filters is 0.8 s. Therefore, it is possible to acquire a set of nine images within 10 s. The integration time can be reduced by optimizing the arrangement of the optical system. The field of view of the system was 2.97 × 2.23 mm² with 772 × 580 pixels. The lateral resolution of the images was estimated to be 3.85 μm. The multispectral reflectance images were stored in a personal computer and analyzed according to the estimation process shown in Fig. 4.1.

4.2.3 Validation using tissue-like optical phantoms

To confirm the validity of the proposed method, I performed experiments using tissue-like optical phantoms. I prepared agar solution by diluting agarose powder (Fast Gene AG01; NIPPON Genetics EUROPE GmbH, Düren, NRW, Germany) with saline at a weight ratio of 1.0%. To make the scattering condition, a mixture of polystyrene latex beads solution with 0.1-μm mean particle size (LB1-15L; Sigma-Aldrich Japan K.K. Tokyo, Japan) and that with 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 was 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 \(2.6 \times 4.5 \text{ cm}^2\). The volume concentration of the oxygenated hemoglobin solution was \(C_{HbO} = 5.0, 10, \text{ and } 20\%\), whereas that of the deoxygenated hemoglobin solution was \(C_{HbR} = 0\%\). Therefore, the tissue oxygen saturation was \(StO_2 = 100\%\), whereas the volume concentration of the total hemoglobin solution was \(C_{HbT} = 5.0, 10, \text{ and } 20\%\). A tissue-like agar gel phantom with fully deoxygenated hemoglobin (\(StO_2 = 0\%\)) has been developed using \(\text{Na}_2\text{S}_2\text{O}_4\) solution for the investigation of a skin tissue model [42]. In the preliminary experiments, I attempted to measure the phantoms with hemoglobin deoxygenated by \(\text{Na}_2\text{S}_2\text{O}_4\) solution. However, the measured diffuse reflectance spectra had a tendency to exhibit unexpected fluctuation and it was difficult to keep the stable condition of the deoxygenated spectra during the experiments. To avoid potential uncertainty of the estimation of the total hemoglobin with the condition of \(StO_2 = 0\%\), the phantoms with hemoglobin deoxygenated by \(\text{Na}_2\text{S}_2\text{O}_4\) solution were excluded from the measurements in this study. I made 19 optical phantoms with the different combinations of \(C_{HbO}, C_{HbR}, a, \text{ and } b\).

As preparatory measurements, I first determined the absorption coefficient \(\mu_a\) and reduced scattering coefficients \(\mu'_a\) 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 \(C_{HbO}, C_{HbR}, a, \text{ and } b\). The spectra of the absorption coefficient \(\mu_a(\lambda)\) and reduced scattering coefficients \(\mu'_a(\lambda)\) are also required to calculate the given values for \(C_{HbO}, C_{HbR}, a, \text{ and } b\). For this purpose, I 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 (LGC1-5L1000; Hayashi Watch Works Co., Ltd) and lens with a spot diameter of 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, while the area of each phantom was $2.6 \times 4.5 \text{ cm}^2$. The phantom was placed between two glass slides having a thickness of 1.0 mm 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 $\mu$m placed at the detector port of the sphere. The fiber transmits the received light into a multi-channel spectrometer (USB2000, Ocean Optics Inc., Dunedin, FL, USA), which measured reflectance or transmittance spectra in the visible to near-infrared wavelength region under the control of a personal computer.

To determine $\mu_a(\lambda)$ and $\mu'_s(\lambda)$ from the measured diffuse reflectance and total transmittance spectra, I utilized the inverse Monte Carlo method (IMC) [76]. In the IMC, the MCS of the reflectance and transmittance spectra was iterated for different values of $\mu_a(\lambda)$ and $\mu'_s(\lambda)$ 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 $\mu_a(\lambda)$ and $\mu'_s(\lambda)$ 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. (4.4) with the estimated absorption coefficient $\mu_a(\lambda)$ and the known values of $\varepsilon_{HbO}(\lambda)$ and $\varepsilon_{HbR}(\lambda)$. The concentration of total hemoglobin $C_{HbT}$ and the tissue oxygen saturation $StO_2$ in each phantom were calculated from Eqs. (4.5) and (4.6), respectively. The scattering amplitude $a$ and the scattering power $b$ for each
phantom were calculated from the estimated $\mu'_s(\lambda)$ based on Eq. (4.1). Those values of
$\mu_a$, $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, $StO_2$, $\mu'_s$, $a$, and $b$ obtained by IMC are used as the given values to
evaluate the validity of the proposed method experimentally.

I also need to have the conversion vectors for the phantoms used in this study. I
generated diffuse reflectance spectra at 500, 520, 540, 560, 570, 580, 600, 730, and 760
nm using the MCS with conditions of the phantoms. For this simulation, the values of
$\mu_a$ and $\mu'_s$ 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. 4.2, I derived the conversion vectors and the corresponding empirical formula
for estimating the volume concentrations of oxygenated hemoglobin $C_{HbO}$, that of
deoxygenated hemoglobin $C_{HbR}$, scattering amplitude $a$, and scattering power $b$.

4.2.4 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 $\alpha$-chloralose (50 mg/kg) and urethane (600 mg/kg) in
seven adult male Wister rats (290–408 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 approximately 20 mm 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.4.3(b). The cranial window was bathed with
normal saline and covered by a transparent glass plate with 0.1-mm thickness. The
measurements were performed during normoxia ($t = 0–5$ min), hyperoxia ($t = 5–10$ min),
and anoxia ($t = 10–40$ min). The respiration state was changed by regulating the fraction
of inspired oxygen (FiO<sub>2</sub>). Hyperoxia (FiO<sub>2</sub> = 95%) was induced by 95%O<sub>2</sub>–5%CO<sub>2</sub> gas inhalation, for which a breathing mask was used under spontaneous respiration, whereas anoxia (FiO<sub>2</sub> = 0%) was induced by 95%N<sub>2</sub>–5%CO<sub>2</sub> gas inhalation. The multispectral reflectance images for the normoxic state and hyperoxic state were acquired at 2 min after the onset of normoxia and that of hyperoxia, respectively. The multispectral reflectance images for anoxia were acquired at 1, 5, 15, and 30 min after the onset of anoxia. During the experiments, the respiration of the rat was confirmed by observing the periodical movement of the lateral region of the abdomen to identify respiratory arrest. The electrocardiogram was monitored using a differential amplifier (ISO-80, World Precision Instruments, Inc., Sarasota, FL, US) to identify cardiac arrest. The two electrodes were inserted subcutaneously into the root of the anterior limbs. The output signal from the amplifier was acquired by a digital storage oscilloscope (TDS1000C-EDU, Tektronix, Inc., Tokyo, Japan).

