Neuronal electrical activity in the brain causes an increase in regional cerebral blood flow, which is the basis for some measurements of functional brain mapping. It remains controversial, however, exactly how neuronal electrical activity is coupled to microcirculation responses together with the relation to energy metabolic changes that are inevitably induced by neural activity. One useful technique that assists an understanding of the biological basis underlying neurovascular and neurometabolic coupling is optical spectroscopy.

Malonek and Grinvald [1] reported the presence of an early increase in deoxygenated hemoglobin (deoxy-Hb), an “initial dip,” which preceded an increase in blood flow with neuronal activation in the cat visual cortex after imaging spectroscopy. The increase in deoxy-Hb implies a rise of oxygen consumption, which suggests the relationship of the aerobic metabolism to the energy supply for neuronal activity. This indicates the possibility that oxygen metabolic events triggered the increase in regional blood flow in the brain activation area. Therefore the possibility has been pointed out that a mapping of the initial deoxygenation may give a more accurate map of the neuronally active area. Early decreases in blood oxygen level–dependent (BOLD) signals detected by functional magnetic resonance imaging (fMRI), corresponding to the increase in deoxy-Hb, have been reported [2–6]. These confirmed the conclusion of optical spectroscopy, but

**Key words:** optical measurements, spectrophotometry, initial deoxygenation, intrinsic signal.

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other experiments showed no initial dip with optical measurement [7] and fMRI [8]. Thus the existence of the initial deoxygenation is still controversial and awaits further investigation.

In our previous report on spectrophotometric measurement of the rat somatosensory cortex, we demonstrated the existence of initial deoxygenation by applying simply linear multicomponent analysis [9], which was the same as that of Malonek and Grinvald [1] and Malonek et al. [10]. This analysis assumed that overall changes in optical attenuation were expressed as the summation of the corresponding absorbance terms of each chromophore and the wavelength-independent light-scattering term, and that the optical path length was independent of the wavelength. In turbid media such as living tissue, however, light scattering because of the tissue causes wavelength dependence of the optical path length and largely distorts the absorption [11, 12]. In consideration of this point, Mayhew et al. [13, 14], Jones et al. [15], and Lindauer et al. [7] analyzed the optical data incorporating the wavelength dependence of the photon path length (differential path length) by the Monte Carlo method. Mayhew et al. [13, 14] again detected initial deoxygenation that agreed with previous reports. In contrast, Lindauer et al. [7] showed that differential path length correction eliminated the existence of the initial deoxygenation. The reason for the discrepancy is unknown, though the kind of stimulation, animal preparation, and anesthesia conditions as well as the analytical model for spectral changes are pointed out as some probable causes [16].

In the present study, we focused again on the existence of the initial deoxygenation and the origins of intrinsic optical signals in the early phase during neuronal activation, where wavelength dependence of the path length is introduced into simple linear multicomponent analysis. This dependency was experimentally assessed not by using the Monte Carlo method, but by measuring the absorption spectra of oxyhemoglobin (oxy-Hb) and deoxy-Hb in a tissue-simulating phantom. By separating the effect of light scattering on the signals into two parts, simple loss of light and modification of the hemoglobin absorption as a result of the wavelength-dependent path length, we also evaluated the contribution of the light scattering to the intrinsic signals. We concluded that initial deoxygenation exists in the rat somatosensory cortex with stimulation of the hind paw, and that tissue scattering changes contribute little to intrinsic signals in the early time after an onset of the stimulus.

Part of this study has already been published in abstract form [17].

**METHODS**

**In vitro experiment.**

Preparation of phantom. To obtain the relative optical path length in the cortex between the various wavelengths, we acquired the absorption spectra of oxy-Hb and deoxy-Hb of the reflectance mode in a phantom model of the rat cortex. For the phantom, we used the blood of a rat as the absorber and 10% Intralipid (Fresenius Kabi AB, Uppsala, Sweden) as the scatterer. Intralipid is widely used as a tissue phantom to investigate the propagation of light in tissue [18] and whose optical properties have been measured in a wide wavelength range. These materials were diluted by the saline with phosphate buffer (5 mM K2HPO4/KH2PO4, pH 7.4) to serve as the appropriate concentration. The reduced scattering coefficient ($\mu_s'$) of the phantom was 0.5–5 mm$^{-1}$ (0.5–5% Intralipid), similar to that of the cortex [19, 20]. The hemoglobin concentration in the hemoglobin base (MW 64500) was 25–125 $\mu$M (0.5–5% red blood cells), according to the mean hematocrit of the rat cerebral hemisphere [21, 22].

