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Cerebrovascular Reactivity Measurement with Functional Near Infrared Spectroscopy

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TITLE:

Cerebrovascular Reactivity Measurement with Functional Near Infrared Spectroscopy

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KEYWORDS

cerebrovascular reactivity, blood flow, brain imaging, functional near infrared spectroscopy, optical imaging, hypercapnia

SUMMARY

Presented here is a protocol for imaging and measurement of cerebrovascular reactivity in humans with functional Near Infrared Spectroscopy (fNIRS). fNIRS is a novel imaging modality that captures the concentration changes of hemoglobin species in the brain's outermost cortex under specific stimuli.

ABSTRACT:

Cerebrovascular reactivity (CVR) is the capacity of blood vessels in the brain to alter the cerebral blood flow (either with dilation or constriction) in response to chemical or physical stimuli. The rate of reactivity in the cerebral microvasculature depends on the integrity of the capacitance vasculature and is the primary function of the endothelial cells. CVR is, therefore, an indicator of the microvasculature's physiology and overall health. Imaging methods that can measure CVR are available but can be costly, require magnetic resonance imaging centers and require technical expertise. In this study, we used fNIRS technology to monitor changes of oxyhemoglobin (HbO)

and deoxyhemoglobin (HbR) in the cerebral microvasculature to assess the CVR of 15 healthy controls (HC) in response to a vasoactive stimulus (inhaled 5% carbon dioxide or CO_2). Our results suggest that this is a promising imaging technology that can offer a non-invasive, accurate, portable, and cost-effective method of mapping cortical CVR and associated microvasculature dysfunction, resulting from a traumatic brain injury or other conditions associated with microvasculopathy.

INTRODUCTION

Vascular health in the cerebral cortex can be measured via the vessels' ability to constrict or dilate under varying physiological conditions. Measuring vascular reactivity can be useful in the diagnosis and management of neurological conditions associated with cerebral microvascular dysfunction, like dementia, traumatic brain injury (TBI) and even aging[1-4]. Additionally, CVR can be used as a predictive and/or pharmacodynamic biomarker for neurological disorders such as Alzheimer[5] or TBI[6-10]. Well-established imaging methods exist to study CVR in human and animal subjects. A typical method includes functional magnetic resonance imaging (fMRI) in conjunction with an exogenous or endogenous stimulus, such as hypercapnia[11], breath holding, or acetazolamide[2] . Lu et al.[12,13] demonstrated that a simple gas delivery system coupled with MRI-Blood Oxygen Level Dependent (MRI-BOLD) imaging generates an accurate whole brain CVR maps.

Disruptions to the cerebral vasculature's blood flow, volume, and metabolic rate of oxygen produces changes in the tissue concentrations of HbO and HbR. Tissue absorption of light at the near infrared range is sensitive to changes in the concentration of hemoglobin species, such as HbO and HbR. Therefore, measuring backscattered light over time can quantify changes of HbO and HbR concentration in the outermost cortex (approximately 2 cm)[15], and can be used to assess temporal hemodynamic variations[16] including cerebrovascular reactivity (CVR) [17].

In our research paradigm, we employ the fNIRS instrument with continuous wave function. The device is composed of 4 sources and 10 detectors, which create 16 source-detector pairs (see **Figure 1**). The source-detector pairs are molded together onto a silicone strap that can easily be set over the forehead and held in place with self-adherent wrap. The device measures light intensity at 730 and 850 nm and has an acquisition frequency of 2 Hz. This system was selected because it is patient-friendly, easy to wear, and collects data from the prefrontal cortex, a brain region particularly vulnerable to TBI. Fortunately, most other fNIRS systems are compatible with our CVR acquisition technique, differing only in the cortical regions measured based on the brain area of interest.

While fMRI is considered the gold standard for the functional brain imaging, fNIRS technology has unique advantages for assessing CVR in comparison to fMRI. The fNIRS imaging technique provides a high temporal resolution (with a granularity of milliseconds) and can quantify changes in both HbO and HbR concentration, while fMRI only measures changes in HbR[18-20]. Moreover, fNIRS instruments are portable, economical, and easier to operate than fMRI. Finally, fNIRS technology better resolves subject motion, which is necessary given that vascular challenges like hypercapnia are often used as cognitive or physical study tasks[21].

In this paper, a hypercapnia challenge, combined with fNIRS technology is presented. We measured CVR values and studied the reproducibility of this method, hoping to offer a reliable alternative to fMRI CVR measures.

PROTOCOL

The participants were recruited under an institutional review board approved protocol (ClinicalTrials.gov NCT01789164). The equipment described in the protocol is ethically approved by our institution.

1. Prepare the Materials used for the Hypercapnia Challenge (Figure 2)

1.1. Inflate a 200 L Douglas bag (Item #1) with a pre-mixed canister of medical-grade gas which comprises of 5% carbon dioxide, 21% oxygen, and 74% nitrogen until full.

1.2. Insert two diaphragms (Item #3) into the two-way non-rebreathing valve (Item #4) to safeguard that the gas will only flow in one direction. Attach one port of the three-way valve (Item #2) to the Douglas bag (Item #1) via the gas delivery tube (Item #5), and the other port to the two-way non-rebreathing valve (Item #4) via a second gas delivery tube (Item #5).

1.3. Fasten the mouthpiece (Item #6) to the connector (Item #7) and then fasten the connector to the two-way non-rebreathing valve (Item #4).

1.4. Insert the capnograph tubing (Item #8) into the hole in the connector (Item #7).

1.5. Attach the air-filter (Item #9) to the capnograph tubing (Item #8).

1.6. Screw the end of the plastic air-filter (Item #9) that isn't connected to the capnograph tubing (Item #8) into the CO_2 (Item #10) monitor.

1.7. Connect the capnograh (Item #10) to a laptop with a cable. Open the data port reader software, select the corresponding USB port and start the data reading. Turn on the capnograph. Data will automatically be displayed on the computer screen.

1.7. Connect the fNIRS box to the computer with a USB cable. Connect the source-detector headband to the FNIRS box. Connect the power adapter to the fNIRS box and turn on the switch.

2. Procedures during the experiment

2.1. Ask the participant to sit on the chair and to make themselves comfortable while setting up the devices. Turn the fNIRS system on.

2.2. Place the source-detector headband on the patient's forehead, over the underlying prefrontal cortex areas (dorsal and inferior frontal cortical areas)[21].

2.3. Check that the source-detector headband is carefully positioned above the eyebrow and in the middle of the forehead. Place the lower detector row approximately 3.5 cm above the nasion or bridge of the nose where the indentation of the upper nose meets the forehead between the eyes.

2.4. Make sure the detectors are firmly adhered to the participant's skin without chromophores (e.g., make-up) or hair interfering. No skin preparation is needed.

2.5. Under "**Device Setting**", set the gain for detectors between 1 and 20. A higher gain will increase the sensitivity of the light detectors. The default value is 20. Set the "**LED Current**" between 5 mA and 20 mA. Larger values will result in brighter light and will increase the signal level generated by the detectors. The default value is 20 mA.

2.6. In the acquisition software, press "**Start Current Experiment**". Sources will send light at 2 wavelengths and light signal intensity detected from each detector will be displayed in real time. In case of saturated (signal>4,000) or low signal (signal <1,000), adjust the contact between the source-detector headband and the skin or the parameters in step 2.3 and 2.4. The exact full procedure has been explained in Ayaz et al.[22].

2.7 Direct the participant to inhale and exhale through their mouth at their normal breathing pace. Fasten a nose clip on the participant's nose and remind them to continue breathing normally through their mouth, and to alert someone if they feel any discomfort or have any difficulty breathing.

2.8. Carefully insert the mouthpiece (item#6) into the participant's mouth so that they can continue to breathe. For increased participant comfort during the procedure, ask the participant to support the non-rebreathing valve (Item #3) with their hand.