4.2.5 Statistical Considerations

A region of interest (ROI) with 500 × 500 pixels was placed in a part of an image for each resultant image in both phantom validation and in vivo animal experiments. Simple linear regression analysis was used to describe the correlation coefficient R between the estimated and given values in validation using tissue-like optical phantoms. An unpaired Student’s t-test was used for statistical analysis when comparing the in vivo results of the normoxic condition with that of the hyperoxic condition or with that of anoxic conditions. The normality of the averaged value over the seven samples for each condition was tested by the Shapiro–Wilk test before the Student’s t-test. A P value < 0.05 was considered statistically significant.
4.3 Results and Discussion

4.3.1 Validation using tissue-like optical phantoms

Fig. 4.4 Comparisons between the estimated and given values for (a) oxygenated hemoglobin $C_{HbO}$, (b) deoxygenated hemoglobin $C_{HbR}$, (c) total hemoglobin $C_{HbT}$, (d) tissue oxygen saturation $StO_2$, (e) scattering amplitude $a$, and (f) scattering power $b$, obtained from the phantom experiments.

To estimate the absorption coefficient and reduced scattering coefficient of the tissue-like optical phantoms, the volume concentrations $C_{HbO}$ and $C_{HbR}$, the scattering amplitude $a$, and the scattering power $b$ were determined from the measured spectral reflectance images based on the proposed method described above. The volume concentration $C_{HbT}$ and the tissue oxygen saturation $StO_2$ were also calculated from the values of $C_{HbO}$ and $C_{HbR}$. Figure 4.4 shows the comparisons between the estimated and given values for (a) oxygenated hemoglobin $C_{HbO}$, (b) deoxygenated hemoglobin $C_{HbR}$,
(c) total hemoglobin $C_{HbT}$, (d) tissue oxygen saturation $StO_2$, (e) scattering amplitude $a$, and (f) scattering power $b$, obtained from the phantom experiments. In Figs. 4.4(a), 4.4(c), 4.4(e), and 4.4(f), the estimated values are well correlated with the given values. Correlation coefficients between the estimated and given values are 0.92 ($P < 0.0001$), 0.95 ($P < 0.0001$), 0.77 ($P = 0.00011$) and 0.51 ($P = 0.027$) for $C_{HbO}$, $C_{HbT}$, $a$, and $b$, respectively. In Figs. 4.4(b) and 4.4(d), all estimated values of $C_{HbR}$ and $StO_2$ are close to 0% 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 method for estimating the volume concentrations $C_{HbO}$ and $C_{HbR}$, the scattering amplitude $a$, and the scattering power $b$. Figure 4.5 shows the comparisons between the estimated and given values for (a) absorption coefficient $\mu_a$ and (b) reduced scattering coefficient $\mu_s'$. Reasonable results were obtained for both $\mu_a$ and $\mu_s'$. Correlation coefficients between the estimated and given values are 0.97 ($P < 0.0001$) and 0.86 ($P < 0.0001$) for $\mu_a$ and $\mu_s'$, respectively. The mean square error in $\mu_a$ is $23 \pm 19\%$, whereas that in $\mu_s'$ is $29 \pm 15\%$. These results demonstrate the validity of the proposed method for estimating absorption and reduced scattering coefficients from the diffuse reflectance spectrum.
Fig. 4.5 Comparisons between the estimated and given values for (a) absorption coefficient $\mu_a$ and (b) reduced scattering coefficient $\mu_s^\prime$, obtained from the phantom experiments.

4.3.2 Animal experiments

Figure 4.6 shows the typical multispectral images of exposed rat brain measured under normoxia. The distribution of blood vessels in the cortex can be clearly observed in the measured reflectance images at 500, 520, 540, 560, 570, and 580 nm. The images at 600, 730, and 760 nm have low contrast between the blood vessel region and parenchyma region, which reflects the lower absorption coefficient of hemoglobin at longer wavelengths.

Figure 4.7 shows the estimated images of exposed rat brain under normoxia obtained from the multispectral reflectance images shown in Fig. 4.6 for (a) oxygenated hemoglobin $C_{HbO}$, (b) deoxygenated hemoglobin $C_{HbR}$, (c) total hemoglobin $C_{HbT}$, (d) tissue oxygen saturation $StO_2$, (e) coefficient $a$ for scattering amplitude, and (f) exponent $b$ for scattering power. In Figs. 4.7(a), 4.7(b), and 4.7(c), the values of $C_{HbO}$,
Fig. 4.6 Typical measured multispectral images of exposed rat brain under normoxia.

$C_{HbR}$ and $C_{HbT}$ in the blood vessel region are higher than those in the parenchyma region, respectively, which indicates the difference in blood volume between the parenchyma and blood vessels. The distribution of $StO_2$ in the blood vessel region in Fig. 4.7(d) is probably due to the difference between an artery and a vein. Besides the obvious blood vessels, there seems some large heterogeneity of both $C_{HbO}$ and $C_{HbR}$, which is probably due to unexpected subdural bleedings. The average values over the
Fig. 4.7 The estimated images of exposed rat brain under normoxia for (a) oxygenated hemoglobin $C_{\text{HbO}}$, (b) deoxygenated hemoglobin $C_{\text{HbR}}$, (c) total hemoglobin $C_{\text{HbT}}$, (d) tissue oxygen saturation $\text{StO}_2$, (e) coefficient $a$ for scattering amplitude, and (f) exponent $b$ for scattering power.

entire region were calculated to be $C_{\text{HbO}} = 2.29\pm0.30\%$, $C_{\text{HbR}} = 3.47\pm0.40\%$, $C_{\text{HbT}} = 5.76\pm0.68\%$, $\text{StO}_2 = 37.43\pm1.67\%$, $a = 122012\pm2242$, and $b = 1.38\pm0.01$.

