2023

Investigating the effects of systemic physiology on Functional Near-Infrared Spectroscopy based resting-state functional connectivity networks

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Introduction:

The use of neuroimaging has enhanced our understanding of the human brain and its associated functions. Research and development of these neuroimaging techniques have made it possible to study the brain in both healthy and patient populations, including neurological and psychiatric conditions (Pinti *et al.,* 2020). The present thesis aims to contribute to the research and development of neuroimaging techniques by improving the functional near-infrared spectroscopy (fNIRS) technology. The objective of this thesis is to improve the research application of fNIRS-based resting state functional connectivity (rsFC) studies and enhance the clinical relevance of fNIRS.

Functional Connectivity

The concept of functional connectivity posits that brain regions exhibiting consistent correlations in neural activity form a neural network (Eickhoff and Müller, 2015). The characteristics of each network depend on several factors and vary with brain disorders, providing important biomarkers of brain health. Robust and reliable functional networks can be extracted in both resting state and task-based protocols. Resting state protocols involve measuring functional connectivity while the subject is at rest and not engaged in a specific task, while task-based protocols involve measuring functional connectivity while the subject performs a specific task. However, task-based protocols may present limitations, such as the exclusion of participants with preoperative cognitive impairments, physical impairments or due to their age, as they require the participant's ability to perform the task (Lemée *et al.,* 2019). These limitations can be circumvented by rsFC protocols, which simply necessitate that the participant remains still without focusing on any specific task. Following pioneering research by Biswal and colleagues, rsFC has gained popularity for its simplicity and potential to serve as a biomarker of brain function (Biswal *et al.,* 1995). Currently, there is ongoing research exploring its potential usefulness as a diagnostic and monitoring tool for various brain disorders (Biswal *et al.,* 1995; Vemuri *et al.,* 2012; Abdalmalak *et al.,* 2021).

Neurovascular Coupling

While functional connectivity refers to the connections between regions that exhibit consistent correlations in neural activity, neurovascular coupling describes the increase in cerebral blood flow following neural activity. Upon brain activation, there is an increase in the brain's metabolic demand for oxygen and glucose leading to local arteriolar vasodilation resulting in an increased regional cerebral blood flow. The increase in cerebral blood flow following neural activity is referred to as neurovascular coupling. The systemic responses induced by neurovascular coupling will eventually result in a rate of oxygen supply to the activated brain region which exceeds the brain's rate of consumption. This disbalance results in an increase in oxygenated hemoglobin (HbO) and a decrease in deoxygenated hemoglobin (HbR), a phenomenon known as the hemodynamic response. This response can be quantified and used to infer brain function with specific neuroimaging techniques, such as functional magnetic resonance imaging (fMRI) (Nippert *et al.,* 2018).

fMRI

Although considered the gold standard in neuroimaging, the fMRI technique does have limitations, including poor temporal resolution and limited suitability for certain populations (Wijeakumar *et al.,* 2017). Specifically, the use of fMRI is limited when it comes to intubated

patients due to its lack of portability. This is because moving such patients to the fMRI room can pose safety risks.

fNIRS

Recent research has revealed that fNIRS can be used as an alternative to fMRI in the assessment of brain activity (Cui *et al.,* 2011). fNIRS presents several advantages over fMRI, including higher temporal resolution, allowing for brain signals to be captured every 0.01 seconds for more precise measurements. Moreover, fNIRS is relatively inexpensive and highly portable when compared to fMRI (Wilcox and Biondi, 2015). The portability of fNIRS allows for bedside monitoring of patients without the need for them to be transported to specific imaging rooms. This feature makes it a useful tool for studying the brain in both healthy and patient populations, including those who are unable to undergo traditional imaging techniques due to medical conditions or physical limitations.

Measuring brain activity using fNIRS involves shining near-infrared light (650-950 nm) onto the scalp. As the light travels through the different cerebral layers, such as the scalp, skin, skull, and cerebrospinal fluid, it interacts with various components, including water, lipids, hemoglobin, melanin, and cytochrome-c-oxidase, each of which has unique absorption and scattering properties at different wavelengths (Scholkmann *et al.,* 2014). Among these components, hemoglobin is the primary absorber of near-infrared light, with different absorption properties for HbO and HbR. HbO absorption is highest for light with a wavelength below 800 nm, while HbR absorption is highest for light with a wavelength greater than 800 nm (Pinti *et al.,* 2020).

The light shined on the scalp diffuses to the biological tissue, reaches the brain cortex, and comes back to the surface, carrying valuable information about the hemoglobin concentration changes from the brain. The reflected light can be measured with a light detector placed apart from the emitter. To assess cortical information, the distance between the source and detector needs to be around 3cm. The combination of a source/emitter and a detector is referred to as a channel. Since the near-infrared light during its path is primarily absorbed by hemoglobin, changes in light attenuation can be used to estimate concentration changes of HbO and HbR. Due to the phenomenon of neurovascular coupling, this is an indirect measure of neural activity in the underlying brain tissue (Delpy and Cope, 1997).

