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# Monitoring angiogenesis noninvasively with near-infrared spectroscopy

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**Abstract.** Near-infrared (NIR) spectroscopy is used to quantify cerebral blood volume (CBV) as a marker of angiogenesis (formation of new blood vessels). Rats are exposed to chronic hypoxia for 3 weeks at half atmospheric pressure to stimulate angiogenesis, and second-differential NIR spectroscopy is used to quantify total cerebral hemoglobin before and after angiogenesis. The cerebral hemoglobin (from broadband NIR spectroscopy), and the large vessel hemoglobin and hematocrit (from blood samples), are used to derive values for the calculation of CBV. The total hemoglobin in brain is  $46.6 \pm 1.9 \mu\text{mol/l}$  (mean  $\pm$  SD,  $n=5$ ) preacclimation and increases by 72% postacclimation. CBV is initially  $3.26 \pm 0.41\%$  v/v and increases by 31% with acclimation. Each individual animal shows a measurable increase in CBV. This study indicates that NIR broadband spectroscopy can be used for repeated measurements of CBV and can be applied as a noninvasive method to study angiogenesis. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3000431]

Keywords: near-infrared spectroscopy; cerebral blood volume; angiogenesis; hypoxia; anoxia; brain.

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## 1 Introduction

Recent developments in such disparate conditions such as cancer, stroke, vascular disease, diabetes, and high altitude illnesses indicate that angiogenesis is a key area of interest, and the equipment that can be used to study angiogenesis longitudinally is needed. For brain studies, cerebral blood volume (CBV) can be used as a measure of angiogenesis following ischemia<sup>1</sup> and hypoxic exposure.<sup>2</sup>

A wide range of options are available for repeated measurements of CBV, which include dynamic contrast-based methods using MRI, CT, and NIR techniques.<sup>3-6</sup> Steady-state methods involving the use of contrast agents are also used.<sup>2</sup>

The need for either expensive instrumentation or an exogenous contrast agent poses an impediment to the use of CBV measurements. While there is a large body of literature relating to the use of NIR spectroscopy to follow qualitative (or occasionally, quantitative) changes in blood volume,<sup>5,7-10</sup> absolute quantification by NIR spectroscopy without exogenous contrast agents is less common.<sup>8,11</sup>

In this study, we hypothesize that the changes in CBV due to angiogenesis can be estimated by a noninvasive measurement of total hemoglobin (Hbt). We implemented a broadband NIR detection system (relatively inexpensive and simple to construct, as it is continuous wave) with quantification using a second differential analysis of the attenuation spectra and an anoxic period for calibration.<sup>12,13</sup>

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We propose to apply this method to estimate CBV as a marker of angiogenesis. Hbt concentration is estimated from the peak concentration of deoxyhemoglobin during anoxia. CBV is then derived from the concentration of Hbt. We applied this system to study angiogenesis in rat brain after stimulation of angiogenesis by chronic hypoxic exposure, which results in increase of blood vessel density by approximately 50%.<sup>14</sup>

## 2 Methods

Male Wistar rats (Charles River, 175 to 200 g, n=5) were examined before (preacclimation) and after 3 weeks exposure to hypobaric hypoxia (postacclimation) using a custom built hypoxic chamber maintained at  $370 \pm 2.1$  mmHg, (mean  $\pm$  SD). Postacclimated animals were studied 24 h after removal to minimize the influence of posthypoxic hypercapnia, which can stimulate cerebral blood flow.<sup>15</sup> Animals were anaesthetized with up to 2% isoflurane with 30% O<sub>2</sub> and spontaneously ventilated. Arterial hemoglobin saturation was between 94 and 98% between subjects and monitored with a Nonin pulse oximeter (Plymouth, Minnesota). Blood (200  $\mu$ l) was obtained from the tail vein and analyzed with a blood gas analyzer (Stat Profile CCX, Nova Biomedical Corporation, Waltham, Massachusetts).

