Video Article

Simultaneous Evaluation of Cerebral Hemodynamics and Light Scattering Properties of the *In Vivo* Rat Brain Using Multispectral Diffuse Reflectance Imaging

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Abstract

The simultaneous evaluation of cerebral hemodynamics and the light scattering properties of *in vivo* rat brain tissue is demonstrated using a conventional multispectral diffuse reflectance imaging system. This system is constructed from a broadband white light source, a motorized filter wheel with a set of narrowband interference filters, a light guide, a collecting lens, a video zoom lens, and a monochromatic charged-coupled device (CCD) camera. An ellipsoidal cranial window is made in the skull bone of a rat under isoflurane anesthesia to capture *in vivo* multispectral diffuse reflectance images of the cortical surface. Regulation of the fraction of inspired oxygen using a gas mixture device enables the induction of different respiratory states such as normoxia, hyperoxia, and anoxia. A Monte Carlo simulation-based multiple regression analysis for the measured multispectral diffuse reflectance images at nine wavelengths (500, 520, 540, 560, 570, 580, 600, 730, and 760 nm) is then performed to visualize the two-dimensional maps of hemodynamics and the light scattering properties of the *in vivo* rat brain.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55399/>

Introduction

Multispectral diffuse reflectance imaging is the most common technique for obtaining a spatial map of intrinsic optical signals (IOSs) in cortical tissue. IOSs observed in the *in vivo* brain are mainly attributed to three phenomena: variations in light absorption and scattering properties due to cortical hemodynamics, variation in absorption depending on the reduction or oxidization of cytochromes in mitochondria, and variations in light scattering properties induced by morphological alterations¹.

Light in the visible (VIS) to near-infrared (NIR) spectral range is effectively absorbed and scattered by biological tissue. The diffuse reflectance spectrum of the *in vivo* brain is characterized by absorption and scattering spectra. The reduced scattering coefficients μ_s' of brain tissue in the VIS-to-NIR wavelength range result in a monotonous scattering spectrum exhibiting smaller magnitudes at longer wavelengths. The reduced scattering coefficient spectrum $\mu_s'(\lambda)$ can be approximated to be in the form of the power law function^{2,3} as $\mu_s'(\lambda) = a \times \lambda^b$. The scattering power b is related to the size of biological scatterers in living tissue^{2,3}. Morphological alterations of the tissue and reduction of the viability of living cortical tissue can affect the size of the biological scatterers^{4,5,6,7,8,9}.

An optical system for multispectral diffuse reflectance imaging can be easily constructed from an incandescent light source, simple optical components, and a monochromatic charged-coupled device (CCD). Therefore, various algorithms and optical systems for multispectral diffuse reflectance imaging have been used to evaluate cortical hemodynamics and/or tissue morphology^{10,11,12,13,14,15,16,17,18}.

The method described in this article is used to visualize both the hemodynamics and light scattering properties of rat cerebral tissue *in vivo* using a conventional multispectral diffuse reflectance imaging system. The advantages of this method over alternative techniques are the ability to evaluate spatiotemporal changes in both cerebral hemodynamics and cortical tissue morphology, as well as its applicability to various brain dysfunction animal models. Therefore, the method will be appropriate for investigations of traumatic brain injury, epileptic seizure, stroke, and ischemia.

Protocol

Animal care, preparation, and experimental protocols were approved by the Animal Research Committee of Tokyo University of Agriculture and Technology. For this methodology, the rat is housed in a controlled environment (24 °C, 12 h light/dark cycle), with food and water available *ad libitum*.

1. Construction of a Conventional Multispectral Diffuse Reflectance Imaging System

1. Mount nine narrowband optical interference filters with center wavelengths of 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm to the filter holes of the motorized filter wheel.
2. Construct a multispectral imaging system using a broadband white light source, a motorized filter wheel with the above set of narrowband interference filters, a light guide, a collecting lens, a video zoom lens, and a monochromatic CCD camera. The layout of optical components, shown in **Figure 2**, can be referred to for this construction procedure.
NOTE: The angle of illumination is approximately 45° with respect to the sample surface.
3. Turn on the halogen lamp light source to illuminate the surface of the sample via an interference filter, the light guide, and the collecting lens.
4. Open the operating software of the CCD camera.

2. Animal Preparation

NOTE: In this protocol, the rat was not used for the future experiments and it was sacrificed immediately after the measurements of multispectral images.