Seed based correlation analysis (SCA).

Seed-based correlation analysis (SCA) is a commonly used method to analyze resting state fNIRS data. SCA involves extracting the hemoglobin time series, the changes in hemoglobin concentrations over the span of the measurement, from a selected seed region, a specific fNIRS channel of the brain. Selection of the seed depends on the purpose of the work and is typically based on prior knowledge of which brain regions or networks are important for a given population. The hemoglobin time-series of the seed channel is then correlated to all the other channels spanning the brain to detect regions with coherent time series. SCA relies on the assumption that functionally correlated neural networks have similar activity and hemodynamic changes, thus being functionally connected. These correlations are then summarized within a correlation matrix which can then be projected onto brain models. The major advantage of SCA

is its relatively straightforward interpretability, which directly shows which regions are most strongly functionally correlated with the seed (Cole *et al.,* 2010).

Signal Contamination by Systemic Physiology

A major critique of fNIRS pertains to its susceptibility to spurious correlations, or false positives, within SCA, which results in the display of non-existent connections. A major culprit for these spurious correlations is the fNIRS signal being highly contaminated by systemic physiology. fNIRS signal contamination by systemic physiology includes both extracortical components and global components such as changes in blood pressure and respiration rate (Abdalmalak et al., 2022; Zhou et al., 2021). The signal contamination caused by systemic physiology leads to two major issues. Firstly, analyzing the fNIRS signals without removing systemic physiology leads to high correlation across the whole brain. Essentially, the fNIRS signal shows that every area of the brain is functionally correlated to every other area covered by the fNIRS system. The second problem is that systemic physiology leads to high levels of intra-subject variability. This means that when measuring a single healthy participant multiple times, the systemic physiology leads to significantly different functional connectivity patterns being produced for each measurement. This is problematic as the functional connectivity patterns of a healthy brain should not change drastically over the span of multiple measurements (Abdalmalak *et al.,* 2022).

Short channel regression

Due to the diffusive character of the fNIRS light propagation in the biological tissue, the distance between the sources and detectors determines the depth sensitivity of each channel (Saager and Berger, 2005). The light detected by a regular fNIRS channel (~ 3cm), probes both the cortical and extracortical layers. Thus, regular fNIRS channel deliver hemodynamic information from both the cortical and extracortical layers. A problem arises from the fact that the extracortical layers are densely vascularized, which leads to majority (~ 94%) of the signal measured from a regular fNIRS channel reflecting hemodynamic information from the extracortical layers, primarily the scalp (Brigadoi and Cooper, 2015). In 2005, Saager and colleagues took advantage of the relationship between the source-detector distance and depth sensitivity by employing the use of a channel with a smaller source-detector distance to obtain a signal primarily from the extracortical layers. They aimed to decrease the distance between the source and the detector to then decrease the depth that the near-infrared light travelled. The signal recorded from this short channel (SC) was then subtracted from the standard fNIRS channel. This was done with the goal of regressing or removing the extracortical components from the fNIRS signal, isolating only the brain's hemodynamic response (Saager and Berger, 2005).

Physiological Measurements

Despite SC regression being a useful method to remove signal contamination caused by systemic physiology, it may not contain all the necessary non-neural information to fully decontaminate regular fNIRS channels (Scholkmann et al., 2014; Caldwell et al., 2016). To address these limitations and increase the sensitivity of the fNIRS signal to the brain, researchers have suggested recording additional physiological measurements (Phys) and regressing them

from the fNIRS signal. Specifically, researchers suggested recording mean arterial pressure (MAP) and end-tidal CO_2 (Caldwell et al., 2016; Tachtsidis and Scholkmann, 2016).

GLM Frameworks

The removal of SCs and Phys is often performed within a General Linear Model (GLM). The GLM is a mathematical framework that enables the analysis of complex signals such as those produced by fNIRS. Within the GLM framework, fNIRS signals are expanded as a linear combination of components called regressors, which are representative of different aspects of the signal. For example, the baseline level of blood flow. The inclusion of nuisance regressors such as SC and Phys measurements can help to decontaminate the fNIRS signal by accounting for non-brain-related physiological changes that may interfere with the signal. By controlling for these effects at the statistical level, the accuracy of the fNIRS technique can be improved (Von Lühmann et al., 2020).