Figure 4.8 shows the reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_a(\lambda)$ and (b) the reduced scattering coefficient spectrum $\mu_s'(\lambda)$ under normoxia. The average values of $\mu_a(\lambda)$ and $\mu_s'(\lambda)$ over the ROIs (white squares) are also shown in Figure 4.9. The wavelength-dependence of $\mu_a(\lambda)$ is dominated by the spectral characteristics of oxygenated hemoglobin [64]. The reduced scattering coefficients $\mu_s'(\lambda)$ have a broad scattering spectrum, exhibiting larger magnitude at shorter wavelengths. The spectral features of $\mu_s'$ correspond to the typical spectrum of brain tissue published in the literature [75].
Fig. 4.8 The reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_a(\lambda)$ and (b) the reduced scattering coefficient spectrum $\mu_s'(\lambda)$ under normoxia.

Fig. 4.9 The estimated spectra averaged over the ROI (corresponding to the entire region of each image in Fig. 4.8) for (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s'(\lambda)$. Error bars show the standard deviations.
Fig. 4.10 Typical images of in vivo results during change in FiO\textsubscript{2} (from top to bottom: reflectance at 500 nm, C\textsubscript{HbO}, C\textsubscript{Hbr}, C\textsubscript{Hbt}, StO\textsubscript{2}, \(\mu_s\)' at 500 nm, and \(\mu_s\)' at 760 nm).

Figure 4.10 shows the in vivo results of \(r(500)\), C\textsubscript{HbO}, C\textsubscript{Hbr}, C\textsubscript{Hbt}, StO\textsubscript{2}, \(\mu_s\)'(500), and \(\mu_s\)'(760) during change in FiO\textsubscript{2}. Figure 4.11 shows the results of C\textsubscript{HbO}, C\textsubscript{Hbr}, C\textsubscript{Hbt}, and StO\textsubscript{2} averaged over the ROI for all seven samples during change in FiO\textsubscript{2}. The value of C\textsubscript{HbO} and that of C\textsubscript{Hbr} were increased and decreased, respectively, during hyperoxia, which caused the increase in StO\textsubscript{2}. The value of C\textsubscript{HbO} and that of C\textsubscript{Hbr} decreased and
increased, respectively, at 1 min after the onset of anoxia. Consequently, the value of $StO_2$ was dramatically decreased. The period between the onset of anoxia and respiratory arrest averaged over all seven samples was $213\pm 76$ s, whereas the period between the onset of anoxia and cardiac arrest averaged over all seven samples was $485\pm 202$ s. After the induction of anoxia, the values of $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, and $StO_2$ were almost constant. The above results for $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, and $StO_2$ are consistent with the well-known hemodynamic responses to the change in the fraction of inspired oxygen.

As shown in Fig. 4.11(d), the average value of $StO_2$ over all seven samples was $38.94\pm 15.75\%$ under normoxia, which is relatively lower than the value of around 60% reported in the literature [74,77]. This may be due to the experimental conditions such as the kind of anesthetic, the anesthetic depth, or uncontrolled body temperature in this study.

Figure 4.12 shows the results of the scattering amplitude $a$, the scattering power $b$, $\mu_s'(500)$, and $\mu_s'(760)$ averaged over the ROI for all seven samples during change in FiO$_2$. The scattering amplitude $a$ and the scattering power $b$ are significantly decreased and increased at 1 min after the onset of anoxia, respectively. Those changes in the scattering parameters decrease the reduced scattering coefficients. On the other hand, the values of $\mu_s'(500)$ and $\mu_s'(760)$ were continuously increased at the period from 5 min to 30 min after the onset of anoxia, which implies morphological changes in brain tissue.
Fig. 4.11 The results of (a) $C_{HbO}$, (b) $C_{HbR}$, (c) $C_{HbT}$, and (d) $StO_2$ averaged over the ROI (corresponding to the entire region of each image in Fig. 4.10) for seven samples during change in $FiO_2$. The error bars show the standard deviations ($n = 7$). *$P < 0.05$.

Mitochondrial respiration is inhibited during the ischemia-like condition by the rapid drop in $O_2$ tension, which causes depletion of the adenosine triphosphate (ATP). Reduced ATP production due to the inhibition of mitochondrial respiration leads to failure of the $Na^+/K^+$ ATPase pump [60]. In such a case, extracellular $Na^+$, $Cl^-$, and $Ca^{2+}$...
rush in, with water following osmotically, causing cell swelling that shrinks the extracellular space [34,55]. As described above, the scattering amplitude $a$ and the scattering power $b$ are related to the scatterer density and the scatterer size, respectively [73,74]. The swelling of cellular and subcellular structures cause decrease in the

Fig. 4.12 The results of (a) the scattering amplitude $a$, (b) the scattering power $b$, (c) $\mu_s'(500)$, and (d) $\mu_s'(760)$ averaged over the ROIs for seven samples during change in FiO$_2$. The error bars show the standard deviations ($n=7$). *$P < 0.05$. 

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scatterer density and increase in the scatterer size.

The spectrum of reduced scattering coefficient $\mu_r(\lambda)$ of biological tissues can be treated as the combination of $\mu_r(\lambda)$ for the cellular and subcellular structures with the different sizes [75]. Generally, the sizes of cellular and subcellular structures in biological tissues are distributed as follows: vesicles, 0.1-0.5μm [78]; lysosomes, 0.1-0.5μm [73]; mitochondrion, 1-2μm [75]; nuclei, 5-10μm [75]; cell, 5-75μm [75]. Figure 4.13 shows the spectra of reduced scattering coefficient $\mu_r(\lambda)$ calculated by the Mie theory for various size spheres. In the Mie-theory-based calculation, the refractive indices of a sphere and the surrounding medium were set to be 1.46 and 1.35 at a volume concentration of 2%. The slope of $\mu_r(\lambda)$ decreases as the diameter of sphere $d$ increases. The whole spectrum of $\mu_r(\lambda)$ decreases as the diameter of sphere $d$ increases in the range of $d=0.4$-10.0 μm. Therefore, the dependence of $\mu_r(\lambda)$ on the particle size shown in Fig. 4.13 implies that the volume increases in the cellular and subcellular structures contributes the decrease in $\mu_r$. If the volume increases in all cellular and subcellular structures occur simultaneously, the net scattering spectrum will be decreased with a greater slope. Therefore, the scattering amplitude $a$ and the scattering power $b$ will be decreased and increased, respectively, by the cell swelling. This assumption is supported by the results shown in Figs. 4.12 (a) and 4.12 (b). Thus, the significant decreases in $\mu_r$ at 1 min after the onset of anoxia shown in Fig. 4.12 are most likely caused by cell swelling due to failure of the Na$^+$/K$^+$ATPase pump. The minor increases in $\mu_r$ after the respiratory arrest are probably caused by the dendritic beading effect, which is indicative of neuronal damage [55]. The necklace-like fine 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 [61-63]. The appearance of beaded fine structures will cause the increase in the scatterer density and the relative decrease in the scatterer size. As a consequence, the scattering amplitude $a$ and the scattering power $b$ will be increased and decreased, respectively, as shown in Figs. 4.12 (a) and 4.12 (b).