1. Connect the inlet port of an induction chamber to the outlet port of an anesthesia machine with a tube. Connect the outlet port of the induction chamber to the inlet port of the anesthesia machine with a second tube.
2. Place the rat into the induction chamber and induce anesthesia with 5.0% isoflurane. Maintain anesthesia at a depth such that the rat does not respond to toe pinches. Lower to 2.0% isoflurane using a rotary knob on the anesthesia machine.
3. Fix the rat head in a stereotaxic frame. Attach a mouthpiece for anesthesia to the stereotaxic frame.
4. Connect the inlet port of the mouthpiece to the outlet port of the anesthesia machine with a tube. Connect the outlet port of the mouthpiece to the inlet port of the anesthesia machine with a tube.
5. Shave the head region beyond the prospective incision site using hair clippers until the skin surface appears.
6. Make a longitudinal incision approximately 20 mm long along the midline of the head using a surgical scalpel (**Figure 1(a)**) and expose the subcutaneous connective tissues (**Figure 1(b)**).
7. Remove the subcutaneous connective tissues using a sharp curette or a pincer and pull it to both sides of the head to expose the skull bone (**Figure 1(c)**).
8. Dig an ellipsoidal ditch on the skull bone inside the cranial sutures (coronal suture, sagittal suture, and lambdoid suture) using a high-speed drill (**Figure 1(d)**).
9. Slowly and homogeneously excavate the skull bone inside the ditch using the high-speed drill.
10. Press lightly on the surface of the thinned skull with the tip of the pincer to estimate the bone thickness and strength after a cerebral blood vessel appears. If the thinned skull region depresses easily, terminate the reduction of the skull bone with the high-speed drill.
11. Cut the ellipsoidal border line of the thinned skull piecemeal using the tip of the pincer or small surgical scissors.
12. Remove the thinned skull from the brain surface slowly and gently using the pincer.
13. Gently bathe the cranial window with physiological saline and cover it with a transparent glass plate approximately 0.1 mm thick.

3. Regulating the Fraction of Inspired Oxygen

NOTE: The respiratory condition can be changed by regulating the fraction of inspired oxygen (FiO_2).

1. Using a tube, connect the first port of a Y-shaped tube connector (connector 1) to the first port of another Y-shaped tube connector (connector 2).
2. Connect the inlet port of the mouthpiece to the second port of tube connector 1.
3. Using a tube, connect the third port of tube connector 1 to an oxygen concentration monitor device.
4. With a tube, connect the second port of tube connector 2 to the outlet port of an anesthesia machine.
5. Using a tube, connect the third port of tube connector 2 to the outlet port of a gas mixture device.
6. Connect one inlet port of the gas mixture device to a high-pressure 95% O_2 - 5% CO_2 gas cylinder using a tube.
7. Connect the other inlet port of the gas mixture device to a high-pressure 95% N_2 - 5% CO_2 gas cylinder using a tube.
8. Change the gas flow rates of O_2 and N_2 using the rotary knobs on the gas mixture device.
9. Check and regulate the FiO_2 using the oxygen concentration monitor device.

4. Acquisition of the Multispectral Diffuse Reflectance Images

1. Acquisition of reference images

NOTE: The optical components used in this experiment, such as the light source, optical fiber, and detectors have their own spectral characteristics. Therefore, the intensity of light passed through these components should be recorded as a reference image. The reference image is an image taken with a standard white diffuser illuminated with the light from the light source.

1. Put the standard white diffuser on the stage horizontally.
2. Focus the camera lens on the surface of the white diffuser by rotating the zoom ring on the barrel.

- Adjust the integration time of the camera by selecting the appropriate value from the drop-down list of integration times in the operating software of the camera so that the greatest amount of light produces a signal that is approximately 75% of the maximum counts. While watching the histogram of pixel values, adjust the integration time until the signal intensity level is approximately 75% of the maximum counts.
- Select the "save" command from the file menu to save an image to a file.
- Change the filter location by rotating the filter wheel.
- Save an image at the other wavelengths according to the process described above. The file name should identify the sample and the wavelength used (e.g., W500, W520, W540 ... W760).

2. Acquisition of sample images

Note: Images of diffusely-reflected light intensity of exposed rat brain at nine wavelengths are captured and saved on the hard drive of a personal computer using the same acquisition conditions.