We also prepared another phantom model that contained the blood of the rat as the absorber and perfluorocarbon (perfluorotributylamine emulsion, PFC) as the scatterer for the experiment to test the validity of our analysis.

Measurement of wavelength dependency of path length. The absorption spectra of phantom models were obtained through a microscope optical system with the reflectance measurements. The phantom, contained in a plastic cell (15 mm $\phi \times$ 10 mm $H$) was set on the stage of an optical microscope (Axioptem, Carl Zeiss, Germany) and epi-illuminated with white light from a stabilized 150 W halogen lamp (KL1500 Electronic; Schott Glaswerke, Germany). Light reflected through the microscope (20× objective lens) was guided to a spectrophotometer (MCPD 2000, Otsuka Electronics, Osaka, Japan) through a quartz light guide (fiber diameter: 2 mm) inserted into the eyepiece segment of the microscope. The optical geometry and measuring system were set to be exactly the same as those of in vivo experiments. For reference, we used the spectra of Intralipid without blood. The oxygen saturation of hemoglobin ($S_O_2$) was changed gradually to 0%, from 100%, by adding yeast (0.1%). The yeast did not affect the spectra under our conditions. A completely deoxygenated state was obtained by the addition of a small amount of sodium hydrosulfit. The absorption spectra of phantom models using PFC instead of Intralipid were also acquired under several $S_O_2$ conditions. Actual $S_O_2$ in each spec-
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A plastic tube whose length was about 2–3 mm shorter than injection needles was vertically fixed on bregma. The tip of the needle was inserted in the sagittal sinus through the tube, and the blood sampling was performed. We chose the range of SO\textsubscript{2} above 30%, where no significant changes of tissue scattering occurred and cytochrome oxidase remained unchanged.

In this experiment, the spectrophotometer was not synchronized with the electrocardiogram and respiration. Nonactivity-dependent change in blood flow by heart beat had no effect in the time resolution of this measurement (the heart beat of rat is about 350 min\textsuperscript{-1}). We canceled the effect of respiration by taking an average of repeatedly measured data.

Spectrophotometric measurements with neural activation. The rats were placed on the stage of a microscope with a cooled charged coupled device (CCD) camera (C4880; Hamamatsu Photonics, Hamamatsu, Japan). First, digitized CCD images at 586 nm before, during, and after stimulus were obtained for each trial at a rate of 500 ms/frame. The changes in the intensity of 586 nm, the isosbestic point of oxy-Hb and deoxy-Hb, reflect changes in the total hemoglobin (total-Hb) concentration. The images were analyzed by pixel-to-pixel subtraction between rest and during and after the stimulus to detect intrinsic signals.

For the spectroscopic analysis, reflected light from the activated area (capillary bed) was guided to a spectrophotometer, and reflection spectra were obtained. A series of reflection spectra (500–650 nm) from the cortex (approximately 100 \mu m in diameter with a 20\times objective lens) was obtained at a rate of 500 ms for each spectrum during 38 s (a 15-s prestimulus period and a 23-s poststimulus onset period). The reference spectrum was taken from the reflected light of white polyvinyl tape placed on the thinned skull window.

Somatosensory electrical stimulation. The stimuli that were used in this study were sequences of electrical pulses to the right hind limb. A pair of needle electrodes was inserted underneath the skin of the plantar and ankle region in the contralateral hind limb, and the posterior tibial nerve was then transcutaneously stimulated with a pulse intensity of 1.7 mA, duration of 0.5 ms, frequency of 5 Hz, and sequence time of 2 s. The interstimulus interval was 4 min.