![Graph showing reduced scattering coefficient $\mu'_s(\lambda)$ calculated by the Mie theory for various size spheres.](image)

Fig. 4.13 Reduced scattering coefficient $\mu'_s(\lambda)$ calculated by the Mie theory for various size spheres.

In the present study, I used a standard multispectral imaging system with the motorized filter wheel to confirm the feasibility of the proposed method for quantitative evaluations of $C_{HbO}, C_{HbR}, C_{HbT}, StO_2, a, b, \mu'_s$ and $\mu_a$. Such a conventional multispectral imaging system is somewhat time-consuming since the filter positions in the wheel were changed mechanically. This means that the imaging system captures each spectral image at a different time-point. It may have little impact on the estimations of absorption and scattering spectra presented in this study because the period between the switching of FiO$_2$ and onset of image acquisition is relatively long in this experiment. However, it
will be insufficient to capture the fast IOSs such as the change in reflectance spectrum due to neuronal activities [79]. It is advantageous that the proposed method can also be applied to the multispectral images acquired by the other rapid imaging systems such as an acousto-optical tunable filter [32], the combination of a lenslets array with narrow-band filters [31], and the reconstruction of multispectral images from a single snap shot of an RGB image [80]. The combinations of the proposed method and the rapid multispectral imaging techniques are promising for evaluating the fast IOSs imaging as well as the clinical situations. This issue should be investigated in future work.

4.4 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 are proposed in the present report. Experiments with phantoms demonstrated the validity of the proposed imaging method for estimating spectral images of the absorption coefficient and the reduced scattering coefficient. In vivo experiments with the exposed brain of rats during a change in the fraction of inspired oxygen confirmed the possibility of the method to evaluate both cortical hemodynamics and changes in tissue morphologies due to loss of tissue viability in brain. Since the method visualizes both the hemodynamic response and the morphological changes in brain tissue, it may be useful for evaluating brain functions and tissue viabilities in neurosurgery as well as in the diagnosis of several neurological disorders, such as neurotrauma, seizure, stroke, and ischemia. I expect to further extend this method in order to investigate the brain functions in cortical spreading depression.
Chapter 5.

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

5.1 Introduction

Evaluating the optical properties of brain tissue is important for 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). Intrinsic optical signals 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 [30], 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 slices [64] based on several light transport models, such as the Kubelka-Munk theory [65], the diffusion approximation to the transport equation [66], the Monte Carlo method [11], and the adding-doubling method [67]. Numerous spectroscopic methods
have been investigated for \textit{in vivo} determination of the scattering and absorption properties in living tissues, including time-resolved measurements \cite{13}, a frequency-domain method \cite{22}, and spatially resolved measurements with continuous wave (CW) light \cite{15-21}.

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. Diffuse reflectance spectroscopy is one of the most promising methods for evaluating the absorption and scattering properties of \textit{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 \cite{81-84}. Several imaging methods based on DRS have been used to investigate cortical hemodynamics based on changes in the absorption properties of brain tissue \cite{68-72}. In order to achieve rapid multispectral imaging, the use of an acousto-optical tunable filter \cite{32} and the combination of a lenslet array with narrow-band filters \cite{31} 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 \cite{85-88}, finite-dimensional modeling \cite{87, 89}, the nonlinear estimation method \cite{90}, and the Wiener estimation method (WEM) \cite{38-40}, 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 Chapter 4, I 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 wavelength from visible to near-infrared based on DRS using a multi-spectral imaging system. Multi-spectral 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 spectrum and the reduced scattering coefficient spectrum 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, I performed \textit{in vivo} experiments using exposed rat brain while changing the fraction of inspired oxygen (FiO$_2$).

5.2 Principle

5.2.1 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

$$v_i(x, y) = \int u_i(\lambda) E(\lambda) S(\lambda) r(x, y; \lambda) d\lambda,$$  \hspace{1cm} (5.1)
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. (5.1) is expressed in discrete vector notation as

$$v = F r,$$

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

$$F = U E S,$$

where $U = [u_1, u_2, u_3]^T$. Column vector $u_i$ denotes the transmittance spectrum of the $i$th filter, and $[^T]$ represents the transposition 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 [38-40] of $r$ is given by

$$\tilde{r} = W v,$$

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

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

(5.5)

From Eqs. (5.4) and (5.5), the minimum square error is rewritten as

$$e = \langle (r - \tilde{r})^T (r - \tilde{r}) \rangle = \langle r'^T r' \rangle - \langle r'^T W v \rangle - \langle W^T v' r' \rangle + \langle W' W v' v \rangle.$$  

(5.6)
The minimization of the minimum square error requires that the partial derivative of $e$ with respect to $W$ be zero, i.e.,

$$\frac{\partial e}{\partial W} = -\langle r' v \rangle + W' \langle v' v \rangle = 0. \quad (5.7)$$

From Eq. (5.7), the matrix $W$ is derived as

$$W = \langle rv^T \rangle \langle vv^T \rangle^{-1} = \langle rv^T \rangle F^T \left( F \langle rr^T \rangle F^T \right)^{-1}, \quad (5.8)$$

where $\langle \cdot \rangle$ is an ensemble-averaging operator. The derivation of matrix $W$ requires the autocorrelation matrix $\langle rr^T \rangle$. In the present study, I determine $\langle rr^T \rangle$ based on 720 different reflectance spectra obtained from in vivo rat brain under various physiological conditions as described in Sec.5.3.2.