- Gently place the rat on the stage and slowly adjust the stage level so that the camera can focus on the surface of the rat brain.
- Select the "save" command from the file menu to save an image to a file.
- Change the filter location by rotating the filter wheel.
- Save an image at the other wavelengths according to the process described above. The file name should identify the sample and the wavelength (e.g., R500, R520, R540 ... R760).

3. Acquisition of dark images

NOTE: The CCD camera can generate a light intensity in response to an electrical signal. However, there is some minor output due to noise in the electric circuits and detectors, even if light does not enter to the detector; this is called dark current noise. To accurately measure the spectral intensity of light, the dark current component should be recorded as a dark image and then subtracted from the measured signal. The dark image is an image taken with the light path blocked.

- Turn off the halogen lamp light source.
- Block the light path to the CCD camera system using a shielding plate.
- Select the "save" command from the file menu to save an image to a file. The file name should identify the sample (e.g., Dark).

5. Visualizing the Hemoglobin Content and the Light Scattering Parameter

NOTE: A set of multispectral diffuse reflectance images is saved to the hard drive of a personal computer and analyzed offline. A multiple regression analysis aided by a Monte Carlo simulation¹⁹ of the multispectral diffuse reflectance images at nine wavelengths (500, 520, 540, 560, 570, 580, 600, 730, and 760 nm) is then performed to visualize the two-dimensional maps of oxygenated hemoglobin concentration, deoxygenated hemoglobin concentration, total hemoglobin concentration, regional cerebral oxygen saturation, and scattering power. The detailed algorithm has been published in the literatures^{17,18}.

- Subtract the dark image from both the reference image and the sample image at each wavelength.
- Normalize the sample image by the reference image at each wavelength λ . Treat the normalized image as the diffuse reflectance image R .
- Calculate the absorbance (or optical density) image A by taking the logarithm of the reciprocal of the diffuse reflectance image R at each wavelength λ :

$$A(\lambda) = \log_{10} \left\{ \frac{1}{R(\lambda)} \right\} \quad (1)$$

- Generate a three-dimensional matrix by stacking the absorbance images in the order of their wavelengths, where the x-y plane shows the structural information obtained for the brain surface and the z-axis shows the spectral information.
- Perform a multiple regression analysis for the absorbance spectrum $A(\lambda)$ at each x-y coordinate.
- Use the absorbance spectrum $A(\lambda)$ as the dependent variable and the molar extinction coefficient spectra of oxygenated hemoglobin $\epsilon_{HbO}(\lambda)$ and deoxygenated hemoglobin $\epsilon_{HbR}(\lambda)$ as the independent variables for step 5.5 (published values for $\epsilon_{HbO}(\lambda)$ and $\epsilon_{HbR}(\lambda)$ are provided in **Table 1**).
- Check the two-dimensional maps (images) of the three multiple regression coefficients a_{HbO} , a_{HbR} , and a_0 .
- Generate a three-dimensional matrix by stacking the images of the multiple regression coefficients in the order a_{HbO} , a_{HbR} , and a_0 , where the x-y plane shows the structural information obtained for the brain surface and the z-axis shows the multiple regression coefficients.
- Calculate the oxygenated hemoglobin concentration C_{HbO} , the deoxygenated hemoglobin concentration C_{HbR} , and the scattering power b from the set of multiple regression coefficients a_{HbO} , a_{HbR} , and a_0 at each x-y coordinate using the following empirical formulae (the values of $\beta_{HbO,i}$, $\beta_{HbR,i}$, and $\beta_{0,i}$ ($i = 0, 1, 2, 3$) are provided in **Table 2**):

$$C_{HbO} = \beta_{HbO,0} + \beta_{HbO,1} \times a_{HbO} + \beta_{HbO,2} \times a_{HbR} + \beta_{HbO,3} \times a_0 \quad (2)$$

$$C_{HbR} = \beta_{HbR,0} + \beta_{HbR,1} \times a_{HbO} + \beta_{HbR,2} \times a_{HbR} + \beta_{HbR,3} \times a_0 \quad (3)$$

$$b = \beta_{b,0} + \beta_{b,1} \times a_{HbO} + \beta_{b,2} \times a_{HbR} + \beta_{b,3} \times a_0 \quad (4)$$

- Check the two-dimensional maps (images) of the oxygenated hemoglobin concentration C_{HbO} , the deoxygenated hemoglobin concentration C_{HbR} , and the scattering power b .
- Calculate a two-dimensional map of the total hemoglobin concentration C_{HbT} by summing C_{HbO} and C_{HbR} at each x-y coordinate.
- Calculate a two-dimensional map of the regional cerebral oxygen saturation rSO_2 by dividing the oxygenated hemoglobin concentration C_{HbO} by the total hemoglobin concentration C_{HbT} at each x-y coordinate.