Rationale and Hypothesis

Previous work by the Owen Lab demonstrated that a combination of SC + Phys regression is a powerful approach to decontaminate fNIRS signals. However, the previous study was limited by acquiring data only once from each participant. In the present study, we aim to build upon this work by collecting data multiple times from each participant to further investigate intra-subject variability. The rationale for this experiment is to identify which regressors or combination of regressors can most effectively remove systemic noise from fNIRS signals to improve the precision of fNIRS signals and reduce intra-subject variability. Ultimately, the goal is to enhance the methodology of fNIRS and improve its research application for resting-state functional connectivity (rsFC) studies, as well as its clinical relevance. We hypothesize that using a combination of SC and Phys regression will improve the localization of fNIRS signals during resting-state data acquisition and reduce intra-subject variability.

Methods:

Experimental Protocol

This study was approved by the Research Ethics Board at Western University. All participants provided written informed consent before participating in the study. Data collection was performed at the University Hospital at Western University, London, Canada. The study recruited fifteen healthy controls (ten males and five females) with no history of severe brain injury. Data was collected three times for each participant, once per session for three different days within the span of a week. To reduce variance, each recording occurred at the same time for each participant. Data collection was conducted in a dimly lit room, where the participant was seated comfortably. Before starting data collection, participants were instructed to close their eyes, stay relaxed, and to not focus their thoughts on anything specific. Each data collection involved a twelve-minute resting state recording. To ensure the participants did not fall asleep, two researchers monitored their behaviour during the recording and then after the recording got verbal confirmation from the participant that they did not fall asleep.

fNIRS Signal Acquisition

Data was collected using a commercially available continuous-wave NIRS system (NIRScout, NIRx Medical Systems) with a sampling rate of 3.9 Hz. A 10–20 standard head cap was used to attach the sources and detectors to the participants' heads. The optical probe utilized in this study was specifically designed by the Owen lab and comprised of 39 detectors and 32 sources, resulting in 121 source-detector combinations at approximately 3 cm (channels) and eight source-detector pairs at 0.8 cm (SC). The sources were LEDs centered at 760 and 850 nm.

fNIRS Preprocessing

A summary of the pre-processing steps is shown in **Figure 1**. To conduct data analysis, the present study used MATLAB scripts developed by the Owen Lab based on existing Homer 2 functions (Huppert *et al.,* 2009). Before analyzing the fNIRS data, preprocessing was necessary to convert light intensity measurements into hemoglobin concentration changes. Firstly, channels with low signal-to-noise ratios (SNRs) were removed, defined as values below 8 based on previous experiments using similar fNIRS systems (Novi *et al.,* 2020). SCs with pink noise (1/f decay) were also excluded. Pink noise is a type of noise with a power spectral density that follows a 1/f power law distribution. Afterward, the modified Beer-Lambert law was employed to estimate hemoglobin concentration changes, a biophysical model that relates measured light intensity to hemodynamics. Finally, band-pass filtering was applied to the hemoglobin time series between 0.009 and 0.08 Hz to mitigate low-frequency drifts and high-frequency physiological noise, such as heart rate. Band-pass filtering is a signal processing technique that allows for the selection of specific frequency bands, in this case, frequencies that do not represent neural signals were removed.