2.9. Press the "**Baseline**" button in associated software. It will measure and automatically record the light signal for the fNIRS baseline for 20s.

2.10. Press "**Record**" before starting the experiment.

2.11. At the beginning of the experiment, start the clock, press "**Manual Marker**" and write on a paper the time displayed by the capnograph. Every minute turn the valve connected to the gas tubing to cycle between room air and room air mixed with 5% CO_2. Again, press "**Manual Marker**" and write on a paper the time displayed by the capnograph each time the inhaled gas mixture is changed (**Figure 3**).

NOTE: Manually marking the time displayed on the capnograph is essential for future synchronization between fNIRS optical signals and capnograph's $EtCO_2$ trace.

2.12. After 7 min, stop the fNIRS recording by clicking the "**Stop**" button. Allow 60 additional seconds of recording for the $EtCO_2$ and save the $EtCO_2$ data as ASCII within the data reader software.

2.13. Notify the participant that the procedure is completed. Carefully take off the nose clip and withdraw the mouthpiece. Offer a tissue to the participant to absorb any accumulated saliva from the procedure.

3. Clean up Procedures

3.1. Dispose of the capnograph tubing (Item #8), filter (Item #9), mouthpiece (Item #6) and nose clip.

3.2. Cleanse the reusable equipment. Detach the two-way valve (Item #4) from the gas-delivery tube (Item #5) and the connector tube (Item #7) and extract the diaphragms (Item #3). Immerse the diaphragms (Item #3), connector tube, and two-way valve (Item #4) in a container full of a medical-grade detergent disinfectant that is phosphate-free and contains surfactants for 20 min. Dilute the detergent with distilled water in a ratio of 1:64.

3.3. Wash items #1,4,7 with distilled water then place them on top of a clean counter with a sterile material such as a chux pad underneath. Allow them to air-dry before re-use.

3.4. Empty the Douglas bag.

4. Data analysis

4.1. Signal processing using fNIRS data processing software

NOTE: Signal processing is the first step of the data analysis. It is done using an fNIRS data processing software (e.g., fNIRSoft) in order to remove noise or artefact in the data due to patient movement. Only data from the acquisition software are needed for this analysis.

4.1.1. In data processing software, click on "**Load File**" to select and then upload the acquired fNIRS data.

4.1.2. Click on "**Refine**" and a pop-up window will appear. Select "**Raw Data**" and press "**Next**".

4.1.2.1. Click on both the median filtering and the sliding window motion artifact rejection (SMAR)[23] tools to recognize and delete both motion artifact and saturated channels from the raw signal. Press "**Apply**".

4.1.2.2. Click on "**Low Pass Frequency**" filter to discard pulse and breathing component (Hanning filter, n=20, cutoff=0.1Hz)[21;24-26]. Press "**Apply**".

4.1.2.3. Click on "**Detrend**" to eliminate the slow temporal variation. Press "**Apply**".

4.1.3. Click on "**OXI**" to transform the light intensity into HbO and HbR concentrations. Click on "**Save**" and then select MATLAB as the save file format.

4.2. Signal processing with MATLAB

NOTE: The second part of the analysis is done using MATLAB in order to correlate the fNIRS signal with the time shifted $EtCO_2$. Data from the previous step (4.1.5) and data from the capnograph ($EtCO_2$ trace, step 2.12) are needed for processing the data.

4.2.1. Import the $EtCO_2$ trace from the capnograph in MATLAB as two columns (one for time and the second for $EtCO_2$ values). Shift the $EtCO_2$ time with pre-calibrated time to correct the delay from the sampling tubing time.

NOTE: This is the time difference between one breath to the mouthpiece and the appearance of that breath on the CO_2 recording. In this set-up, it was 15 s.

4.2.2. Use the first time point recorded from the capnograph at the beginning of the experiment, step 2.11 as the starting point (t=0). Convert the $EtCO_2$ into seconds.

4.2.3. Import the oxy and deoxyhemoglobin data from step 4.1.3 into MATLAB.

4.2.4. Calculate the physiological delay between $EtCO_2$ (measured in the mouth) and the fNIRS signal (measured in the brain) by finding the higher correlation coefficient between these two signals at varying time shifts. (see MATLAB script attached for step 4.2.3 to step 4.2.6). The time shift with the higher correlation coefficient is considered the optimal time.

4.2.5. Shift the $EtCO_2$ time course by the optimal time (obtained in step 4.2.4). Keep the time points that have both fNIRS and $EtCO_2$ data. The two-time series should have the same length.

4.2.6. Calculate CVR values for each channel, which is the solution of the linear equation between HbO (or HbR) and $EtCO_2$ using the Cholesky decomposition in MATLAB.

REPRESENTATIVE RESULTS

fNIRS was performed with hypercapnia challenge on 15 healthy participants. Exclusion criteria were history of TBI, pre-existing disabling disorders or pregnancy. The participants had a mean age of 37.7 ± 16 years (range 20-55) and 20% were female. As shown in a similar fMRI study[28], a 60 s inhalation of 5% CO_2 was accompanied by an increase in $EtCO_2$ pressure as measured by capnography. In our study, the $EtCO_2$ trace was accompanied by an increase of HbO and a decrease of HbR (**Figure 4**).

Physiologically, HbO and HbR are out of phase[14]. In **Figure 4**, which represents the fNIRS signal of one participant, we observed that the HbR signal precedes the HbO signal by 3.5 s (a precise

measurement can be derived from the time shift for each signal). On an average, across all participants, it was observed that the HbO signal increases 2.3 ±2.6 s after the HbR signal decreases. This implied that the time shift for HbO and HbR were different and needed to be estimated before calculating a participant's CVR. For this same reason, we also needed to estimate the time shift between the $EtCO_2$ tracing and Hb-diff (the difference between HbO and HbR signals). The Hb-diff parameter gave us the strongest amplitude between the two conditions.

On an average, in our HC group, the increase of HbO appeared 2.3 ± 2.6 s before the HbR decrease was noted. Because of this delay between HbO and HbR, the time shift between the $EtCO_2$ tracing and HbO signal was not the same as the time shift between the $EtCO_2$ tracing and HbR signal. In addition, also calculated was the time shift between the $EtCO_2$ tracing and Hb-diff (difference between HbO and HbR signal). The Hb-diff parameter gave us the strongest amplitude between the two conditions.

After shifting the $EtCO_2$ trace for HbO, HbR, and Hb-diff, we measured the Pearson correlation between the shifted $EtCO_2$ traces and HbO, HbR, and Hb-diff. $EtCO_2$ trace highly correlated with fNIRS signals (Pearson's correlations of 0.94, -0.98 and 0.91 for HbO, HbR and Hb-diff, respectively; $p<0.0001$). (**Figure 5**).

We explored the CVR inter- and intra- variability between all 15 participants and all source/detector pairs. Averaging the CVR between the source/detector pair for each participant, we assessed the CVR from HbO, HbR, and Hb-diff (difference between HBO and HbR). On average between all participants, CVR values were 13.1 ± 4.7 µM/mmHg using HbO, -14.6 ± 10.2 µM/mmHg using HbR, and 12.4 ± 3.7 µM/mmHg using Hb-diff (**Table 1**). Variability between the channels within each participant was also analyzed. On average, the intra-variability of CVR assessed with Hb-diff was lowest (30%), appeared to be the best parameter to investigate CVR using fNIRS.

Finally, we can overlay the CVR values on an anatomical template or directly on the structural image of the patient's brain, as available for better visualization.[27]

FIGURE AND TABLE LEGENDS:

Figure 1: Optical sensor pad schematic. It is composed of 4 sources (large red circles), and 10 detectors (small red dots), which form 16 source/detector pairs having 2.5 cm separation. The sensor pad is positioned on the volunteer's forehead. The numbers indicate the position of the 16 source/detector pairs on the sensor.