Spectral analysis. Multicomponent analysis. In turbid systems, the attenuation of incident light is caused by absorption and scattering. Light absorption can be assumed to be expressed as a linear combination of each component that follows the Beer-Lambert law when changes in...
the concentrations of absorbers are small [23]. Optical density (O.D.) is represented by Eq. 1.

\[
\text{O.D.}_\lambda = - \log \{ I_\lambda / I_\lambda^0 \} = \sum \varepsilon_i C_i L_\lambda + K_\lambda
\]  
(1)

where \( I_\lambda \) is reflected light intensity with the absorber and scatterer at wavelength \( \lambda \); \( I_\lambda^0 \) is incident light intensity at \( \lambda \); \( \varepsilon_i \) is the molar extinction coefficient of the \( i \)-th absorber at \( \lambda \); \( C_i \) is the concentration of the \( i \)-th absorber in the media; \( L_\lambda \) is the mean optical path length at \( \lambda \); and \( K_\lambda \) is the light attenuation caused by scattering at \( \lambda \).

Thus the change in optical density is approximately represented by the summation of the above-mentioned factors. The change at time \( t \) after the onset of the stimulus at each wavelength is therefore given by the following equation:

\[
\Delta \text{O.D.}_\lambda(t) = \log \{ I_\lambda(t) / I_\lambda^0(t) \} - \log \{ I_\lambda^0 / I_\lambda^0 \} \\
= \log \{ I_\lambda(t) / I_\lambda(t) \} \\
= L_\lambda \left( \varepsilon_\lambda^{\text{oxy}} \Delta [\text{oxy-Hb}(t)] \right) + \varepsilon_\lambda^{\text{deoxy}} \Delta [\text{deoxy-Hb}(t)] + \Delta K_\lambda(t)
\]  
(2)

where \( I_\lambda \) is reflected light intensity at wavelength \( \lambda \) in the prestimulus state, and \( I_\lambda(t) \) is reflected light intensity at \( t \) (s) after the onset of the stimulus. \( L_\lambda \) is kept almost constant with the changes in the hemoglobin concentration under the range of physiological conditions [23]. \( \Delta [\text{oxy-Hb}(t)] \) and \( \Delta [\text{deoxy-Hb}(t)] \) are changes in concentrations of the oxy-Hb and deoxy-Hb components, respectively. In actual measurements, reflected light intensity of the reference spectrum was used as \( I_\lambda^0 \) instead of the intensity of incident light.

In our previous study [9], a least-squares curve fitting was performed according to Eq. 2 by the use of the extinction coefficients of oxy-Hb and deoxy-Hb in a clear solution, where \( L_\lambda \) was assumed to be wavelength independent.

**Wavelength dependence of path length.** Wavelength dependence of the path length was incorporated into the spectral analysis as follows. Equation 2 can be written as

\[
\Delta \text{O.D.}_\lambda(t) = a_\lambda^{\text{oxy}} \Delta [\text{oxy-Hb}(t)] \\
+ a_\lambda^{\text{deoxy}} \Delta [\text{deoxy-Hb}(t)] + \Delta K_\lambda(t)
\]  
(3)

where \( a_\lambda^{\text{oxy}} \) and \( a_\lambda^{\text{deoxy}} \) are the apparent absorption coefficients of oxy- and deoxy-Hb in turbid media at wavelength \( \lambda \), respectively. These include the path length at wavelength \( \lambda \) i.e., \( a_\lambda = \varepsilon_\lambda L_\lambda \). When a multicomponent least-squares curve fitting is performed based on Eq. 3, we can obtain the time courses of \( \Delta [\text{oxy-Hb}] \) and \( \Delta [\text{deoxy-Hb}] \) from the relative value of \( a_\lambda^{\text{oxy}} \) at each wavelength instead of its absolute value. In this study, \( a_\lambda^{\text{oxy}} \) and \( a_\lambda^{\text{deoxy}} \) as relative ratios in the wavelength range of 500–650 nm were experimentally determined by measuring the absorption spectra of oxy- and deoxy-Hb in the tissue-simulating phantom. \( \Delta K_\lambda(t) \) was assumed to be wavelength-independent in this analysis to emphasize the effect of the wavelength dependence of path length on analytical results.