NIR spectroscopic measurements were made using a custom built system. A broadband 100-W quartz-tungsten halogen lamp (model 77501, Oriel Instruments Incorporated, Stratford, Connecticut) was used for illumination via a borosilicate fiber bundle (3 mm in optode diameter) with peak transmission range of 700 to 900 nm (Techen, Milford, Massachusetts). Attenuated light was transmitted via a second identical fiber bundle to a spectrograph (Shamrock 303i, Andor Technology Incorporated, Northern Ireland) with a 10- $\mu$ m slit. The grating (1501 lines/mm to 300-nm spacing, providing a wavelength range of 716.53 to 983.86 nm) dispersed the spectra onto a charge-coupled device (CCD) camera (DU420-BR-DD, Andor Technology Incorporated, Northern Ireland). Data were acquired using custom written MATLAB software (MathWorks, Natick, Massachusetts). The pair of optodes was pressed into the scalp above the midline after removing the fur, aligning with stereotaxic marks at 5 mm ahead of the interaural line. Probes were positioned 7 mm apart. Light intensity for calibration and studies was adjusted using the source diaphragm to prevent saturation in the CCD camera.

Wavelength calibration was done using a reference neon spectrum. Reference (single-beam) spectra were measured for intensity calibration before each study. The reference spectra  $I_{ref}$  were measured for light transmitted directly across the input-output optode pair. The attenuation spectra  $A_{obj}$  of interest was calculated by:  $A_{obj} = -\log(I_{obj}/I_{ref})$ , where  $A_{obj}$  is the desired pseudoabsorbance spectrum of the object, and  $I_{obj}$  is the measured single-beam intensity spectrum passing through the object (rat brain).

The Hbt was determined by assuming that a brief pulse of anoxia would convert all hemoglobin to the deoxy form and so Hbt would equal deoxyhemoglobin (Hb). The Hbt obtained was used in the calculation of CBV:  $CBV(vol/vol\%) = ([Hbt]k/HR) \times 10^{-4}$ , where  $k=64500$  g/mol (molecular

weight of hemoglobin),  $H$  is the large vessel hemoglobin content obtained from the blood sample, and  $R$  is the ratio of small to large vessel hematocrit set at 0.61.<sup>16</sup>

A differentiation program including Savitzky-Golay smoothing (SAVGOL)<sup>17</sup> was used to calculate the second derivative spectrum from the pseudoabsorbance spectra. The spectrum from 720 to 840 nm was analyzed. The region from 810 to 840 nm was assigned to water and that of 740 to 810 nm to Hb. The water feature was used to standardize the Hb concentration to water content<sup>8</sup> at 80%.<sup>18</sup> This assumes that there is no change in water content in the study group. It has been shown that water content in rat brain does not change with chronic hypoxia.<sup>14</sup>

For CBV measurements, the inspired gas was set to zero oxygen. The concentration of oxygenated hemoglobin (HbO) is converted to Hb.<sup>13,19</sup> Hb was determined by the second derivative of the attenuation spectra, carrying out a least-squares fit with the two component spectra (second derivative spectra of Hb and water) that contribute between 716.53 and 983.86 nm. The measured maximum Hb concentration (as determined via the method of Matcher and Cooper<sup>12</sup>) during anoxia then equates to the Hbt, and the HbO concentration may thus be evaluated as Hbt-Hb. Mean cerebral hemoglobin saturation (SmcO<sub>2</sub>) in the microvasculature was calculated as HbO/Hbt.

Pre- and postacclimation CBV and other physiological parameters were compared using a paired t-test, where  $p < 0.05$  was considered significant. Experiments were approved by the Animal Care Committee of the University of Calgary and conform to the guidelines established by the Canadian Council on Animal Care.