Figure 2: Diagram of the Gas Delivery System. (**1**) Douglas bag. (**2**) Three-way valve. (**3**) Diaphragms. (**4**) Two-way non-rebreathing valve. (**5**) Gas delivery tube. (**6**) Mouthpiece. (**7**) Connector. (**8**) Gas sampling tube. (**9**) Hydrophobic filter. (**10**) EtCO2 monitor. (**11**) fNIRS system.

Figure 3: Timing and marker of the breathing paradigm. Every minute, the three-way valve switched between the two gases. A marker signal was sent to the fNIRS software to sort each period with the appropriate gas inhalation.

Figure 4: Example of HbO and HbR concentration measures under a 5% CO_2 challenge of one participant. Each fNIRS curve is the average of 16 channels. The red curve represents the variation of HbO during 60s of room air and 60s under 5% of CO2. The blue curve represents the variation of HbR under the same conditions. The curves were time shifted in order to match the EtCO2 (black curve). Each HbO and HbR curves are the average of 3 challenges.

Figure 5: Correlation between the $EtCO_2$ and the HbO, HbR, oxygenation, in one channel for one participant. HbO, HbR and oxygenation were time shifted in order to temporally match the EtCO2 trace. Pearson's correlations is 0.94, -0.98 and 0.91 for HbO, HbR and Hb-diff, respectively ($p<0.0001$)

Table 1: Inter-subject and inter-channel variability of CVR values for 15 HC. CVR variability was calculated with 3 physiological signals: HbO, HbR and oxygenation (HbO-HbR).

DISCUSSION

We were able to measure CVR using fNIRS and a CO_2 gas inhalation technique in 15 healthy volunteers. The CVR value measured is the correlation between the acquired fNIRS signal and the $EtCO_2$. The challenge is to accurately align the temporal $EtCO_2$ trace with the fNIRS signal. In other words, to account for the time that it takes for blood to travel from the pulmonary vascular system to the heart and then to the cerebral vasculature. The inter-channel variability is low (30%) and shows a uniform CVR value in the cortex which correlates with previous fMRI results [12]. Our data show the inter-participant variability in CVR among HC is lowest for the oxygenation signal but increases significantly when using HbO or HbR. For future group difference studies with HC, we recommend using CVR values with the least variability, i.e., the CVR values from the oxygenation signal.

The critical step of the technique is the same as any fNIRS experiment: the placement of the sources and light detectors on the head. A loose connection can lead to a displacement of the sensors during the experiment and artefacts in the signal. Too much artefact will corrupt the signal and disrupt the analysis.

CVR is mostly measured in humans by 2 different techniques: Doppler ultrasound and fMRI. This third method, fNIRS, give us a precise and accurate measure of CVR under gas inhalation. Like ultrasound, it gives excellent temporal resolution, but in addition, it also provides a 2D map of CVR (cortical values). While these images are low in resolution compared to CVR measured with fMRI, fNIRS provides both measures inexpensively and non-invasively, and can be easily carried out in the outpatient clinical setting. In addition, fNIRS can measure the two components of hemoglobin (HbO and HbR), which has potential benefits for vascular research. Depending on the application, this method of measuring CVR via fNIRS can be carried out over a single area of interest or over a multi-band array for a 2D map of the brain. Cerebrovascular using hypercapnia

measures changes of blood flow across the brain with fNIRS, as opposed to a cognitive task which would only allow for detection of changes in blood flow in the motor cortex.

Because this procedure can be performed safely, inexpensively, and without side effects in a clinic setting, this method is well suited for both research and clinical use, especially for neurovascular diseases like vascular dementia or traumatic brain injury (TBI). In Alzheimer's disease, early evidence of cerebrovascular reactivity deficits is detectable[28]. In the same way, traumatic cerebrovascular injury is one endophenotype of TBI[29] where endothelial cells are disrupted and lead to cerebrovascular pathology. Clinical trials measuring an intervention with a pharmacodynamic biomarker for vascular injury will need to assess CVR with fMRI or fNIRS to provide information on proof of mechanism.

DISCLOSURES

The authors have no conflicts of interest to disclose.

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Figure 1

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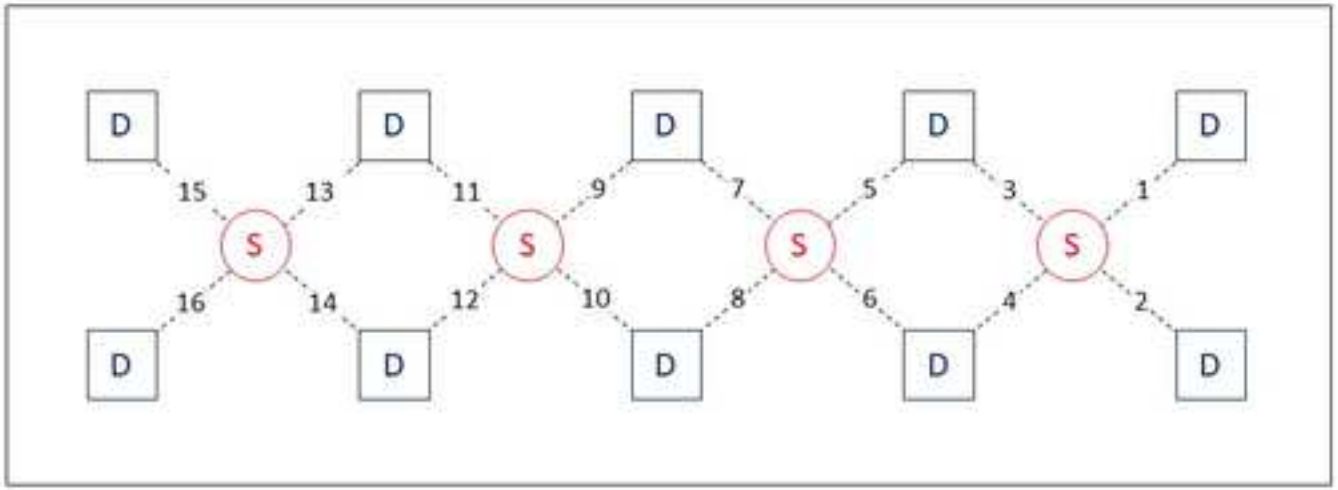