Changes in the total hemoglobin (total-Hb) concentration were calculated as follows:

\[
\Delta [\text{total-Hb}] = \Delta [\text{oxy-Hb}] + \Delta [\text{deoxy-Hb}]
\]  
(4)

We selected three different wavelength ranges for curve fitting—500–650 nm, 500–586 nm, and 586–650 nm—to test the validity of the fitting procedure.

**Statistical analysis.** The spectrophotometric data were time-locked to the onset of the hind limb electrical stimulation, and the times in the data denote the time after which the data collection started with respect to the onset of stimulation. Averages and standard deviations of the changes in O.D., hemoglobin concentrations, and \( K \) were calculated and tested for significance with the paired \( t \)-test. Unless otherwise noted, we show \( p \) values on the basis of data at the stimulus onset (0 s).

**RESULTS**

**Hemoglobin absorption spectra in tissue phantom**

Figure 1 shows the representative absorption spectra (500–650 nm) of oxy- and deoxy-Hb measured in clear solution (0% of scatter concentration) (a), in the phantom with different Intralipid concentrations of 0.5, 1, and 5% (50 \( \mu \)M Hb) (b), and different Hb concentrations of 25, 50, and 100 \( \mu \)M (1% Intralipid) (c). In Fig. 1(b), the spectra in the scattered medium were significantly distorted compared with the spectra of the clear solution. For example, the relative heights of absorption peaks at 558 and 578 nm to 600 nm were smaller in the scattered medium than in the clear solution. This was mainly caused by the wavelength-dependence of optical path length in turbid media. In Fig. 1(b) and (c), all spectra in the presence of scattering resembled one another. The wavelength dependency of the optical path length was insensitive to the change in \( \mu_s' \) and \( \mu_a' \) in the range of 500–650 nm under our experimental conditions. Figure 2 compares the difference spectra obtained from the phantom (\( \mu_a' = 1 \text{ mm}^{-1}; \) Hb concentration: 50 \( \mu \)M), Hb clear solution, and a rat cortex. The difference spectrum of the rat cortex was achieved by a subtraction of spectra of
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Fig. 1. Absorption spectra (500–650 nm) of oxyhemoglobin (oxy-Hb) and deoxyhemoglobin (deoxy-Hb). (a) Spectra measured in clear solution and (b) those in the phantom with different Intralipid concentrations of 0.5, 1, and 5%. Hemoglobin concentrations of this solution and these phantoms were adjusted to be the same (50 μM). (c) Spectra measured in the phantom with different Hb concentrations of 25, 50, and 100 μM (1% Intralipid). In (b) and (c), spectra were normalized to the peak value of each. Insets show raw spectra obtained with the same reference.

Fig. 2. Difference spectra of hemoglobin at two conditions (different oxygen saturation) obtained from a hemoglobin clear solution (Hb concentration 50 μM), the tissue-simulating phantom (μ₄ₐ: 1 mm⁻¹; Hb concentration: 50 μM), and a rat in vivo cortex. The different spectrum of rat cortex was equivalent to the difference of oxygen saturation by about 30%, which was achieved by a subtraction of spectra of 30% SO₂ from that of 60%. Those of the phantom and solution were equivalent to the difference of oxygen saturation by 25%.

30±2% SO₂ from that of 60±2%. The spectra of the phantom and solution were equivalent to a change of SO₂ of 25%. The spectrum of the phantom was nearly identical to that of rat cortex in contrast to that of clear solution. The magnitude of these difference spectra was relatively small at a shorter wavelength (<600 nm) than that at a longer one (>600 nm).