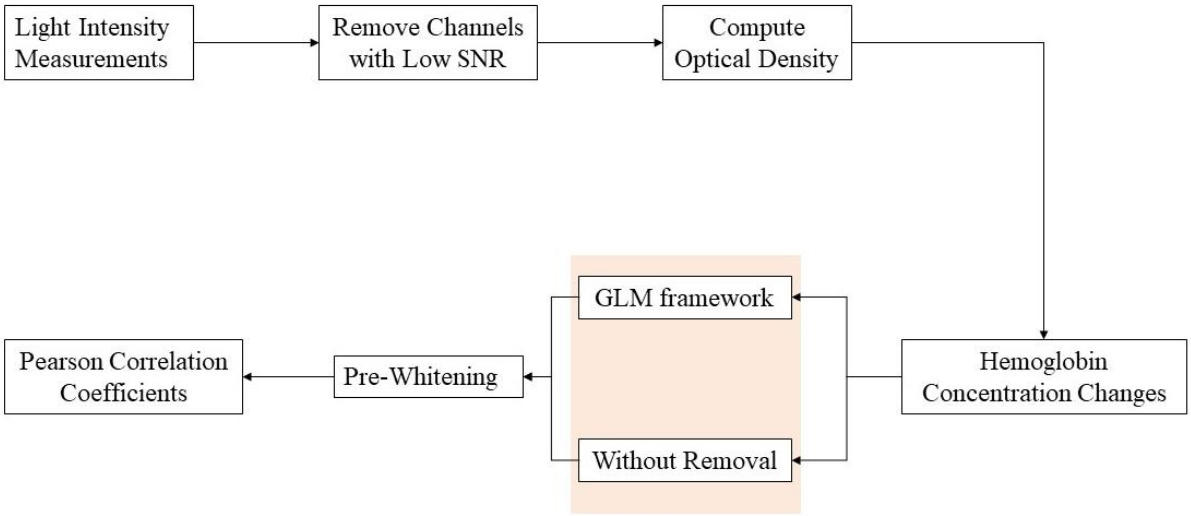

Figure 1. Flow chart summarizing the pre-processing steps for extracting hemoglobin concentration changes and Pearson Correlation Coefficients from the functional near-infrared spectroscopy (fNIRS) data used in this study. The highlighted part shows the focus of this work. The first step involves the removal of channels with low signal to noise ratio (SNR) from the light intensity measurements. Following this, the optical density is computed, which is then used to extract the hemoglobin concentration changes. The hemoglobin concentrations can either be manipulated within the general linear model (GLM) to remove the systemic physiology regressors or kept without removal of the regressors. The concentrations are then pre-whitened before calculating the Pearson Correlation Coefficients. Adapted from (Abdalmalak *et al.*, 2022).

Physiological Recordings and Preprocessing

In addition to the fNIRS data, physiological information was acquired simultaneously. MAP was measured using a Caretaker system (Caretaker Medical, United States of America) which was affixed to the participants' left arm. The Caretaker system allowed for continuous monitoring of these systemic physiological changes at a sampling rate of 1.5 Hz. End-tidal CO_2 was measured using a cannula connected to a capnograph (Oxigraph, Inc., United States) at a sampling rate of 804 Hz. The Caretaker system required calibration before data acquisition; therefore, it was started 1 minute prior to the start of the experiment. The capnograph was started 30 seconds before the beginning of the experiment, and the first minute of Caretaker data and the first 30 seconds of end-tidal CO_2 data were excluded from analysis. This exclusion occurred to synchronize the physiological data with fNIRS data. The Caretaker and capnograph data were then resampled to match the fNIRS acquisition frequency (3.9 Hz) and band-pass filtered between 0.009 and 0.08 Hz.

Removal of Systemic Physiology

The systemic physiology was removed from the fNIRS signal within the GLM framework. A linear model for each hemoglobin time series was used: $Y_{Hbx} = X\beta + \varepsilon$, which consisted of the design matrix (X), model parameters (β), and an error term (ε). The design matrix (X) contains explanatory variables, such as SC data (X_{SC}), physiological measurements (X_{Phys}), and a constant offset array (X_C) which corrects for the baseline of the data. To analyze the impact of each regressor, the data was analyzed using three distinct GLM models: SC

regression only ($X \equiv [X_C, X_{SC}]$), systemic physiology only ($X \equiv [X_C, X_{Phys}]$), and both SC and physiology ($X \equiv [X_C, X_{SC + Phys}]$), in which $X_{SC + Phys} \equiv [X_{SC}, X_{Phys}]$. X_{SC} includes only the good SCs, as defined in the fNIRS preprocessing section, to account for heterogenous scalp hemodynamics, and both HbO and HbR from the SCs were included in the GLM following previous works (Kirilina *et al.,* 2012). The X_{Phys} submatrix used MAP and end-tidal CO_2 measurements. To account for the difference in transit time between systemic physiology and fNIRS signal acquired from different brain regions, a maximum time shift of ±20 s was allowed for each fNIRS channel and each physiological regressor. The transit time is described as the time it takes for the changes in the physiology measured in the periphery to affect the hemodynamics in the brain. The optimum time lag was determined as the time shift that yielded the highest correlation between the regressor and the regular fNIRS channel. This time shift also accounted for any possible synchronization errors between the different acquisition systems (i.e., NIRScout, Caretaker and capnograph). Finally, the model parameters (β) were estimated to minimize the use of robust regression by the GLM. Despite robust regression reducing the impact of outliers within the dataset, it can reduce the accuracy of the analysis leading to incorrect conclusions. The filtered signal was written as: $Y_{filtered} = Y_{Hbx} - X\beta$.

Correlation Analysis and Seed-Based Networks -

To reduce spurious correlations across the brain due to autocorrelation in the time-series, pre-whitening was applied in the filtered HbO and HbR concentration time series via an autoregressive model. Next, the total hemoglobin (HbT) concentration was estimated as the sum of HbO and HbR, and the Pearson correlation coefficient across the regular fNIRS channels was computed. The impact of the regression techniques on the correlation distributions for each participant was investigated. To do this, group analysis via the concatenation of the correlation distributions of each participant was performed. In addition to the correlation distributions, the most common rsFC networks that have cortical contributions were extracted using the previously validated seed-based method (Biswal *et al.,* 1995). The correlation coefficients were converted to Z-scores via Fisher's transformation, then averaged across participants to compute an average correlation matrix per hemoglobin. Finally, a seed (i.e., regular channel) was selected per network then back-projected its Z-scores to the brain. The chosen seeds were located at the left precentral gyrus over the primary motor cortex (sensorimotor network), left superior temporal gyrus (auditory network), and left middle frontal gyrus (FPC network). These specific seeds were chosen to their high clinical significance (Biswal *et al.,* 1995) (Beckmann *et al.,* 2005). To visualize the seed-based networks, the sensitivity profile of each channel computed through Monte Carlo (MC) simulations was used to project to the cortex the correlation value of that specific channel with the chosen seed (Aasted *et al.,* 2015). Codes for creating these figures were available in the Owen Lab fNIRS library.