Figure 2

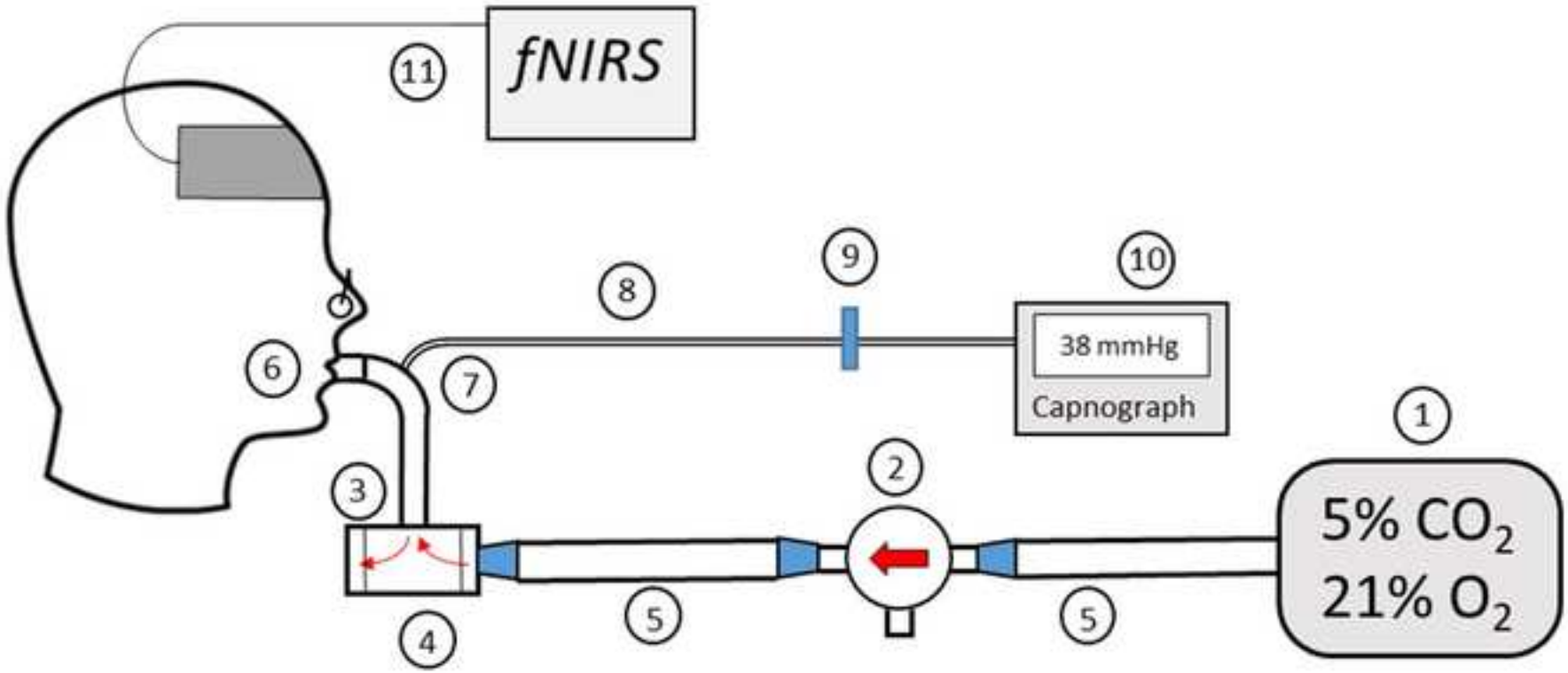


Figure 3

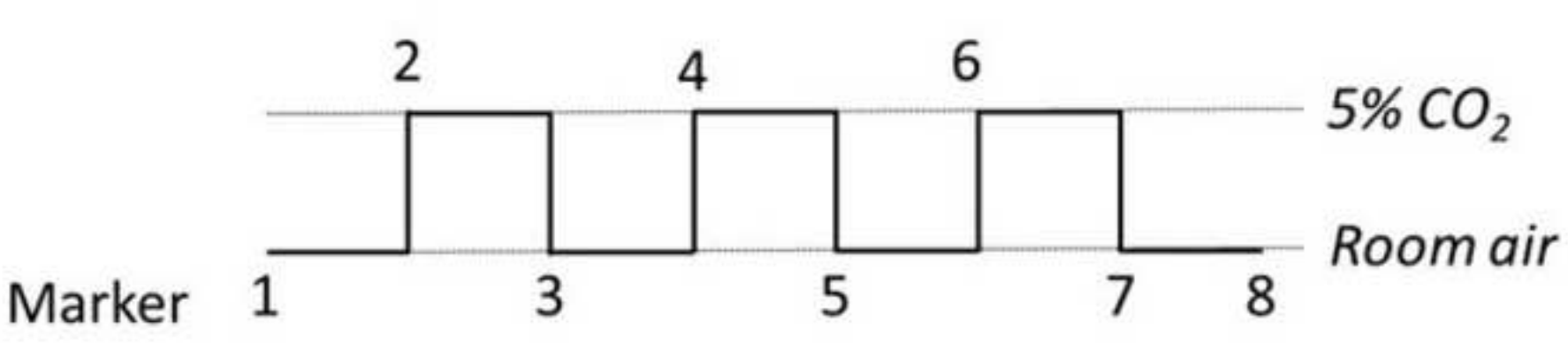


Figure 4

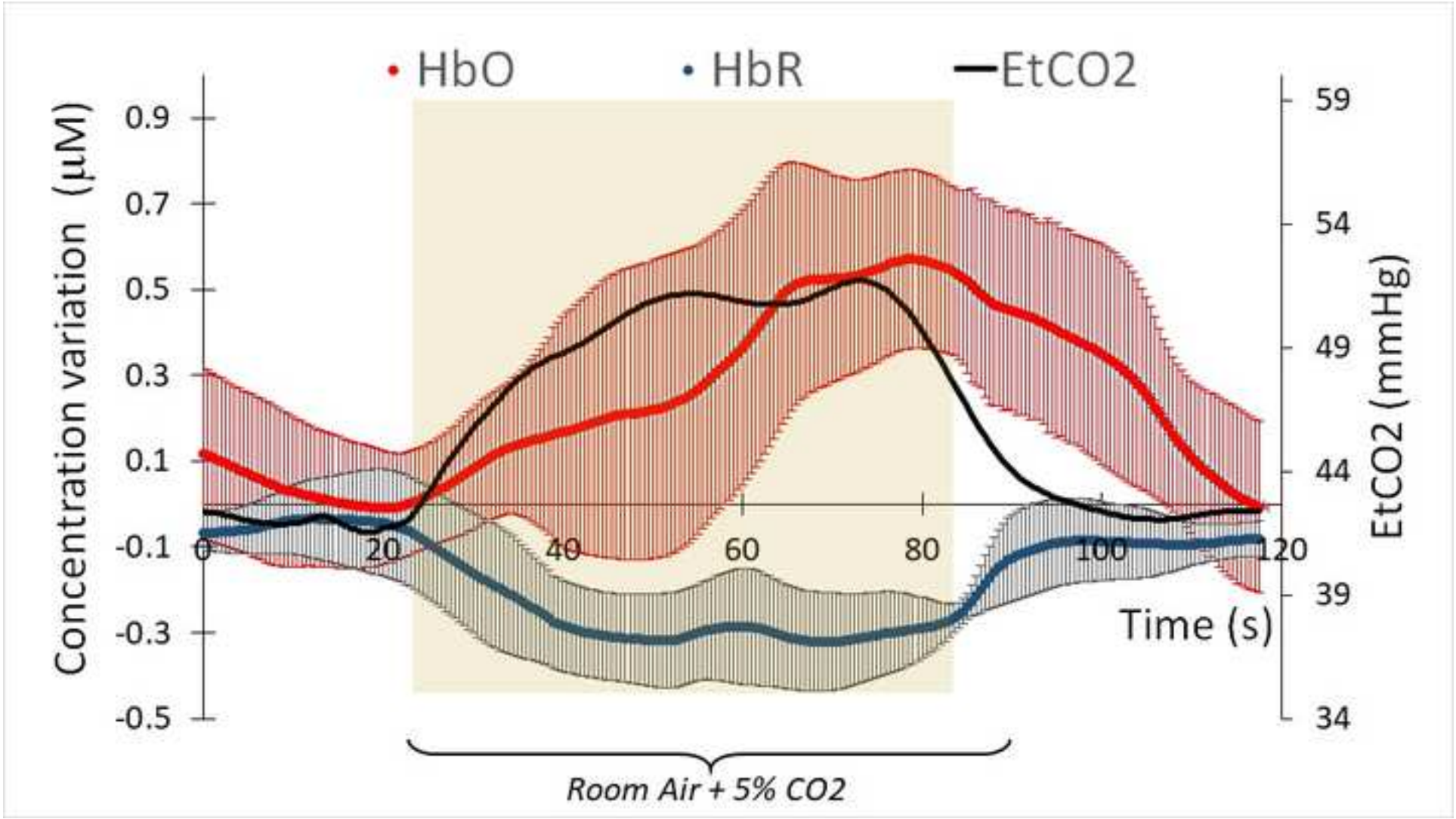
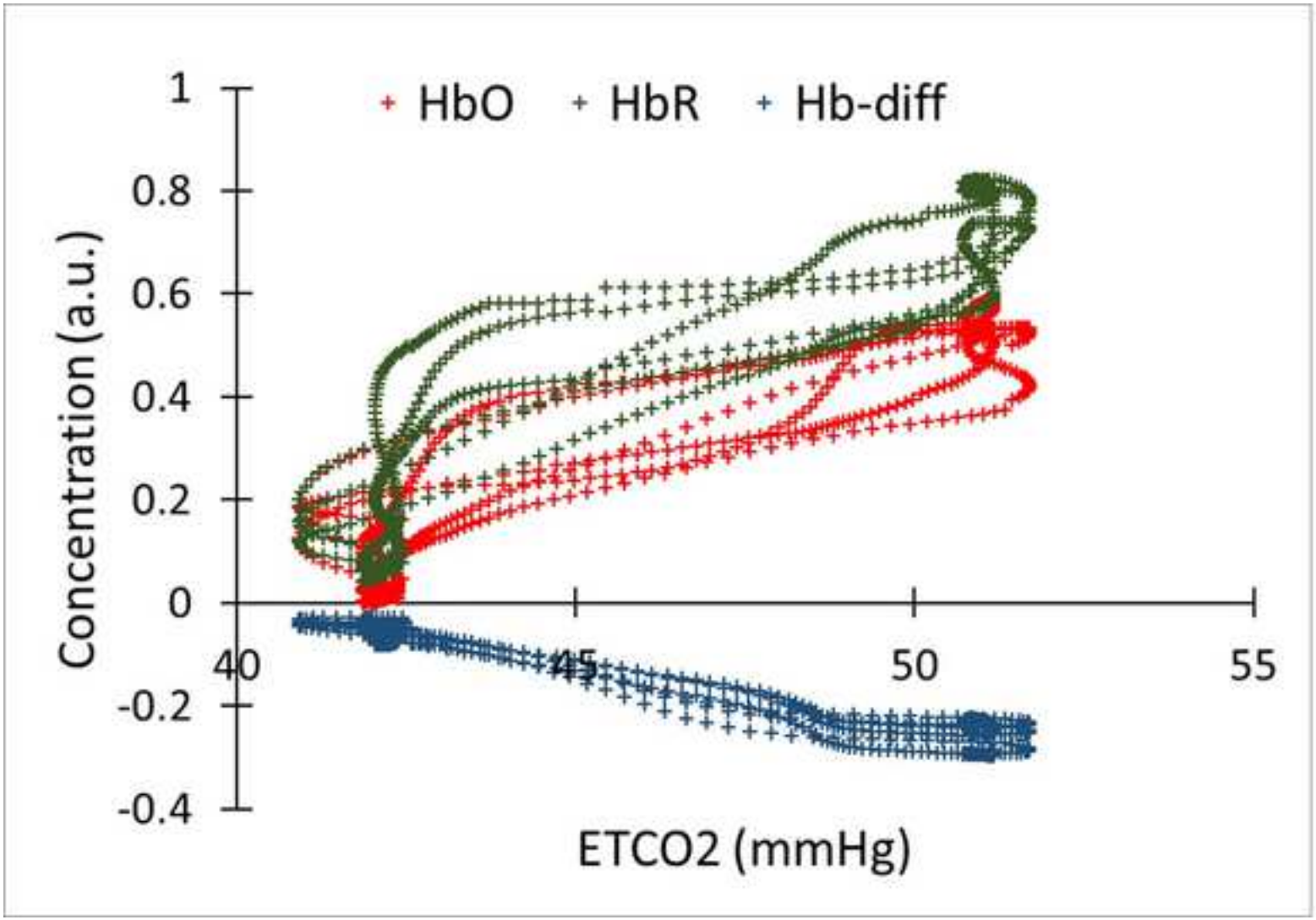