Oxygen saturation measurements in vitro

To confirm the validity of our analytical method, which incorporated the wavelength-dependent path-length effect estimated by the phantom, we first applied Eq. 3 to in vitro experiments. We successively measured the hemoglobin absorption spectra of the phantom containing 1 vol% red blood cells (50 μM Hb concentration) as the absorber and PFC as the scatterer, with various degrees of SO₂ obtained by changing the oxygenated state from 100 to 0%. The spectra in each stage of SO₂ were then simulated according to Eq. 2 with the spectra of the clear solution or to Eq. 3 with the phantom spectra including the wavelength-dependent path length. The comparison between the calculated SO₂ levels and the measured one with and without correction for the scattering effect are shown in Fig. 3. The results by Eq. 2 (Fig. 3(b)) gave no mirror image of the concentration changes in oxy- and deoxy-Hb. ΔK of the scatter term significantly changed with SO₂, which was expected to be un-
changed during the measurements. In contrast, Eq. 3 (Fig. 3(a)) gave a mirror image of the oxy-Hb and deoxy-Hb changes, and ΔK was almost constant. It seems that the change in ΔK in Eq. 2 included the residuals derived from a lack of consideration of path length effect on absorption.

**Measurements of blood flow change in hypercapnia**

We next applied Eq. 3 to an in vivo experiment. We analyzed the changes in reflection spectra by Eqs. 2 and 3 when rats inhaled 5% CO₂–30% O₂–65% N₂ gas (P_	ext{aO}_2: 90–110 mmHg, P_	ext{aCO}_2: 62–67 mmHg). Figure 4 shows the time courses of the contributions of oxy-Hb, deoxy-Hb, and K to the O.D. changes at 600 nm together with the measured O.D. change after starting the CO₂ inhalation. The ΔO.D. of each component represents the product of the Δconcentration obtained by analyses and each extinction coefficient,

![Figure 3. The analytical results of some hemoglobin spectra, changing oxygen saturation from the 100% oxygenated state to 0%, with the analysis of wavelength-dependent path length (a) and the analysis of constant path length (b). Vertical axes represent the concentration changes of oxy- (●) and deoxy-Hb (■) and the change in K (×) to that of 50% saturation. K represents the wavelength independent component of Eqs. 2, and 3 in the text. These hemoglobin spectra were obtained from a red blood cell (1%) suspension in fluorocarbon (perfluorotributylamine) emulsion. Broken lines show the best fit to the data.](image)

![Figure 4. The time courses of the contribution of each component to the O.D. changes at 600 nm after starting the CO₂ inhalation with the analysis of wavelength-dependent path length (a) and with the analysis of constant path length (b). The value in the horizontal axis indicated the time when the data collection started with respect to the onset of the gas (5% CO₂–30% O₂–65% N₂) exchange. The contribution value (CV) of each component was calculated as follows: oxy-Hb-CV = a^oxy × Δ[oxy-Hb]; deoxy-Hb-CV = a^deoxy × Δ[deoxy-Hb]; K-CV = ΔK; a^oxy, and a^deoxy represents the absorbance of the phantom model (1% Intralipid, 50 μM Hb concentration) at each wavelength in each oxygenated state. The dotted line (——) represents the change in measured O.D.](image)
including path length of 600 nm. As seen in Fig. 4(a) analyzed by Eq. 3, when inspired gas was changed, oxy-Hb gradually increased to reach a plateau. Deoxy-Hb successively decreased up to 200 s and reached a plateau. The scattering term $K$ was increased slightly with the increase in oxy-Hb. In contrast, as seen in Fig. 4(b) analyzed by Eq. 2, the changes in $\Delta K$ were very large, though the profiles of the oxy- and deoxy-Hb were similar to those of Fig. 4(a). In hypercapnia, cerebral blood flow generally increases compared with normocapnia. Therefore the increase in oxy-Hb and the decrease in deoxy-Hb are reasonable. However, large scattering changes because of the volume changes of neurons and glia did not occur in this condition. Thus an analysis with the wavelength-dependent path length was also appropriate for the data obtained by *in vivo* experiments.