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) [91]. The GFC is based on the inequality of Schwartz and it is described as

$$\text{GFC} = \frac{\left| \sum_j r_{\text{mes}}(\lambda_j) r_{\text{est}}(\lambda_j) \right|}{\sqrt{\left[ \sum_j r_{\text{mes}}(\lambda_j) \right]^2 \sqrt{\sum_j r_{\text{est}}(\lambda_j)^2}}}, \quad (5.9)$$

where $r_{\text{mes}}(\lambda_j)$ is the measured original spectral data at the wavelength $\lambda_j$ and $r_{\text{est}}(\lambda_i)$ is the estimated spectral data at the wavelength $\lambda_i$. Hernández-Andrés et al. [91] suggested that colorimetrically accurate $r_{\text{mes}}(\lambda_i)$ requires a GFC $> 0.995$; a “good” spectral fit requires a GFC $\geq 0.999$, and GFC $\geq 0.99999$ is necessary for an “excellent” spectral fit.
5.2.2 Estimation of the absorption coefficient and the reduced scattering coefficient

In this Chapter 5, the absorption coefficient spectrum and the reduced scattering coefficient spectrum are reconstructed from estimated reflectance spectra by using empirical formulas based on Monte Carlo simulation. I described detail the principle in Sec.4.2.1.

5.3 Experiments

5.3.1 Imaging system

Fig.5.1 (a) Schematic diagram of the experimental system, and (b) representation of the rat skull with an RGB image of the exposed rat brain.
Figure 5.1(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 of 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 Nicols 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, FL, 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 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 mm × 6.98 mm with 1,024 × 768 pixels. The lateral resolution of the images was estimated to be 9.1 μm. The multi-spectral 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 \( C_{HbO} \), \( C_{HbR} \), \( a \), \( b \), \( \mu_s(\lambda) \), and \( \mu'_s(\lambda) \) according to the process described in Fig. 4.1.

5.3.2 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 \( \alpha \)-chloralose (50 mg/kg) and urethane (600 mg/kg) in four adult male Wister 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 approximately 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. 5.3(b). In order to confirm the change in reflectance spectra of exposed rat brain, I 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 \( \text{FiO}_2 \). Hyperoxia (\( \text{FiO}_2 = 95\% \)) was induced by 95%\( \text{O}_2 \)-5%\( \text{CO}_2 \) gas inhalation, for which a breath mask was used under spontaneous respiration, whereas anoxia (\( \text{FiO}_2 = 0\% \)) was induced by 95%\( \text{N}_2 \)-5%\( \text{CO}_2 \) 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,
I 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 $\Delta S = \{(S - S_c)/S_c \} \times 100$. The above calculation was applied to the time series of $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, $StO_2$, $a$, $b$, and $\mu_{t}^{'}(\lambda)$. The time of RA can differ from sample to sample. Therefore, I 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 were averaged over all five samples.

5.3.3 Statistical Considerations

A region of interest (ROI) of $40 \times 40$ 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.

5.4 Results and Discussion

Figure 5.2 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 higher wavelengths.

![Reflectance Images](image)

Fig. 5.2 Typical estimated multispectral images \( r(\lambda) \) of the exposed rat brain under normoxia.

Figure 5.3 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 spectrum for a blood vessel and that for parenchyma in each graph of Fig. 5.3 are 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 5 samples summarized in Table 5.1 in the revised manuscript indicate the accurate spectral reconstruction by WEM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Region</th>
<th>Mean</th>
<th>±SD</th>
<th>Max</th>
<th>Min</th>
<th>Number of spectra</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Parenchyma</td>
<td>0.9992</td>
<td>0.0003</td>
<td>0.9998</td>
<td>0.9980</td>
<td>121</td>
<td>Good</td>
</tr>
<tr>
<td>#2</td>
<td>Parenchyma</td>
<td>0.9982</td>
<td>0.0006</td>
<td>0.9992</td>
<td>0.9964</td>
<td>361</td>
<td>Colorimetrically accurate</td>
</tr>
<tr>
<td>#3</td>
<td>Parenchyma</td>
<td>0.9975</td>
<td>0.0015</td>
<td>0.9994</td>
<td>0.9947</td>
<td>361</td>
<td>Colorimetrically accurate</td>
</tr>
<tr>
<td>#4</td>
<td>Parenchyma</td>
<td>0.9990</td>
<td>0.0005</td>
<td>0.9998</td>
<td>0.9975</td>
<td>361</td>
<td>Good</td>
</tr>
<tr>
<td>#5</td>
<td>Parenchyma</td>
<td>0.9997</td>
<td>0.0002</td>
<td>0.9999</td>
<td>0.9985</td>
<td>361</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>Blood vessel</td>
<td>0.9984</td>
<td>0.0013</td>
<td>0.9999</td>
<td>0.9935</td>
<td>361</td>
<td>Colorimetrically accurate</td>
</tr>
</tbody>
</table>

Fig. 5.3 Comparison of the estimated reflectance spectra by the WEM and the reference spectra measured by spectrometer for (a) normoxia, (b) hyperoxia, and (c) anoxia.
Fig. 5.4 Estimated images of exposed rat brain obtained using the proposed method under normoxia 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 $b$.

Figure 5.4 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. 5.4(a), 5.4(b), and 5.4(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 the blood vessels and the parenchyma. The distribution of $StO_2$ in the blood vessel region in Fig. 5.4(d) is probably due to the difference between artery and vein. The average values over the entire region were calculated to be $C_{HbO} = 150.08 \pm 50.61$ μM, $C_{HbR} = 40.11 \pm 12.12$ μM, $C_{HbT} = 190.19 \pm 52.05$ μM, $StO_2 = 75.52 \pm 7.62\%$, $a = 134,458 \pm 3,634$, 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 $C_{HbO} = 41.16 \pm 3.13$ µM, $C_{HbR} = 42.34 \pm 5.51$ µM, $C_{HbT} = 83.49 \pm 6.52$ µM, $StO_2 = 49.29 \pm 4.19\%$, $a = 136,811 \pm 2,298$, and $b = 1.32 \pm 0.01$.

![Typical estimated images of exposed rat brain obtained using the proposed method under normoxia for (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s'(\lambda)$.](image)

Fig.5.5 Typical estimated images of exposed rat brain obtained using the proposed method under normoxia for (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s'(\lambda)$.

![Estimated spectra averaged over the ROI (white square in Fig.5.6) for (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s'(\lambda)$.](image)

Fig.5.6 Estimated spectra averaged over the ROI (white square in Fig.5.6) for (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s'(\lambda)$.