Figure 5



Table

	Mean CVR between patient	Variability between channels
HbO	13.1 +/- 4.7	41%
HbR	-14.6 +/- 10.2	85%
Hb-diff (HbO-HbR)	12.4 +/- 3.7	30%

Table of Materials

Click here to access/download;Table of Materials;table of materials.xlsx

Item	Catalog Number	Vendor
Blue cuff	22254	Vacumed
CO2-Air Gas Mixture Size 200	R012000 2003	Roberts Oxygen
Diaphragm (Size: medium, Type: spiral)	602021-2608	Hans Rudolph
Douglas bag (200-liters capacity)	500942	Harvard Apparatus
Gas delivery Tube	1011-108	Vacumed
Gas sampling Tube	T4305	QoSINA
Hydrophobic filter	9906-00	Philips Medical Systems
Male luer	11547	QoSINA
Mouth piece (Silicone, Model #9061)	602076	Hans Rudolph
Nose clip (Plastic foam, Model #9014)	201413	Hans Rudolph
Three-way valve (100% plastic)	CR1207	Hans Rudolph
Two-way non-breathing valve (22mm/ 15mm ID)	CR1480	Hans Rudolph

April 15th, 2020

Ref: JoVE61284R1

Dear Editor,

I am pleased to send you our revised manuscript "Measure of Cerebrovascular Reactivity with functional Near Infrared Spectroscopy" for your consideration. We thank you for your careful editing.

Following your recommendations, we edited our manuscript. The responses to your comments can be found in the document below.

Sincerely,

Franck AMYOT, Ph.D.
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Editorial comments

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Response: We used the document for revision

2. Please address all the specific comments marked in the manuscript.

Response: Full response of editorial comments are below. Original comments were kept.

3. Please ensure that there is a minimum of one page and a maximum of 2.75 pages of highlighting including headings and spacings.

Response: The highlighting is in a separate document

4. Please proofread the manuscript well before submitting it.

Response: We proofread the manuscript carefully before re-submitting.

5. Please reword lines 123-128, 208-211, 227-229, 234-236 as it matches with previously published literature.

Line 123-128. Previously:

2.5. Set the LED current between 5mA to 20mA. LED drive current defines how bright each LED shines. The default value for LED drive current is 20mA. This value may have to change based on the skin pigmentation and other characteristics of the participant.

2.6. Set the initial gain. Default value for gain for all channels is 20. Suggested values for gain are 1,5,10,15,20.

Now reads:

Under “Device Setting”, set the gain for detectors between 1 and 20. A higher gain will increase the sensitivity of the light detectors. The default value is 20. Set the “LED Current” between 5mA and 20mA. Larger values will result in brighter light and will increase the signal level generated by the detectors. The default value is 20mA.

Line 208-211. Previously:

4.2.3. Identify the physiological delay between EtCO2 (measured in the lungs) and the fNIRS signal (measured in the brain) by calculating the correlation coefficient between these two time courses at varying time shifts. The shift value that yields the higher correlation coefficient is considered the optimal time.

Now reads:

Calculate the physiological delay between EtCO2 (measured in the mouth) and the fNIRS signal (measured in the brain) by finding the higher correlation coefficient between these two signals at varying time shifts. (MATLAB script attached for step 4.2.3 to step 4.2.5). The time shift with the higher correlation coefficient is considered the optimal time.

Line 227-229. Previously:

On average, in our HC group, the increase of HbO appeared 2.3 + 2.6 s before the HbR decrease was noted. Because of this delay between HbO and HbR, the time shift between the EtCO2 tracing and HbO signal is not the same as the time shift between the EtCO2 tracing and HbR signal.

Now reads:

Physiologically, HbO and HbR are out of phase14. In Figure 4, which represents the fNIRS signal of one participant, we observed that the HbR signal precedes the HbO signal by 3.5 seconds (a precise measurement can be derived from the time shift for each signal). On average across all participants, we observed that the HbO signal increases 2.3 +2.6 s after the HbR signal decreases.