**Spectral analyses with wavelength-dependent path length**

A total of 51 spectrophotometric measurements of the activated area (capillary bed) were obtained from 5 rats. The time courses of changes in the $\Delta O.D.$ (poststimulus−prestimulus) at 500–590 nm were

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**Fig. 5.** The time courses of relative changes in the oxy-Hb, deoxy-Hb, and total-Hb concentrations and wavelength-independent term $\Delta K$ at the capillary bed in the wavelength range of 500–650 nm (mean ± SD of 51 trials in 5 rats), analyzed with (a) wavelength-dependent path length of Eq. 3 and (b) constant path length of Eq. 2. Stimulus parameters: 500 μs, 1.7 mA pulse, 5 Hz, 2 s.
monophasic. At 590–650 nm, the change showed a biphasic pattern. The results were consistent with those of a previous report [9] on the optical measurement of activated cortex. All physiologic variables were stable during data acquisition.

The changes in the concentrations of oxy-, deoxy-, and total-Hbs were calculated with a multicomponent analysis based on Eq. 3 by the standard spectra of scattered media of Fig. 1 (1% Intralipid, 50 μM Hb). We first examined the correlation coefficients between the measured spectrum from the rat cortex and the ones reproduced by each analysis, with Eqs. 2 and 3. The correlation coefficient of the analysis with Eq. 3 was 0.995±0.002, and with Eq. 2 it was 0.961±0.002. The residual of the curve fitting was markedly reduced by the present analysis of Eq. 3. Figure 5 shows the time courses of relative changes in the oxy-, deoxy-, and total-Hb concentrations and ΔK at the capillary bed in the wavelength range of 500–650 nm, where the results of Eq. 3 (a) are compared with those of Eq. 2 (b).

In the analysis of Fig. 5(a) with the wavelength-dependent path length of Eq. 3, the oxy-Hb slightly decreased at 0.5–1.5s (1.0s, p=0.0055) and increased to the maximum value at 4.0s (2.0s, p=0.018); it then returned to the baseline level 8.0s after the stimulus onset. The deoxy-Hb increased in the early stage, peaked at 1.5s (1.0s, p=0.0097; 1.5s, p=0.0057), and decreased largely in the late phase to the minimum level at 8.0s. Total-Hb, the sum of oxy-Hb and deoxy-Hb, started to increase at 1.5s (1.5s, p=0.0025), peaked at 3.0s, then returned to the baseline level. Because of the presence of basal hemodynamic fluctuations, we examined the Hb concentration changes in the resting state up to 15s before stimulus onset. No significant changes were found in Hb concentrations at any point in comparison to 1.0, 1.5, and 2.0s before (p>0.09). The change in ΔK showed a simple monophasic pattern; it started to increase at 2.5s and returned to near the baseline at 7.0s. For the ΔK in the early phase (<2.0s), no statistical significance was found.

When the results were compared with those in Fig. 5(b) with the constant path length of Eq. 2, the profiles of changes in oxy-, deoxy-, and total-Hbs were very similar to Fig. 5(a), though the relative amplitudes were different. The profile of ΔK obtained by Eq. 2 was very different from that of Fig. 5(a), which was a biphasic pattern with a large increase that peaked at 2.5s and decreased in the late phase. The early increase in ΔK overlaid the early increase in deoxy-Hb.

Figure 6 shows the time courses of the contributions of each component to the O.D. change at 600 nm. The contribution value (CV) of each component—oxy-Hb-CV (——), deoxy-Hb-CV (— — —), and K-CV (——)—was calculated by the same equation in Fig. 4. The dotted line (——) represents the change in measured O.D.

Effect of the wavelength range for curve fitting

To examine the effect of the wavelength range on the results of analysis, as Mayhew et al. [13] pointed out, we performed curve fitting with the wavelength ranges of 500–586 and 586–650 nm as well as with the whole range of 500–650 nm. Figure 7 shows the changes in oxy- and deoxy-Hbs concentrations at the three different fitting ranges obtained by Eq. 3 (a) and Eq. 2 (b). Figure 7(a) shows very similar behaviors of oxy- and deoxy-Hbs within the limits of experimental error. Those of deoxy-Hb were identical. The profiles of ΔK obtained from different fitting ranges were also similar to one another, having a flat baseline in the early phase (data not shown). For comparison, the results obtained with Eq. 2 are also given (Fig. 7(b)). The profiles were changed by the fitting range. In particular, the peak time and peak ratio (positive peak vs. negative one) of the change in deoxy-Hb were significantly different. The profile of ΔK was very divergent (data not shown).
DISCUSSION