Representative Results

Representative spectral images of diffuse reflectance acquired from *in vivo* rat brains are shown in **Figure 3**. The images at 500, 520, 540, 560, 570, and 580 nm clearly visualize a dense network of blood vessels in the cerebral cortex. The deterioration of contrast between blood vessels and the surrounding tissue observed in the images at 600, 730, and 760 nm reflects the lower absorption of light by hemoglobin at longer and NIR wavelengths.

Figure 4 shows representative estimated images of an exposed rat brain for oxygenated hemoglobin concentration, deoxygenated hemoglobin concentration, total hemoglobin concentration, regional cerebral oxygen saturation, and scattering power. As expected from the diffuse reflectance images at shorter wavelengths in **Figure 3**, the total hemoglobin concentration in the blood vessel region is higher than that in the surrounding tissue region. On the other hand, the oxygenated hemoglobin concentrations in arterioles are higher than those in venules due to the hemoglobin in arterial blood being much more oxygenated than in venous blood. Therefore, the distribution of arterioles and venules can be clearly distinguished in the estimated image of regional oxygen saturation.

Representative estimated images of an exposed rat brain during changes in FiO_2 for diffuse reflectance at 500 nm $r(500)$, concentration of oxygenated hemoglobin C_{HbO_2} , concentration of deoxygenated hemoglobin C_{HbR} , concentration of total hemoglobin C_{HbT} , regional cerebral oxygen saturation $r\text{SO}_2$, and scattering power b are shown in **Figure 5**. The value of $r\text{SO}_2$ increased under hyperoxic conditions and decreased remarkably after the induction of anoxic conditions. The value of b was slightly increased during the period from the onset of anoxia until respiratory arrest, whereas it continuously decreased during the period from 5 min to 30 min after the onset of anoxia. These changes in the value of b were indicative of morphological changes, such as the swelling and shrinkage of cellular and subcellular structures, induced by the loss of tissue viability in brain.

Figure 1: Steps in the Surgical Exposure of the Rat Cerebral Cortex. [Please click here to view a larger version of this figure.](#)

Figure 2: Schematic Diagram of the Experimental Apparatus for Administering Anesthesia and Changing the Fraction of Inspired Oxygen. [Please click here to view a larger version of this figure.](#)

Figure 3: Representative Multispectral Diffuse Reflectance Images at 500, 520, 540, 560, 570, 580, 600, 730, and 760 nm, Obtained from an *In Vivo* Rat Brain. [Please click here to view a larger version of this figure.](#)

Figure 4: Representative Estimated Images of an Exposed Rat Brain. (a) Concentration of oxygenated hemoglobin C_{HbO_2} , (b) concentration of deoxygenated hemoglobin C_{HbR} , (c) concentration of total hemoglobin C_{HbT} , (d) regional cerebral oxygen saturation rSO_2 , and (e) scattering power b . [Please click here to view a larger version of this figure.](#)

Figure 5: Representative Results of an Exposed Rat Brain During Changes in FiO_2 . Images of *in vivo* rat cortical tissue during changes in FiO_2 for diffuse reflectance at 500 nm $r(500)$, concentration of oxygenated hemoglobin C_{HbO} , concentration of deoxygenated hemoglobin C_{HbR} , concentration of total hemoglobin C_{HbT} , regional cerebral oxygen saturation $r\text{SO}_2$, and scattering power b . [Please click here to view a larger version of this figure.](#)

Wavelength λ nm	$\epsilon_{\text{HbO}} (\lambda)$	$\epsilon_{\text{HbR}} (\lambda)$
500	113.03712	112.6548
520	130.69296	170.58384
540	287.4744	251.5968
560	176.11128	290.4552
570	240.2784	243.3888
580	270.5616	199.908
600	17.28	79.25688
730	2.106	5.95188
760	3.1644	8.36201

Table 1: The Values of ϵ_{HbO} and ϵ_{HbR} used for the Multiple Regression Analysis. The molar extinction coefficients of oxygenated hemoglobin ϵ_{HbO} and deoxygenated hemoglobin ϵ_{HbR} at each wavelength λ .