Line 234:236. Previously:

After applying the appropriate temporal shift, we measured the Pearson correlation between EtCO2 and HbO, HbR, and Hb-diff. fNIRS signals are highly correlated with EtCO2.

Now reads:

After shifting the EtCO2 trace for HbO, HbR, and Hb-diff, we measured the Pearson correlation between the shifted EtCO2 traces and HbO, HbR, and Hb-diff. We found that EtCO2 are correlated with fNIRS signals.

6. If reusing previously published figures, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: no previously published figures were used.

Comments and responses:

Editor's comment: Please refer figures in order.

Author's response: Line 62: Changed the order of the figures. Figure 1 is now figure 2 and figure 2 is now figure 1. Legend numbers have been adjusted accordingly.

Editor's comment: Please ensure all steps are written in imperative tense as if suggesting someone how to do the experiment with all specific details. There should be a cohesive story from the beginning to the end.

For each step, please include how it is done. Please include all the button clicks, knob turns etc.

Once done, please highlight 2.75 pages of the protocol section to be used for filming purpose.

Author's response: The protocol steps have been reordered and additional steps and instructions have also been added for clarity. Imperative tense has been incorporated in the steps. These modified and expanded steps are the following:

Line 87---Under heading **"Protocol"**: 1. "Preparing" changed to imperative tense, "Prepare". Now reads: **"<u>Prepare</u> the Materials used for the Hypercapnia Challenge (Figure 2)**."
<u>Subsection 1.7, Line 107-109—Modified subsection 1.7 Previously read:</u> Previously said "Turn on the capnograph and then connect to a laptop for data recording. …
Etc…
<u>Revised for more detail to read:</u> (Line 107) "Connect the capnograh (Item #10) to a laptop with an RS232 cable. Open the data port reader software, select the corresponding USB port and start the data reading. Turn on the capnograph. Data will automatically be displayed on the computer screen."

Author added subsection 1.8, line 112: "1.8 Connect the fNIRS box to the computer with a USB cable. Connect the source-detector headband to the FNIRS box. Connect the power adapter to the fNIRS box and turn on the switch."

Author removed turn on the capnograph in step 2.1 (line 116)

Section 2 Procedures during the experiment

Section 2.3, line 124:
Revised the subsection 2.3 which uses the term "rigid mount". Previously read: "Check that the <u>rigid mount</u> is carefully positioned above the eyebrow and in the middle of the forehead. Place the lower detector row approximately 3.5 cm above the nasion."

<u>Editor's comment</u>: Where is this (rigid mount) in the figure? These are detectors for fNIRS measurement?"

<u>Author response</u>: The "rigid mount" was changed to "<u>source-detector headband</u>", the term used in subsection 2.2, for clarity that this is the headband with light detectors and is referred to in Figure 1 (previously Figure 2).
Beginning on line 124, the subsection now includes a more detailed position description of nasion: "or bridge of the nose where the indentation of the upper nose meets the forehead between the eyes." The revised sentences read, "Check that the *<u>source-detector headband</u>* is carefully positioned above the eyebrow and in the middle of the forehead. Place the lower detector row approximately 3.5 cm above the nasion, *or <u>bridge of the nose where the indentation of the upper nose meets the forehead between the eyes</u>*.*<u>"</u>*

2.4, Line 127
<u>Editor's comment:</u> So you do not clean the skin area with 70% alcohol?

Author's response: This is correct. There is no alcohol required for cleaning the skin.
Revised the instruction so that it currently reads:

> "Make sure the detectors are firmly adhered to the participant's skin without chromophores (e.g. make-up) or hair interfering. No skin preparation is needed." (added e.g. make-up to give an example of a chromophore)

2.5, Line 130
Editor's comments: (Referring to the LED current.) Where is this in the figure? How do you set this? Significance of this? Why do you need LED light? Where are LED lights placed? How many of these are present? Additional Editor's comment about this same subsection regarding the last sentence "skin pigmentation and other characteristics of the participant": What is being looked for here?
Original paragraph: "Set the LED current between 5mA to 20mA. LED drive current defines how bright each LED shines. The default value for LED drive current is 20mA. This value may have to change based on the skin pigmentation and other characteristics of the participant. "

Author's response: Combined the information from 2.5 and 2.6 which is now 2.5.

> Revised the paragraph to now read: "Under "Device Setting", set the gain for detectors between 1 and 20. A higher gain will increase the sensitivity of the light detectors. The default value is 20. Set the "LED Current" between 5mA and 20mA. Larger values will result in brighter light and will increase the signal level generated by the detectors. The default value is 20mA."

2.6, Line 141
Editor's comments: What kind of signal is obtained here? What is being tested? Please reword for clarity. How is the signal obtained? What form?
Original sentences: "*In case of saturated* (raw signal>4000) or low signal (raw signal <1000), adjust the corresponding *sensor* placement or the parameter in step 2.5 and 2.6. The exact full procedure has been explained by Ayaz et al. [22]

Author's response: Modified the subsection for clarity and more detailed information about the signal obtained to read:

> 2.6."In CobiStudio, press "Start Current Experiment". Sources will send light at 2 wavelengths and light signal intensity detected from each detector will be displayed in real time in CobiStudio. In case of saturated (signal>4000) or low signal (signal <1000), adjust the contact between the source-detector headband and the skin or the parameter in steps 2.3 and 2.4. The exact full procedure has been explained by Ayaz et al. [22]

2.8, line 148

Editor's comment: Significance of this? (regarding, why the participant holds the non-rebreathing valve which is the extension of the mouthpiece and the non-rebreathing valve) Original sentence, "Carefully insert the mouthpiece (item#6) into the participant's mouth so that they can continue to breathe. During the procedure, ask the participant to support the *non-rebreathing valve* (Item #3) with their hand."

Author's response: Revised the sentences by adding "for increased participant comfort" and referring to Section 1.2 where the non-rebreathing valve is described.

> The revised paragraph reads: "Carefully insert the mouthpiece (item#6) into the participant's mouth so that they can continue to breathe. *For increased participant comfort* during the procedure, ask the participant to support the non-rebreathing valve (*described in Section 1.2,* Item #4) with their hand.

2.9, 2.10, and 2.11, line 150-162 (formerly 2.10)
Original sentence: Start recording the *end-tidal carbon dioxide ($EtCO_2$)* pressure with the capnograph.
Editor's comment: How is this done? (refers to recording the EtCO2)

Author's response: Recording is in CobiStudio computer program.
Omit the sentence about the capnograph in the former 2.9 subsection. It will be discussed in the new subsection 2.11.

Subsection 2.9, line 150
Subsection formerly 2.11 now 2.9: Original sentence: "*Record the fNIRS baseline for 20s.*"
Editor's comments: Is this connected to a computer and is there an associated software? Do you press the record button on the fNIRS?

Author's response: The selections occur on the laptop computer in CobiStudio. The start button for the baseline and the record button for the experiment are in CobiStudio.

> Revised the sentences in the next paragraphs/subsections: subsection 2.9, line 159 now reads:
> "Press "Baseline" *button in CobiStudio*. It will *measure and automatically record the light signal* for the fNIRS baseline for 20 *seconds*. "
>
> Author added instructions: 2.10, line 153 Reworded the sentence: "Start to record the experiment (press record). " Author: New sentence reads: "*Press Record before starting the experiment."*

2.11, line 155

Editor's comments: What is new marker with respect to your experiment? How do you send it?

Author's response: Revised section 2.11 to give more detailed directions and explanation about the "Manual Marker" which is a selection in the computer program CobiStudio. The manual marker in CobiStudio allows you to synchronize the signal and the stimulus (type of gas). The time the valve is switched is "marked" by pushing "Manual Marker".