## 3 Results

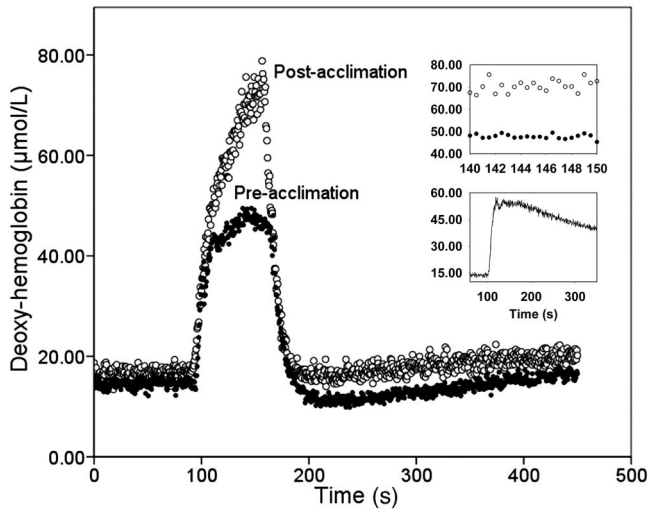
Figure 1 shows deoxyhemoglobin concentrations derived from NIR spectra during a representative anoxic pulse to obtain Hbt. After a stable baseline reading was obtained, the inspired gas was changed to 0% O<sub>2</sub> for 50 s to generate anoxia. The two time lines shown, one from the preacclimation condition and one from the same animal after acclimation, serve to show the significant increase in Hbt in the same rat brain before and after acclimation to hypoxia.

The increase in Hbt reflects increases in both blood hemoglobin concentration and CBV (Table 1). The mean Hct (from tail vein blood samples) increased by 39% and the Hbt increased by 72% with acclimation to hypoxia. Table 1 summarizes physiological data and the mean CBV values before and after acclimation to hypoxia. Repeated measurements in individual animals showed an increase in CBV in all subjects (Fig. 2), with the group mean increasing by 30% and coefficient of variation of approximately 12%.

## 4 Discussion

To validate a method for studying angiogenesis using CBV as a marker, we used a model where angiogenesis could be stimulated in a reproducible fashion. After 3 weeks of exposure to  $\frac{1}{2}$  atm pressure, cerebral capillary density has been shown to increase by approximately 50%.<sup>2</sup>

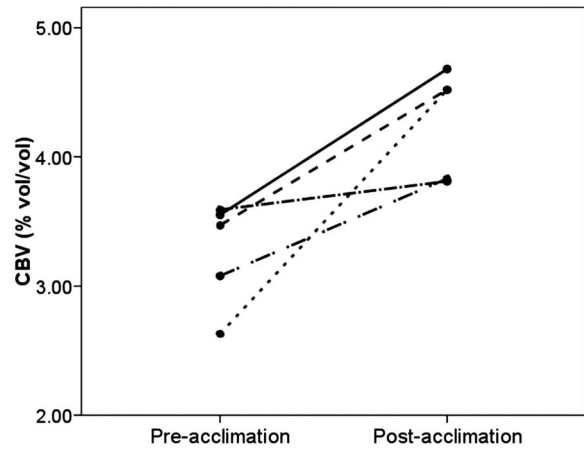
One of the assumptions is that the anoxia pulse is sufficient to convert microvascular hemoglobin to deoxyhemoglobin.



**Fig. 1** Changes in deoxyhemoglobin (Hb) during the calibration pulse of anoxia. The inspired gas was set to zero O<sub>2</sub> for 50 s. Total hemoglobin (Hbt) concentration is postulated to be equal to the maximum Hb concentration, under the assumption that all hemoglobin is deoxygenated. The top insert shows the last 10 s of the 50-s anoxia pulse. The bottom insert shows Hb from a subject where the anoxia pulse was extended to 250 s. These show that the maximum Hb occurs during a relatively stable period around 50 s, and more extended periods result in a reduction of Hb. The two traces show the Hb time courses for the same animal before and after acclimation to hypoxia. The large increase in Hbt postacclimation is due to a combination of increased hematocrit and increased CBV.