Phantom model and analysis. In the present study, we incorporated the wavelength dependence of the path length into a simple linear multicomponent analysis for intrinsic optical signals during neuronal activation. The wavelength dependency of the path length was experimentally determined by using the optically phantom-simulated rat cortex instead of numerical simulation based on the Monte Carlo method [14, 24]. The method used here was essentially the same as those of our multiwavelength near-infrared spectroscopy algorithm [25]. Here it must be noted that the reflection spectra with the microscopic measurements contained not only the scattering properties of cerebral tissue itself, but also the optical geometry and other instrumentation parameters. The examples of these parameters are the distance and aperture of the lens, which picks up the light only within a very narrow angle range, though the reflected light from the tissue surface has strong angle dependence, which is also wavelength-dependent because of the scattering. Therefore it was more effective when the standard spectra were obtained with exactly the same optical geometry as in the animal experiments that we performed.

To incorporate the wavelength dependence of the path length into spectral analysis, we employed the spectra of turbid media, where $\mu_s$ was chosen to be $1 \text{ mm}^{-1}$ and the hemoglobin concentration was 50 $\mu\text{M}$. The value of $\mu_s$ was based on the data obtained from various brains near 600 nm [19, 20], the directly measured data for piglet brains at 760 nm [26, 27] and for human brains [28]. The value of $\mu_a$ was based on the report that the mean red blood cell (RBC) concentration of the cerebral hemisphere is about 0.9 vol% [21, 29]. This value is transformed to a hemoglobin concentration of 45 $\mu\text{M}$, since the concentration of hemoglobin in rat RBC is 5.0 $\mu\text{M}$ on average. In spectrophotometric measurements of this study, we selected the area of capillary bed avoiding large vessels in rat cortex. The validity of this approximation was confirmed by the difference spectrum of our phantom model very closely resembling that of the rat cortex (Fig. 2). We also confirmed it by analyzing the data by

![Graph showing changes in Δ[oxy-Hb] and Δ[deoxy-Hb] at the capillary bed determined by two analyses with (a) wavelength-dependent path length of Eq. 3 and (b) constant path length of Eq. 2 at three different wavelength ranges: 500–586, 586–650, and 500–650 nm.](image_url)
other spectra with $\mu_r$ of 0.5 and 5 mm$^{-1}$ (50 $\mu$M Hb) and Hb concentration of 25 and 125 $\mu$M ($\mu_r$; 1 mm$^{-1}$). The profiles of $\Delta$[oxy-Hb] and $\Delta$[deoxy-Hb] by other spectra were found to be nearly identical with each other.

The validity of Eq. 3 was also certified by the constancy of the results with the fitting of different wavelength ranges of Fig. 7, where Eq. 2 gave different profiles of hemoglobin with different wavelength ranges. This was because the supposition of the constancy of $L_k$ in all wavelengths could not be maintained when the fitting range was changed.

Furthermore, the similarity of the profile between the fitting range at 586–650 nm and that of 500–586 nm showed that a third component, redox change of cytochrome oxidase, can be eliminated under our conditions, in which the absorption maximum of reduced cytochrome oxidase was at around 600 nm [10, 13, 30–32]. Thus the presence of the redox shift of cytochrome oxidase during the neuronal activation is questioned and awaits further experiments.

**Contribution of light scattering to intrinsic signals.** In Eq. 2, $\Delta K$ includes not only absorption-independent light attenuation, but also the residuals derived from lack of consideration of the scattering effect on absorption, i.e., the wavelength dependence of the path length. In contrast, $\Delta K$ in Eq. 3 simply gives the changes in absorption-independent tissue scattering, since the wavelength dependency of the path length was incorporated into the standard absorption spectra. The difference in $\Delta K$ with and without the wavelength dependency of the path length was further supported by the results for hypercapnia shown in Fig. 4.