> The revised sentences read: "At the beginning of the experiment, start the clock, press "Manual Marker" in CobiStudio, and write on paper the time displayed by the capnograph. Every minute, turn the valve connected to the gas tubing to cycle between room air and room air mixed with 5% CO_2. Again, press "Manual Marker" in CobiStudio and write on paper the time displayed by the capnograph each time the inhaled gas mixture is changed (**Figure 3**). Manually marking the time displayed on the capnograph is essential for future synchronization between fNIRS optical signals and capnograph's $EtCO_2$ trace." Omit lines 172-177 which have been incorporated in the revised section 2.12.

2.12, line 163. Original sentences: "After 7 min, stop the fNIRS recording. Allow 60 additional seconds of recording for the $EtCO_2$ and synchronize the $EtCO_2$ trace with the fNIRS signal. Save the data."

Author revised these sentences for further clarification:

> "After 7 min, stop the fNIRS recording *by clicking on "Stop" in CobiStudio*. Allow 60 additional seconds of recording for the $EtCO_2$ and *save the EtCO2 data as ASCII within the data port reader software."*

Section 4 Data analysis (Line 187)

4.1, line 189. Signal Processing using fNIRSoft (Changed Signal Processing to Signal Processing using fNIRSoft)
Original NOTE: Signal processing is the first step of the data analysis. It's done under an fNIRS data processing software in order to remove noise or artefact in the data due to patient movement.

> Modified NOTE: Signal processing is the first step of the data analysis. *It is* done *using* an fNIRS data processing software *(fNIRSoft)* in order to remove noise or *artifact* in the data due to patient movement. *Only data from CobiStudio are needed for this analysis.* 4.1 *In fNIRSoft, Click on "Load File" to select and then upload the acquired fNIRS data.*

Original subsection 4.1.1, line 195: Select both the median filtering and the sliding window motion artifact rejection (SMAR)[23] tools to recognize and delete both motion artifact and saturated channels from the raw signal.

Editor's comment: How and where are these tools selected. E.g., in the fNIRS acquitision system collect all data save. Now go to the edit mode and click on "xxx" to select the median filtering.

Author's response: The author added more steps, including pop-up window and buttons to click.

> Added "fNIRSoft" and the sentence "Only data from CobiStudio are needed for this analysis," for clarification and new, more detailed instructions in response to the Editor's question.
>
> Added: Subsection 4.1.2, line 197 added "Click on "Refine" and a pop-up window will appear. Select "Raw Data" and press "Next".
>
> Added subsection 4.1.2.1: Click on both the median filtering and the sliding window motion artifact rejection (SMAR).[23] tools to recognize and delete both motion artifact and saturated channels from the raw signal. Press "Apply".
>
> Revised subsection 4.1.1.2, line 202: Original sentence in 4.1.2: "Select the low pass frequency filter to discard pulse and breathing component (Hanning filter, n=20, cutoff=0.1Hz). 21;24-26 ".

Editor's comment: How is this done?
Author's response: revised sentence to read:

> *Click on* "Low Pass Frequency" filter to discard pulse and breathing component (Hanning filter, n=20, cutoff=0.1Hz).[21;24-26] *Press "Apply*.

Original sentence in 4.1.3 line 204: Select the detrend option by clicking on xxx to eliminate the slow temporal variation.
Editor's comment: Please include

Author's response: Now 4.1.2.3 line 204:

> *Click on* "Detrend" to eliminate the slow temporal variation. *Press "Apply*". Omitted "Select", "clicking on xxx", and added "Press "Apply" for clarification.

Original sentence in 4.1.4 (Now 4.1.3, line 206): Transform the light intensity into HbO and HbR concentrations. Save the data into a MATLAB format.
Editor's comment: Please include as supplementary file.

Author's response: Revised in 4.1.2 with more specific detail to read:

Click on "OXI" to transform the light intensity into HbO and HbR concentrations. Click on "Save" and then select MATLAB as the save file format.

4.2 Signal processing with MATLAB Line 209

4.2 originally read: **Signal processing**. "The second part of the analysis is done under MATLAB in order to correlate the fNIRS signal with the time shifted $EtCO_2$."

For clarification, the title was expanded: **4.2**. "**Signal processing *with MATLAB*"**

Also, more information was added: The new paragraph reads:

(line 210) "NOTE: The second part of the analysis is done using MATLAB in order to correlate the fNIRS signal with the time-shifted $EtCO_2$. *Data from the previous step (4.1.5) and data from the capnographe ($EtCO_2$ trace, step 2.12) are needed for processing the data."*4.2.1 Originally (formerly 4.1.1) read: *"*Open the $EtCO_2$ trace from the capnograph in MATLAB. Shift the $EtCO_2$ time with the sampling tubing time delay (use the pre-calibrated time difference between one breath to the mouthpiece and the appearance of that breath on the CO_2 recording).

Editor's comment: How is this done?

Author's response: *Revised: 4.2.1, line 214 to read:*

Import the $EtCO_2$ trace from the capnograph in MATLAB as two columns (one for time and the second for $EtCO_2$ values). Shift the $EtCO_2$ time with pre-calibrated time to correct the delay from the sampling tubing time (This is the time difference between one breath to the mouthpiece and the appearance of that breath on the CO_2 recording. In this set-up, it was 15 seconds). Use the first time point recorded from the capnograph at the beginning of the experiment, step 2.11 as the starting point (t=0). Convert the $EtCO_2$ into seconds.

Added 4.2.2, line 222: as follows: Import the oxy and deoxyhemoglobin data from step 4.1.3 into MATLAB.

Subsection 4.2.3, line 224: Original sentences: Identify the physiological delay between $EtCO_2$ (measured in the lungs) and the fNIRS signal (measured in the brain) by calculating the correlation coefficient between these two time courses at varying time shifts. The shift value that yields the higher correlation coefficient is considered the optimal time.

Editor's comment: Please include how each step is done. Please include all the button clicks in the software, command lines, knob turns etc. Scripts used can be included as supplementary files.

Author's response: Revised the paragraph with more detailed instructions. **4.2.3, line 224** now reads:

"*Calculate* the physiological delay between $EtCO_2$ (measured in the *mouth*) and the fNIRS signal (measured in the brain) by *finding the higher* correlation coefficient between these two *signals* at varying time shifts. (*MATLAB script attached for step 4.2.1 to step 4.2.2*) The *time* shift *with* the higher correlation coefficient is considered the optimal time."

Original manuscript these subsections were omitted and revised/incorporated into 4.2.3 above.

4.1.1. Based on the synchronizing marker (step 2.13), segment the $EtCO_2$ data to keep only the recording from 30 sec prior to the first marker to 60 sec after the data acquisition entry.

Editor's comments: Please include how each step is done. Please include all the button clicks in the software, command lines, knob turns etc. Scripts used can be included as supplementary files.

Author's response: See 4.2.3.

Originally 4.1.2 read "Identify the physiological delay between $EtCO_2$ (measured in the lungs) and the fNIRS signal (measured in the brain) by calculating the correlation coefficient between these two time courses at varying time shifts. The shift value that yields the higher correlation coefficient is considered the optimal time." Included in 4.2.3 currently (detailed above).

REPRESENTATIVE RESULTS

Editor's comments: For the representative result, please also include your inference from the observations as well. Please also include how do these results show that the technique is effective.

Author's comments: Some precision has been added in paragraph line 241 to compare this result to validated fMRI study. It now reads" As shown in a similar fMRI study[28], a 60- second inhalation of 5% CO_2 was accompanied by an increase in $EtCO_2$ pressure as measured by capnography. In our study the $EtCO_2$ trace was accompanied by an increase of HbO and a decrease of HbR (**figure 4**)."