i	$\beta_{HbO,i}$	$\beta_{HbR,i}$	$\beta_{b,i}$
0	-8.3302	-5.85271	-0.76587
1	4405.877	-143.23	53.34134
2	2740.622	3798.067	124.4656
3	-4.40454	-2.81699	-1.36919

Table 2: The Values of $\beta_{HbO,i}$, $\beta_{HbR,i}$ and $\beta_{b,i}$ ($i = 0,1,2,3$) used in the Empirical Formulae for C_{HbO} , C_{HbR} , and b . Note that the units of C_{HbO} and C_{HbR} derived from these empirical formulae are the volume concentration, in which the hemoglobin concentration of whole blood with a hematocrit reading of 44% is taken to be the 100% volume concentration of hemoglobin. The empirical formulae for hemoglobin concentrations can be derived from the diffuse reflectance spectra calculated by the Monte Carlo simulation of light transport¹⁹. The detailed process for the derivation of the empirical formulae has been described in the literature^{17,18}.

Discussion

The most critical step in this protocol is the removal of the thinned skull region to make the cranial window; this should be performed carefully to avoid unexpected bleeding. This step is important for obtaining high-quality multispectral diffuse reflectance images with high accuracy. The use of a stereomicroscope is recommended for the surgical procedure if possible. Small pieces of gelatin sponge are useful for hemostasis.

The optical system described in this article passes a monochromatic light through an interference filter located in front of the light source. This can be modified by placing the filter wheel in front of the video camera lens or CCD camera. In this case, however, the focal plane can be variable if interference filters with different thicknesses are used, and this will cause a deterioration of the image quality. It is necessary to remove the glass plate from the cranial window if a recording electrode is inserted into the cortical tissue for electrophysiology measurements, such as measurements of the electrical local field potential. In this case, the imaging system can detect undesirable specular reflection from the cortical surface. This problem can be avoided by using a set of polarization plates with a crossed Nicols alignment.

The conventional multispectral imaging apparatus demonstrated in this article is somewhat time-consuming to use, since the filter positions in the wheel are changed mechanically. This means that the imaging system captures each diffuse reflectance image sequentially at a different wavelength-point. Because of this limitation, this system is inadequate to capture fast IOSs, such as changes in the reflectance spectrum due to neuronal activities²⁰. Although oxygenated hemoglobin and deoxygenated hemoglobin are the main chromophores in the living brain tissue, the other chromophores, such as cytochrome c oxidase, flavin adenine dinucleotide and nicotinamide adenine dinucleotide, also contribute to the absorption coefficient in the visible wavelength region. Therefore, the estimated values of C_{HbO} , C_{HbR} , C_{HbT} , rSO_2 , and b can be affected by the minor chromophores. Moreover, this approach integrates all information along the depth direction because it relies on diffuse reflection. Therefore, the imaging system does not perform depth-resolved measurements.

It is advantageous that the algorithm used for the present system can also be applied to multispectral diffuse reflectance images captured by other rapid spectral imaging techniques, such as an acousto-optical tunable filter²¹, a multi-aperture lenslet array with interference filters²², and the spectral reconstruction images from an RGB image^{17,23}. Using the proposed algorithm and rapid spectral techniques together is a promising approach for evaluating fast IOS imaging, as well as for use in clinical situations.

Most multispectral brain imaging techniques to date have mainly focused on cortical hemodynamics and tissue metabolism, such as cerebral blood volume, regional cerebral oxygen saturation, and cerebral metabolic rate of oxygen^{10,11,12,13,14}. Several existing approaches evaluate the scattering amplitude under the assumption that the scattering power is constant^{15,16}. However, morphological alterations of tissues due to pathophysiological changes and a reduction of viability in living cortical tissue can affect the size of biological scatterers^{4,5,6,7,8,9}. Therefore, it is important to estimate the scattering parameter of b quantitatively to evaluate the tissue morphologies of the brain. The significance of the present technique with respect to existing methods is its ability to simultaneously measure the spatiotemporal changes in cerebral hemodynamics and cortical tissue morphology.

In terms of future applications, this algorithm can be used for monitoring brain function, vitals, and viability in the cortical tissue of various brain disorder animal models, such as traumatic brain injury, epileptic seizure, stroke, and ischemia.

Disclosures

The authors have nothing to disclose.

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