Figure 5.5 shows the reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_a(\lambda)$ and (b) the reduced scattering coefficient spectrum $\mu_s'(\lambda)$ under normoxia. The average values of $\mu_a(\lambda)$ and $\mu_s'(\lambda)$ over the ROIs
(White squares in Figs.5.5(a) and 5.5(b)) are also shown in Fig.5.6. The error bars mean the standard deviations for the all pixels in the ROI. The wavelength dependence of $\mu_a$ ($\lambda$) is dominated by the spectral characteristics of hemoglobin [92]. 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 [75].

![Graph showing measured and predicted reflectance spectra](image)

Fig. 5.7 Comparison between the measured reflectance spectrum and the predicted reflectance spectrum for the $\mu_a$ and $\mu_s'$ shown in Fig.5.6.

Figure 5.7 shows the comparison between the measured reflectance spectrum and the predicted reflectance spectrum for the $\mu_a$ and $\mu_s'$ shown in Fig.5.8. The predicted reflectance spectrum is comparable to the measured spectrum. The value of GFC in this case was 0.9974, which shows the good agreement between the measured reflectance spectrum and the predicted reflectance spectrum.
Figure 5.8 shows the typical in vivo results while varying FiO2 while varying FiO2. Fig.5.9 shows the time courses of $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, and $StO_2$ averaged over the area for the ROI in the parenchyma, as shown in Fig.5.6, while varying FiO2. The average values of $StO_2$ during normoxia over the ROI were $47.69 \pm 0.64\%$, which is lower than the value of around 60% reported in the literature [74,
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. 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 one minute after RA, which is indicative of an increase in blood flow for compensating hypoxia. Immediately after RA, the values of both $C_{HbO}$ and $C_{HbT}$ decreased rapidly.

Fig. 5.9 Time courses of $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, and $StO_2$ averaged over the ROI in the parenchyma region (white square in Fig. 5.8) while varying $FiO_2$. 

77].
Minor fluctuations in $C_{HbO}$, $C_{HbR}$, and $C_{HbT}$ while varying FiO$\textsubscript{2}$ are probably caused by the changes in blood volume due to constriction and dilatation of microvasculature. The time courses of $C_{HbO}$, $C_{HbR}$, $C_{HbT}$, and $StO\textsubscript{2}$ while varying FiO$\textsubscript{2}$ are consistent with the well-known hemodynamic responses to the change in the fraction of inspired oxygen.

![Diffuse reflectance images](image)

Fig. 5.10 Diffuse reflectance images at 500 nm for (a) normoxia, (b) hyperoxia, (c) anoxia before RA, and (d) anoxia after RA.

Figure 5.10 shows the diffuse reflectance images at 500 nm for (a) normoxia, (b) hyperoxia, (c) anoxia immediately before RA, and (d) anoxia after RA. There is a depression of reflectance for each condition that is supposed to be artery. Under the anoxia immediately before RA, the diameter of artery was markedly increased, and then, it was decreased after RA. This change in the diameter of artery is associated with the compensation for an oxygen supply deficit at this condition.
Figure 5.11 shows the time courses of $\mu_s'(500)$, $\mu_s'(600)$, and $\mu_s'(760)$ averaged over the area corresponding to the ROIs in the parenchyma region (white square in Fig. 5.8) while varying FiO$_2$.

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 O$_2$ tension, which causes depletion of the adenosine triphosphate (ATP). Reduction of ATP production due to the inhibition of mitochondrial respiration leads to failure of the Na$^+$/K$^+$ ATPase pump [60]. In such a case, extracellular
Na⁺, Cl⁻, and Ca²⁺ rush in, with water following osmotically, causing cell swelling [34, 55]. Thus, the slight decreases in $\mu_s'$ in Figs.5.8 and 5.11 are most likely caused by cell swelling due to failure of the Na⁺/K⁺ATPase pump. The continuous increases in $\mu_s'$ after RA are probably caused by the dendritic beading effect, which is indicative of neuronal damage [34]. 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 [61-63].

5.5 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 an RGB image using the WEM was presented. In vivo experiments using exposed rat brain while changing the fraction of inspired oxygen confirmed the feasibility of the proposed method for visualizing both hemodynamics-related changes and morphological changes in cortical tissue due to loss of tissue viability in the brain.

Since the proposed method can visualize 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.
Chapter 6.

In vivo multispectral imaging of scattering and absorption properties of exposed rat brain during cortical spreading depression using a digital red-green-blue camera

6.1 Introduction

Determining optical properties of brain tissue is important for application of light in clinical diagnosis, surgery, and therapeutic procedure in brain disorders. Changes in optical properties of the brain have been used to deduce spatial and/or temporal changes of neuronal activity and tissue viability in the brain as intrinsic optical signal (IOSs). It is believed that IOSs in the brain are mainly caused by the following three processes: changes in absorption and scattering originating from hemodynamics, changes in absorption due to redox states of cytochromes in mitochondria, and changes in scattering generated by cell swelling or shrinkage caused by water movement between intracellular and extracellular compartments [30]. IOSs also have potential for evaluating pathophysiology of brain, such as cortical spreading depression (CSD). CSD is a wave of neuronal and glial depolarization propagating at 2 to 4 mm/min over cerebral cortex followed by the temporal changes in cerebral blood flow [28]. CSD is an important disease model for migraine and is related to other neurological disorders, such as neurotrauma, seizure, and ischemia. Several imaging techniques based on the diffuse optical spectroscopy have been used to investigate the hemodynamics of in vivo exposed brain tissue [68-70]. Diffuse reflectance spectroscopy (DRS) is one of most
promising technique for evaluating optical properties of in vivo brain tissue. Several imaging techniques based on the diffuse optical spectroscopy have been used to investigate the cortical hemodynamics [68-70] and tissue viability [71].

In Chapter 6, I proposed a simple and rapid multi-spectral imaging of reduced scattering coefficients $\mu'_s(\lambda)$ and the absorption coefficients $\mu_a(\lambda)$ of in vivo exposed brain tissues in the range from visible to near-infrared wavelength (500-760 nm) based on DRS using a digital red-green-blue camera. Multi-spectral reflectance images of in vivo exposed brain are reconstructed from the digital red, green blue images using the Wiener estimation algorithm [38-40]. The Monte Carlo simulation-based multiple regression analysis for the absorbance spectra is then used to specify the absorption and scattering parameters of brain tissue. In order to confirm the possibility of the method to evaluate changes in absorption and scattering properties of cerebral cortex, I performed in vivo experiments for exposed rat brain during CSD evoked by the topical application of KCl.