Figure 1 is consistent with this, since it shows, in a preacclimated subject, stabilization of hemoglobin into the deoxygenated form after 50 s of anoxia, and that longer periods of anoxia cause a decrease in Hb (possibly due to cardiovascular impairment). In a preliminary study, we verified that 50 s was in the range where the Hb was maximum, after which it began to decline. Since these validation studies were done on preacclimated animals, and since postacclimated animals have a higher Hbt, it is possible that a steady state was not reached in the postacclimated animals. This would serve to underestimate the CBV of the postacclimated subjects. The similarity of the coefficients of variation for CBV values pre- and post-acclimation (12.6% versus 9.8%, respectively) lends evidence that the calibration is consistent for pre- and postacclimation measurements.

The broadband second differential method provided good reproducibility in CBV measurements, as evidenced by the consistency among the preacclimation values (Table 1). Hypoxia-induced variation in the differential path length



**Fig. 2** Changes in CBV for the five individual animals measured pre- and postacclimation to chronic hypoxia. Each line represents data from one subject.

could confound the results.<sup>20</sup> The fact that a ratio between water and deoxyhemoglobin is used both during normoxia and hypoxia will minimize any potential problem. We also fit only water and hemoglobin in the second differential spectra, between the wavelengths of 710 and 840 nm, as previously recommended.<sup>20</sup> Finally, as we applied the same calibration in all animals, any systematic error would not influence the proportion of change between studies. The study was done in a minimally invasive fashion to facilitate repeated measurements. Only the hair was removed, the animal anesthetized, and a few µl of blood was obtained. The SmcO<sub>2</sub> increased from 72% preacclimation to 80% postacclimation on average. This indicates that the acclimation-induced changes in the brain included an increase in brain oxygenation. This is consistent with an increase in brain tissue pO<sub>2</sub> measured after acclimation reported in the same model.<sup>21</sup>

The initial CBV of 3.26% is consistent with the value of 3% measured using MRI and contrast agents,<sup>2,22</sup> 3.8% measured with radio-labeled red blood cells and plasma,<sup>23</sup> and slightly higher than 2.2 ml/100 g measured estimating CBV using small changes in hemoglobin saturation.<sup>11</sup> The 31% increase in CBV is lower than the increase of 120%, as determined previously using MRI measurements with a vascular contrast agent.<sup>2</sup> One possible reason is that the MRI study focused on layers 5 and 6, while the NIR will be more sensitive to the surface of the cortex. A previous study showed layer dependence on the angiogenic response, with most of the changes in layers 5 and 6, and no change in the superficial layer. Also, some of the CBV change in the MRI was due to

**Table 1** Average physiological parameters and cerebral blood volume pre- and postacclimation to chronic hypoxia (mean±SD, n=5). SmcO<sub>2</sub>% is the microvessel oxygen saturation.

Condition	SmcO <sub>2</sub> % <sup>1</sup>	Hbt µmol/l	Hct %	Hbt vein g/l	CBV% v/v
Pre-acclimation	72±5.4	46.6±1.9	44.4±2.3	152.6±17.4	3.26±0.41
Post-acclimation	*80±12 <i>p</i> <0.05	***80.3±3.4 <i>p</i> <0.001	**61.8±5.4 <i>p</i> <0.01	*199.8±15.5 <i>p</i> <0.05	*4.27±0.42 <i>p</i> <0.05

changes in vessel diameter. It may be that the NIR measurement, with contrast contained in cells versus plasma, is relatively more sensitive to change in length, which are on the order of 50% or less.<sup>14,24</sup>

We are not aware of any other study using NIR to quantify angiogenesis in the same brain over time, although there are numerous studies with NIR being applied in some fashion to obtain some index of CBV.<sup>5,7-10</sup> In conclusion, this work has illustrated that the changes in CBV with angiogenesis can be quantified over a time course in the brains of individual animals using NIR spectroscopy.

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