Intra-Subject Variability

Finally, the effect of systemic physiology on the intra-variability of the extracted seed-based rsFC networks was investigated. To this end, the individual variability was quantified by computing the Euclidian distance between the seed-based maps extracted at the first and second days of measurements. Statistical differences were inferred using two-sided t-tests to compare the variability distributions with and without the regression techniques and considered significant if $p<0.05$.

Results:

To address the research question and test the hypothesis of the present thesis, we acquired a very rich dataset in which fNIRS data with 8 SCs and additional systemic physiology were simultaneously measured from 15 participants at three different days at the same time within the same week. The physiological data includes continuous measurements of MAP, end-tidal CO_2, and oxygen saturation. To our knowledge, there are no published works with such a complete dataset as ours. However, due to time constraints, only a subset of the data was analysed. Specifically, we decided to focus on the measurements from the first and second days or runs from the first 12 participants, and we limited the analysis to SCs, MAP, and end-tidal CO_2. Although we did not explore everything we could with the acquired data, we highlight that this subset is enough to evaluate our original hypothesis.

Systemic Physiology Inflates Correlation Distributions

Figure 2 shows the correlation distributions for HbO, HbR and HbT across the first 12 participants when fNIRS signals were not regressed, after SC regression, and after SC+Phys regression. Notably, the different preprocessing steps has a similar effect on both runs. Specifically, both regression techniques (SC only and SC+Phys) were able to shift the HbO and HbT correlation distributions towards smaller values reducing the global correlation of the fNIRS signal across the brain. This indicates that the global correlations (false positives) seen before regression for HbO and HbT are not caused by actual neural activity but rather from the contamination of extracerebral hemodynamics in standard fNIRS signals. For HbR, the extracerebral oscillations did not significantly impact the correlations.

After applying the regression techniques, the distribution mode, which indicates the most frequently occurring value in a distribution of correlation coefficients and corresponds to the peak of the distribution curve, exhibited a substantial decrease for both HbO and HbT in both run 1 and run 2. Specifically, for run 1, the distribution mode without regression was 0.755 for HbO and 0.714 for HbT. This decreased to 0.143 for HbO after both SC only and SC+Phys regression and 0.143 and 0.102 for HbT after SC only and SC+Phys regression, respectively. Whereas, for run 2, the distribution mode without regression was 0.755 for HbO and 0.714 for HbR. This decreased to 0.143 for HbO after both SC only and SC+Phys regression and 0.102 and 0.143 for HbT after SC only and SC+Phys regression, respectively. In both runs, the extracerebral oscillations did not significantly impact the correlations performed using HbR. Interestingly, for both runs, the addition of physiological measurements as a regressor (SC+Phys regression) within the GLM model did not considerably alter the distribution mode HbO and HbT distribution modes. However, it decreased the spread of correlation coefficients by eliminating additional covariance unaccounted for by the SC only regression. Specifically, for run 1, variance decreased from 0.328 to 0.309 for HbO and 0.336 to 0.309 for HbT between the SC only and SC+Phys regression. Therefore, SC + Phys regression decreased the variability of the correlations resulting in a greater level of measurement precision.