6.2 Materials and method

6.2.1 Estimation of spectral diffuse reflectance images by Wiener estimation

In this Chapter 6, Multi-spectral reflectance images of in vivo exposed brain are reconstructed from the digital red, green blue images using the Wiener estimation algorithm. I already described detail the principle in Sec.5.2.1.
6.2.2 Estimation of absorption coefficient and reduced scattering coefficient

In this Chapter 6, the absorption coefficient spectrum and the reduced scattering coefficient spectrum are reconstructed from estimated reflectance spectra by using empirical formulas based on Monte Carlo simulation. I already described detail the principle in Sec. 4.2.1.

6.3 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 three adult male Wister rats (80-286 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 about 20 mm 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 (8.0 mm-major axis and 6.0 mm-minor axis). The cranial window was bathed with normal saline. A burr hole (2 mm diameter) was drilled in the ipsilateral frontal bone as a site for contacting cortex with 3M KCl. CSD was induced via topical application of KCl to the burr hole.

Figure 6.1 shows a schematic illustration of experimental system used in this study. A white light emitting diode (LED) (LA-HDF158A, Hayashi Watch Works Co., Ltd, Tokyo, Japan) illuminated 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-21BF04, 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 set to be a crossed Nicols alignment in order to reduce specular reflection from the sample surface. A standard white diffuser was used to regulate the camera white balance. To evaluate accuracy of WEM, the reflectance spectra of skin were simultaneously measured by a fiber-coupled spectrometer (USB2000, Ocean Optics Inc., Dunedin, FL, USA) at 110 ms of integration time as reference data. Before the measurements of RGB images and reflectance spectra, a measured area by the spectrometer was confirmed by projecting light from a halogen lamp on the skin surface via one lead of bifurcated fiber, lens, and beam splitter. The RGB image of sample including the spot of light illuminated by the halogen lamp was stored into the PC, and then, the size and coordinates of the spot were specified as the measured area by spectrometer. After the halogen lamp was turned off, the measurements of RGB images and reflectance spectra were simultaneously performed. The region of interest, or ROI on the image of skin surface was selected to be the same as the measured area by the spectrometer. Using WEM, reflectance images ranged from 500 to 760 nm at intervals of 10 nm were reconstructed from a RGB image acquired at 65 ms of exposure time. This means that the spectral images at 27 wavelengths can be obtained with a temporal resolution of 15 fps. Images at 9 wavelengths (500, 520, 540, 560, 570, 580, 600, 730, and 760 nm) were then used to estimate the images of $C_{HbO_2}$, $C_{HbR}$, $a$, $b$, $\mu_a(\lambda)$, and $\mu_s'(\lambda)$ according to the above process.

To evaluate the magnitude of signal $P$ induced by CSD, I calculated the change of the signal based on the time series data. The signal at the beginning of the measurement was selected as a control $P_c$. The difference between two successive signals $\Delta P = (P_q - P_{q-1})$ was normalized by dividing by the control $P_c$, where $q$ denotes a
signal number. The change of the signal is expressed as $\Delta P/P_c$. The above calculation was applied to the time series of $r(500)$, $\mu_s'(500)$, and $C_{HbT}$.

Fig. 6.1 Experimental setup

6.4 Results and Discussion

Before the CSD experiments, I compared the diffuse reflectance spectra obtained from the WEM with the measured spectra by the spectrometer under the normoxia, hyperoxia and hypoxia. Figure 6.2 shows an example of the estimated images obtained from the in vivo rat under the normoxia. Spectral reflectance images of cerebral cortex were successfully obtained by the method. Average values of the diffuse reflectance spectra over the regions of interest, or ROIs (white and black squares for blood vessel and tissue, respectively), are comparable to the measured spectra by the spectrometer, as shown in Figure 6.3. The estimated spectra under the hyperoxia and hypoxia are dominated by the spectral characteristics of oxygenated hemoglobin and deoxygenated hemoglobin, respectively.
Figure 6.4 shows the typical estimated images of exposed rat brain obtained by 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 $a$ for scattering amplitude and (f) exponent $b$ for scattering power. Figure 6.5 shows the reconstructed images of the exposed rat brain for (a) the absorption coefficient spectrum $\mu_a(\lambda)$ and the reduced scattering coefficient spectrum $\mu_s'(\lambda)$ under the normoxia.

Fig. 6.2 Typical images of the estimated reflectance spectra $r(\lambda)$ for normoxia.

Fig. 6.3 Comparisons of the estimated reflectance spectra averaged over the ROIs in Fig. 6.2 (white squares) and the reference spectra measured by spectrometer for (a) normoxia, (b) hyperoxia, and (c) hypoxia.
Average values of $\mu_a (\lambda)$ and $\mu_s' (\lambda)$ over the ROIs (white squares) are also shown in Figure 6.6. The wavelength-dependence of $\mu_a (\lambda)$ is dominated by the spectral characteristics of hemoglobin [92]. The reduced scattering coefficients $\mu_s' (\lambda)$ have a broad scattering spectrum, exhibiting larger magnitude at shorter wavelengths. The spectral features of $\mu_s'$ correspond to the typical spectrum of brain tissue published in the literature [75].

Fig. 6.4 Typical estimated images of exposed rat brain obtained by the proposed method under the normoxia for (a) oxygenated hemoglobin $C_{HbO}$, (b) deoxygenated hemoglobin $C_{HbR}$, (c) total hemoglobin $C_{HbT}$, (d) tissue oxygen saturation $StO_2$, (e) coefficient $a$ for scattering amplitude and (f) exponent $b$ for scattering power.

Fig. 6.5 Typical estimated images of exposed brain of rat obtained by the proposed method under the normoxia for (a) the absorption coefficient $\mu_a (\lambda)$ and the reduced scattering coefficient $\mu_s' (\lambda)$. 
Fig. 6.6 The estimated spectra averaged over the ROIs in Fig. 6.5 (white squares) for (a) absorption coefficient $\mu_a(\lambda)$ and (b) reduced scattering coefficient $\mu_s' (\lambda)$.

Fig. 6.7 Sequential images of the percent changes in (a) the diffuse reflectance at 500nm, (b) the reduced scattering coefficient at 500nm, and (c) the total hemoglobin during cortical spreading depression (CSD). Tri-phasic change (phase i: initial increase, phase ii: profound decrease, phase iii: secondary increase) can be observed in reflectance images. The phase i corresponds to the increase in scattering. The phase ii and phase iii correspond to the increase and decrease in total hemoglobin, respectively.