More

Regarding discussion on the observation can be found in the discussion. Discussion on CVR variability and correlation with previous CVR study is described in the first discussion paragraph. (Line 310): The inter-channel variability is low (30%) and shows a uniform CVR value in the cortex which correlates with previous fMRI results [12]. Our data show the inter-participant variability in CVR among HC is lowest for the oxygenation signal but increases significantly when using HbO or HbR. For future group difference studies with HC, we recommend using CVR values with the least variability, i.e. the CVR values from the oxygenation signal.

Editor's comments: For the representative result, please also include your inference from the observations as well. Please also include how do these results show that the technique is effective.

We carried out fNIRS with hypercapnia challenge on 15 HC.

Editor's comments: Please expand. Please include clinical characteristic table along.

Author's response: Revised the section in response as follows:

> Our exclusion criteria were: history of TBI, pre-existing disabling disorders or pregnancy. The participants had a mean age of 37.7 ± 16 years (range 20-55) and 20% were female. A 60 second inhalation of 5% CO_2 was reliably accompanied by an increase in $EtCO_2$ pressure as measured by capnography. The $EtCO_2$ trace was accompanied by an increase of HbO and a decrease of HbR (**figure 4**).

Editor's comment: Is this figure a representative of one participant?

Author's response: One participant. The legend specifies it now as shown below:

> On average, in our HC group, the increase of HbO appeared 2.3 ± 2.6 s before the HbR decrease was noted. Because of this delay between HbO and HbR, the time shift between the $EtCO_2$ tracing and HbO signal is not the same as the time shift between the $EtCO_2$ tracing and HbR signal. In addition, we also calculated the time shift between the $EtCO_2$ tracing and Hb-diff (difference between HbO and HbR signal). The Hb-diff parameter gave us the strongest amplitude between the two conditions.

Editor's comment: Where is this data?

Author's response: Data are from the average of 15 patients. The second paragraph has been rephrase for better clarity, as follows:

> Physiologically, HbO andHbR are out of phase[14]. In figure 4, which represents the fNIRSsignal of one participant, we observed that the HbR signal precedes the HbO signalby 3.5 s (a precise measurement can be derived from the time shift for eachsignal). On average across all participants, we observed that the HbOsignal increases 2.3 ±2.6 s after the HbR signal decreases. This impliesthat the time shift for HbO and HbR are different and need to be estimatedbefore calculating a participant's CVR. For this same reason, we also need toestimate the time shift between the $EtCO_2$ tracing and Hb-diff (the differencebetween HbO and HbR signals). The Hb-diff parameter gave us the strongestamplitude between the two conditions.

Editor's comment: Please upload the table as .xlsx file separately to your editorial manager account.

Author's response: This has been done.

1. Prepare the Materials used for the Hypercapnia Challenge (Figure 1)

1.1. Inflate a 200 L Douglas bag (Item #1) with a pre-mixed canister of medical-grade gas comprised of 5% carbon dioxide, 21% oxygen, and 74% nitrogen until full.

1.2. Insert two diaphragms (Item #3) into the two-way non-rebreathing valve (Item #4) to safeguard that the gas will only flow in one direction. Attach one port of the three-way valve (Item #2) to the Douglas bag (Item #1) via the gas delivery tube (Item #5), and the other port to the two-way non-rebreathing valve (Item #4) via a second gas delivery tube (Item #5).

1.3. Fasten the mouthpiece (Item #6) to the connector (Item #7) and then fasten the connector to the two-way non-rebreathing valve (Item #4).

1.4. Insert the capnograph tubing (Item #8) into the hole in the connector (Item #7).

1.5. Attach the air-filter (Item #9) to the capnograph tubing (Item #8).

1.6. Screw the end of the plastic air-filter (Item #9) that isn't connected to the capnograph tubing (Item #8) into the CO2 (Item #10) monitor.

2. Procedures during the experiment

2.1. Ask the participant to sit in the chair and to make themselves comfortable while setting up the devices. Turn the fNIRS system on.

2.2. Place the source-detector headband on the patient's forehead, over the underlying prefrontal cortex areas (dorsal and inferior frontal cortical areas)21.

2.3. Check that the source-detector headband is carefully positioned above the eyebrow and in the middle of the forehead. Place the lower detector row approximately 3.5 cm above the nasion or bridge of the nose where the indentation of the upper nose meets the forehead between the eyes.

2.6. In CobiStudio, press "start current experiment". Sources will send light at 2 wavelengths and light signal intensity detected from each detector will be displayed in real time in CobiStudio. In case of saturated (signal>4000) or low signal (signal <1000), adjust the contact between the source-detector headband and the skin or the parameters in step 2.3 and 2.4. The exact full procedure has been explained by Ayaz et al. 22

2.7 Direct the participant to inhale and exhale through their mouth at their normal breathing pace. Fasten a nose clip on the participant's nose and remind them to continue breathing normally through their mouth, and to alert someone if they feel any discomfort or have any difficulty breathing.

2.9. Press the "Baseline" button in CobiStudio software. It will measure and automatically record the light signal for the fNIRS baseline for 20s.

2.10. Press "Record" before starting the experiment.

2.11. At the beginning of the experiment, start the clock, press "Manual Marker" in CobiStudio and write on paper the time displayed by the capnograph. Every minute turn the valve connected to the gas tubing to cycle between room air and room air mixed with 5% CO2. Again, press "Manual Marker" in CobiStudio and write on paper the time displayed by the capnograph each time the inhaled gas mixture

is changed (Figure 3). Manually marking the time displayed on the capnograph is essential for future synchronization between fNIRS optical signals and capnograph's EtCO2 trace.

2.12. After 7 min, stop the fNIRS recording by clicking on "Stop" in CobiStudio. Allow 60 additional seconds of recording for the EtCO2 and save the EtCO2 data as ASCII within the data reader software.

2.13. Notify the participant that the procedure is completed. Carefully take off the nose clip and withdraw the mouthpiece. Offer a tissue to the participant to absorb any accumulated saliva from the procedure.

4. Data analysis

4.1.1. In fNIRSoft, Click on "Load File" to select and then upload the acquired fNIRS data.

4.1.2. Click on "Refine" and a pop-up window will appear. Select "Raw Data" and press "Next".

4.1.2.1. Click on both the median filtering and the sliding window motion artifact rejection (SMAR).23 tools to recognize and delete both motion artifact and saturated channels from the raw signal. Press "Apply".

4.1.2.2. Click on "Low Pass Frequency" filter to discard pulse and breathing component (Hanning filter, n=20, cutoff=0.1Hz).21;24-26 Press "Apply".

4.1.2.3. Click on "Detrend" to eliminate the slow temporal variation. Press "Apply".

4.1.3. Click on "OXI" to transform the light intensity into HbO and HbR concentrations. Click on "Save" and then select MATLAB as the save file format

4.2.1. Import the EtCO2 trace from the capnograph in MATLAB as two columns (one for time and the second for EtCO2 values). Shift the EtCO2 time with pre-calibrated time to correct the delay from the sampling tubing time (This is the time difference between one breath to the mouthpiece and the appearance of that breath on the CO2 recording. In this set-up, it was 15 seconds). Use the first time point recorded from the capnograph at the beginning of the experiment, step 2.11 as the starting point (t=0). Convert the EtCO2 into seconds.

4.2.2. Import the oxy and deoxyhemoglobin data from step 4.1.3 into MATLAB.

4.2.3. Calculate the physiological delay between EtCO2 (measured in the mouth) and the fNIRS signal (measured in the brain) by finding the higher correlation coefficient between these two signals at varying time shifts. (MATLAB script attached for step 4.2.3 to step 4.2.5). The time shift with the higher correlation coefficient is considered the optimal time.

4.2.4. Shift the EtCO2 time course by the optimal time (obtained in step 4.2.3). Keep the time points that have both fNIRS and EtCO2 data. The two time series should have the same length.

4.2.5. Calculate CVR values for each channel, which is the solution of the linear equation between HbO (or HbR) and EtCO2 using the Cholesky decomposition in MATLAB.