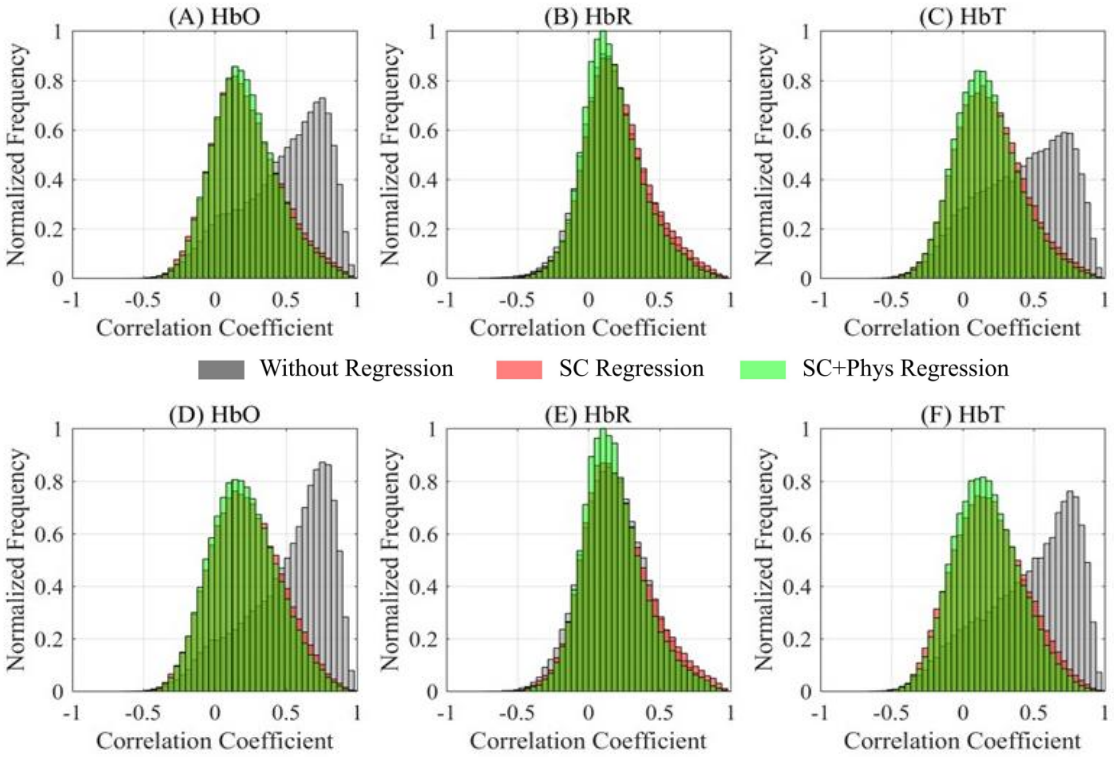

Figure 2. Combined correlation distributions for hemoglobin concentration time series from Runs 1 and 2 of the first 12 participants (n=12) using three different preprocessing approaches. The three hemoglobin types shown are oxyhemoglobin (HbO), deoxyhemoglobin (HbR), and total hemoglobin (HbT). Preprocessing approaches include no regression (gray), short channel regression (SC, red), and short channel and systemic physiology regression (SC+Phys, green). The normalized frequency was determined by dividing the counts of each distribution by the highest count value. Panels (**A**)-(**C**) show the combined correlation distributions for Run 1 of the first 12 participants for HbO, HbR, and HbT, respectively. Panels (**D**)-(**F**) show the combined correlation distributions for Run 2 of the first 12 participants for HbO, HbR, and HbT, respectively. Extracerebral regression has a significant impact on the removal of spurious correlations (false positives). Graphs were generated using MATLAB.

Removal of Systemic Physiology Localizes Functional Connectivity Maps

Figure 3 illustrates the group average rsFC networks when using the HbT time series for the differing regression techniques. The HbT time series was selected as it accounts for both HbO and HbR, and previous studies have shown that using HbT provides higher reproducibility than using the two chromophores separately (Sheth et al., 2004; Novi et al., 2016; Culver et al., 2005; Abdalmalak et al., 2022). **Figure 3a** and **Figure 3b** represents the rsFC networks formed using data collected during run 1 and run 2, respectively. Notably, the different preprocessing steps has a similar impact on the rsFC networks generated for both runs. When rsFC networks are generated before applying any regression techniques to remove the impact of global systemic physiology on the fNIRS signal, there is a high degree of correlation observed throughout the brain. This is apparent in **Figure 3**, where the "without regression" rsFC networks for the sensorimotor and frontoparietal cortex (FPC) regions show correlations with most of the other brain regions covered by the montage. After applying the regression techniques (SC only and SC + Phys), the rsFC networks for these regions become more localized. This finding demonstrates that removal of systemic physiology from the fNIRS signals, eliminates most irrelevant brain connections, leaving only the expected connections for these two networks. Interestingly, this trend was not seen in the auditory rsFC networks. The rsFC networks generated "without regression" show very similar correlations to those generated after applying the regression techniques.