Scattering changes with neural activation seem to originate from the changes in the morphology of vasculature and the change in the number of red blood cells [33] in the microcirculatory system (vasodilation), and also in cell conformation and volume changes of neurons and/or glia with excitation such as ion transport and the release of neurotransmitters [34–40]. As for the factor of cell volume changes, the changes in light scattering signals were observed in the blood-free preparations (hippocampal slices and isolated brain). As shown by the comparison in Fig. 5, the early change (≤2 s after stimulus onset) of $\Delta K$ observed in analysis with a clear solution of our previous paper [9] and that of Malonek et al. [10] disappeared in the present analysis. Therefore in the in vivo system of the rat cortex, tissue scattering changes, which seem to be relatively small, contribute little to intrinsic signals in the early time. The $\Delta K$ change in the late time (>2 s) obtained by the present analysis of in vivo experiments (in a blood-involved system) is inferred to mainly represent the change in tissue scattering caused by morphology changes of vasculature and changes in the number of red blood cells because its time course was almost parallel to that of regional cerebral blood flow [9, 10, 41].

**Initial deoxygenation.** In the present study, the presence of initial deoxygenation became clear. In previous studies, the small change of initial deoxygenation might have been masked by changes in the large fitting parameter of $\Delta K$. This uncertainty was overcome in the present analysis, where $\Delta K$ was unchanged in this period. In contrast, others [7, 24] analyzed the optical data with the wavelength dependence of the photon path length (differential path length) estimated by Monte Carlo simulation. They showed that the differential path length correction eliminated the existence of the initial deoxygenation. Moreover, their measurements of phosphorescence lifetimes did not detect the early deoxygenation. They also showed the analytical results with constant path length. The time courses of concentration changes with constant path length analysis and with wavelength-dependent path length was different from ours, especially for $\Delta$[oxy-Hb]. Therefore the discrepancy in the change in the deoxy-Hb concentration by each method of the wavelength dependent path length seems to be ascribable to the difference of experimental procedures and to the difference in the analytical models [16]. The stimulus and area of brain function might cause differences in oxygen consumption and cerebral blood flow changes quantitatively and temporally.

**The origin of the intrinsic signals at representative wavelengths in the early phase.** It is generally accepted that the absorbance (O.D.) changes (increase at an early time) at 600–610 nm can be mainly assigned to the changes in deoxy-Hb or scattering that directly reflect neural activation [38, 40, 42]. The contribution of the changes significantly depends on the time after the stimulus onset. It is noted that the oxy-Hb component rather than the deoxy-Hb one mainly contributed to the peak of the O.D. change at 600 nm (at about 2 s after stimulus onset) in our experimental conditions. The deoxy-Hb component was predominant in the early time just after starting to change (at 1–1.5 s after stimulus onset) (Fig. 6). On the other hand, the scattering changes with neural activation hardly contributed to the O.D. change within 1–1.5 s after the stimulus onset in the presence of blood. This was confirmed by the PFC-substituted rat of Nomura et al. [43]. It is suggested that when we perform optical measurement at a wavelength of 600 nm up to 1.5 s after stimulus onset,
we can obtain mapping that closely reflects neuronal activation as the deoxy-Hb change, eliminating the microcirculation changes without multiv wavelength spectrophotometric analysis in each area. In contrast, the change in O.D. at 558 nm, the wavelength of the deoxy-Hb peak, is not suited for a mapping of the early phase, since the O.D. changes were smaller than that of 600 nm in this period.

In summary, by incorporating the wavelength dependence of optical path length in the analysis, we found that the changes in the scattering component become negligible in the early phase after stimulus onset. Therefore the interference of tissue scattering can be successfully removed from the changes in intrinsic signals in the early phase. Thus we confirmed again that in the rat somatosensory cortex, there is an increase in the concentration of deoxy-Hb (initial deoxygenation) in the early time after the onset of electrical stimulation of the hind limb.

REFERENCES