Figure 6.7 shows an example of in vivo resultant images of (a) $\Delta r(500)$, (b) $\Delta \mu_s'(500)$, and (c) $\Delta C_{HbT}$ during CSD. The wave spreading over the cerebral cortex can
be observed in each image. The propagation speed of the wave was 3.8 mm/min, which corresponds to the typical value of CSD reported in the literatures [28]. The wave form in $\Delta r$ (500) shows triphasic change of increase, decrease and increase. CSD is usually followed by a profound hyperemia and post-CSD oligemia [28]. This pattern of hemodynamic response to CSD can be clearly observed in temporal changes in $\Delta C_{HbT}$ in Fig. 6.7(c). It is said that the DC shift of LFP is coincident with a rise in extracellular potassium and can evoke cell deformation generated by water movement between intracellular and extracellular compartments, and hence the light scattering by tissue [34, 35]. Therefore, the increase in $\Delta \mu'_e$ (500) before the profound increase in $\Delta C_{HbT}$ is indicative of changes in light scattering by tissue. The Phase 1 in reflectance change coincide increases in scattering. The Phases 2 and 3 in reflectance change correspond to the increase and decrease in total hemoglobin, respectively. This shows the possibility of the method to evaluate both hemodynamics and changes in tissue morphology due to electrical depolarization.

6.5 Conclusion

In summary, demonstrated in Chapter.5 is a method for imaging of reduced scattering coefficients $\mu'_e(\lambda)$ and the absorption coefficients $\mu_a(\lambda)$ of in vivo exposed brain tissues based on spectral reflectance images reconstructed from a single snap shot of an RGB image by WEM. In vivo experiments with exposed brain of rats during CSD confirmed the possibility of the method to evaluate both hemodynamics and changes in tissue morphology due to electrical depolarization.
Chapter 7.

Concluding Remarks

The present dissertation has been concerned with the studies on the rat brain tissue using intrinsic optical signals measurement in physiological condition change. The rat brain tissue change in physiological condition change has been investigated from two points of view. One is the \textit{in vitro} brain slices perfused with artificial cerebrospinal fluid (aCSF), enable evaluation of changes in the optical properties of living brain tissue, which was discussed in Chapter 3. Another is the \textit{in vivo} measurement of the brain surface using multispectral imaging, which has been studied in Chapters 4-6 for the visible to near-infrared diffuse reflectance spectra from the rat brain tissue.

Chapter 2 was devoted to the description of fundamental theories and principles treated through this dissertation. The Optical parameters have been presented in Section 2.1. The algorithm of the Monte Carlo simulation for the light transport in tissue has been mentioned in Section 2.3. The analysis technique of the multiple regression analysis has been mentioned in section 2.5.

Chapter 3 presented reduced scattering coefficients $\mu_s(\lambda)$ and absorption coefficients $\mu_a(\lambda)$ of rat cerebral cortical tissue slices perfused with aCSF. In this chapter, I used the experimental setup that \textit{in vitro} brain slices perfused with artificial cerebrospinal fluid (aCSF) enable evaluation of changes in the optical properties of
living brain tissue, while maintaining tissue viability and without obstructing hemodynamics. I presented the simultaneous measurement of the diffuse reflectance spectrum and transmittance spectrum for in vitro healthy rat brain slices perfused with aCSF. And the spectra of $\mu'_s(\lambda)$ and $\mu_a(\lambda)$ for the brain slices are estimated based on the Inverse Monte Carlo method in Section 3.3.

Chapter 4 proposed a simple spectral imaging of reduced scattering coefficients $\mu'_s(\lambda)$ and the absorption coefficients $\mu_a(\lambda)$ of in vivo exposed brain tissues in the range from visible to near-infrared wavelengths based on DRS using a multispectral imaging system. Multispectral diffuse reflectance images of in vivo exposed rat brain are acquired by interference filter. The Monte Carlo simulation-based on multiple regression analysis for the absorbance spectra is then used to specify the concentrations of oxygenated and deoxygenated hemoglobin, scattering amplitude, and scattering power. In Section 4.3, I presented the experiments with phantoms demonstrated and in vivo experiments with the exposed brain of rats. I confirmed the validity of the proposed imaging method for estimating spectral images of the absorption coefficient and the reduced scattering coefficient by phantoms demonstration. And, I confirmed the possibility of the method to evaluate both cortical hemodynamics and changes in tissue morphologies due to loss of tissue viability in brain by in vivo experiments.

Chapter 5, I proposed the 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 an RGB image using the Wiener estimation method. In Section 5.4, I mentioned that the proposed method can visualize both the hemodynamic response and the morphological changes in brain tissue.

Chapter 6, I extended the study presented in Chapter 5. In Chapter 6, I
performed the Cortical Spreading Depression (CSD) evoked by the topical application of KCl. CSD experiments were presented in Chapter 6.4. I observed the triphasic change of increase, decrease and increase in optical properties. And, this change related the DC shift of LFP and Cortical Blood flow. The Chapter 6 confirmed the possibility of the method to evaluate both hemodynamics and changes in tissue morphology due to electrical depolarization.

As I was shown in the present dissertation, the method for evaluating quantitatively the rat brain tissue using intrinsic optical signals measurement in physiological condition change may become the aid of brain surgery. The proposed approach for estimating the reduced scattering coefficients $\mu_s' (\lambda)$ and the absorption coefficients $\mu_a (\lambda)$ by RGB-camera is useful for the surgery. And this technique is low cost and high time resolution, which can measure the change of optical values in the brain more simply in brain surgery and emergency medical field. The author expects that the results obtained in this dissertation will provide new possibilities for development of non-invasive practical instrumentations.
Acknowledgement

I am deeply grateful to Associate Professor I. Nishidate whose enormous support and insightful comments were invaluable during the course of my study. I am also indebted to Professor T. Iwai whose meticulous comments were an enormous help to me. I would like to extend my profound thanks for Assistant Professor K. Nakano for his constant encouragement. Without their help this dissertation has not been completed. My hearty thanks are also extended to Professor S. Sato of National Defense Medical College for fruitful discussions and advices on rat brain imaging. The author want to express his gratitude and deepest appreciation to other members of the Biomedical photonics Laboratory, Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, for various supports with which the present dissertation has been successfully accomplished.

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