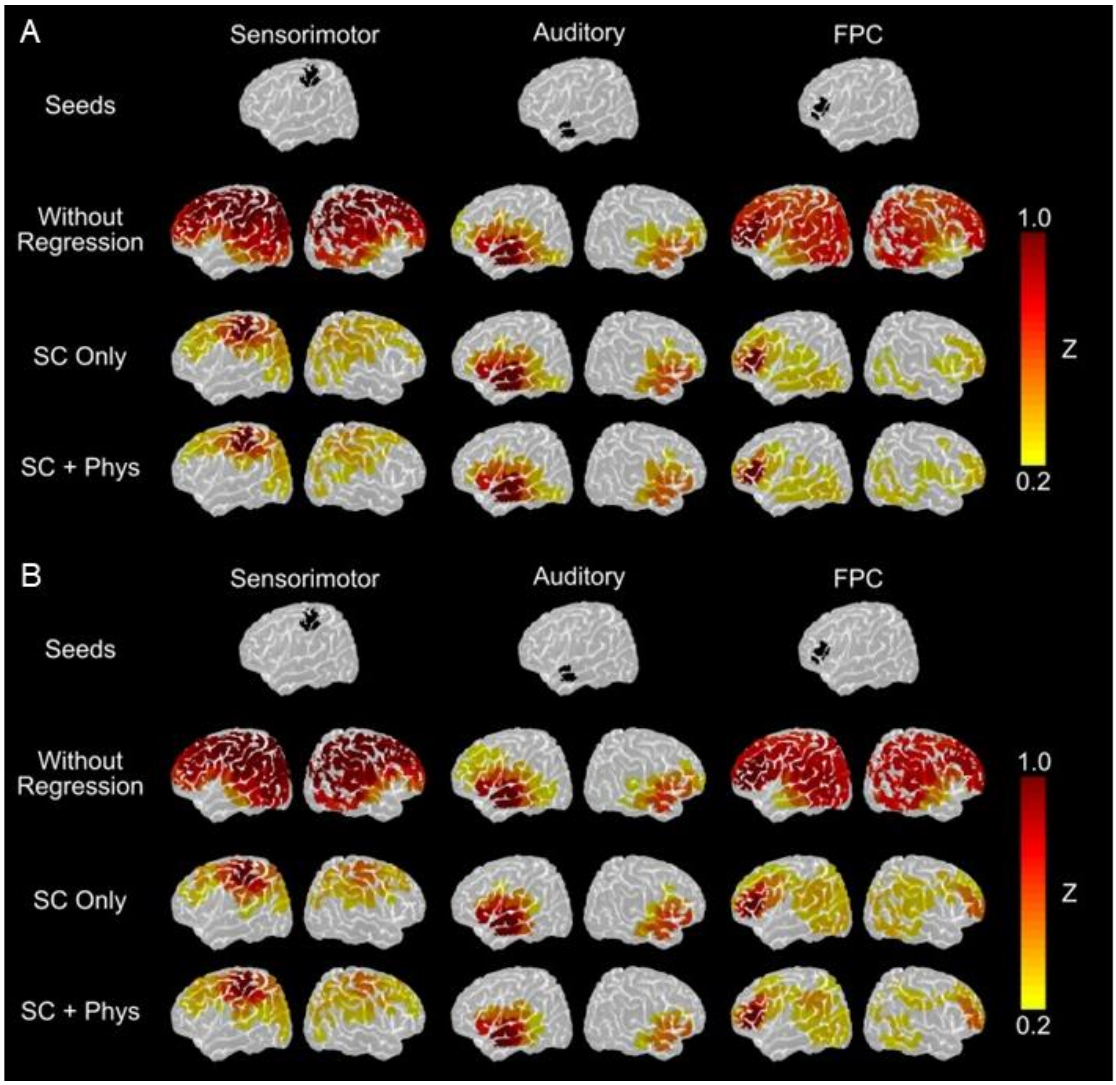

Figure 3. Sensorimotor, Auditory, and Frontal Parietal Cortex (FPC) seed-based networks extracted from the average HbT correlation matrix of Run 1 and Run 2 for the first 12 participants (n=12). Each seed's location is shown in the top row, followed by the resulting network without regression, with SC regression only, and with SC + Phys regression. Panels (**A**) and (**B**) show seed-based networks generated from correlation matrices obtained during runs 1 and 2, respectively. Z-scores are a standardized measure that allows for comparison of the strength of functional connectivity between a brain region and the selected seed. Regression of systemic physiology improves network localization and increases agreement with the fMRI literature. rsFC networks generated using MATLAB.

Decrease of Intra-Subject Variability

To assess the effect of regressing systemic physiology on intra-subject variability in fNIRS rsFC, Euclidean distances between the correlation matrices obtained from Run 1 and Run 2 were calculated for each participant. **Figure 4** depicts the normalized distances for the sensorimotor, auditory, and FPC networks, with normalization performed by dividing the distances by the maximum values across all preprocessing approaches within each network. For the sensorimotor network both SC only ($p = 0.0099$) and SC + Phys ($p = 0.0022$) regression significantly decreased intra-subject variability. Similarly, for the FPC network both SC only ($p = 0.0214$) and SC+Phys ($p = 0.0059$) regression significantly reduced intra-subject variability. These findings indicate that removal of systemic physiology significantly increases the intra-subject reliability across the sensorimotor and FPC networks. However, SC + physiology regression did not significantly decrease ($p > 0.05$) the intra-subject variability compared to SC-only regression for these two regions. Despite not being statistically significant, the SC+Phys regression appeared to alter the intra-subject variability values, suggesting that the alterations observed may provide additional information beyond what is already captured by the SC regression. Additionally, the removal of systemic physiology did not significantly decrease ($p > 0.05$; Figure 4) the intra-subject variability for the auditory network. Overall, the decrease in intra-subject variability after physiological removal using SCs must be considered prior to comparing results between different runs; otherwise, any differences among the runs could be due to systemic physiology and may not have been originated from actual neural sources.

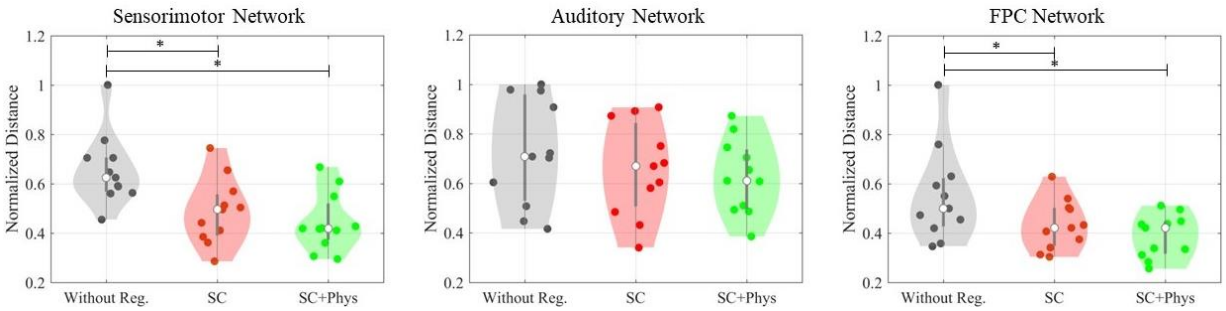

Figure 4. Intra-subject variability of seed-based networks estimated through Euclidean distance between networks from Run 1 and Run 2 of the first 12 participants (n=12), shown as violin plots. The solid dots represent actual distance values, and statistical differences between variability distributions are denoted by asterisks (*), indicating a significance level of $p<0.05$ with a two-sided t-test. To compare the impact of each regression technique on intra-subject variability, distances were normalized by the maximum value across all regression techniques for each network. In the Sensorimotor and FPC networks, the application of the regression techniques resulted in a significant reduction in intra-subject variability. However, there was no significant difference observed between the two regression techniques. Removal of systemic physiology did not significantly decrease the intra-subject variability for the auditory network. Graphs were created with MATLAB and statistical analyses were conducted using Excel.

Discussion:

The goal of this study was to investigate the impact of systemic physiological variables on fNIRS-based rsFC networks by acquiring resting-state data with SCs and additional physiological measurements. Our findings corroborate previous studies, indicating that systemic physiology can lead to false positives (**Figure 2**), overestimating rsFC (Mesquita et al., 2010; Kirilina et al., 2012; Scholkmann et al., 2014; Caldwell et al., 2016; Tachtsidis and Scholkmann, 2016). In contrast to prior studies, we evaluated the effectiveness of various regression techniques and their effects on the reproducibility of rsFC networks. We found that SC regression and SC + Phys regression techniques localized rsFC networks (**Figure 3)** allowing for more precise mapping of brain regions involved in specific cognitive functions. Notably, while SC regression reduced the contribution of extracerebral hemodynamics, the remaining signal still contained systemic information associated with the MAP and end-tidal CO_2 data. Moreover, removal of systemic physiology decreases intra-subject variability (**Figure 4**). Thus, highlighting the potential for systemic physiology to hinder the detection of changes in rsFC and impair single-subject longitudinal interpretations. Consistent with earlier research, we also found that fNIRS could extract rsFC networks that are typically identified with fMRI, but only after eliminating systemic physiology and selecting appropriate channels as seeds (Duan *et al.,* 2012).

The findings presented in **Figure 2** demonstrate that extracerebral physiology has a greater impact on HbO and HbT than on HbR. Specifically, HbO and HbT exhibit a reduction in correlation following SC only and SC + physiology regression, while this trend is not observed for HbR. These results support earlier studies that have similarly shown HbR to be less sensitive to systemic physiology (Franceschini et al., 2003; Tachtsidis and Scholkmann, 2016). This is likely because the autonomic nervous system preferentially affects arterioles more than venules during vascular drainage. Arterioles are the small blood vessels that supply oxygenated blood to the capillaries in the brain, while venules are the small blood vessels that collect deoxygenated blood from the capillaries. During autonomic nervous system activity, arterioles are more likely to constrict, which reduces blood flow and leads to a decrease in HbO levels. In contrast, the venules are more likely to dilate, which increases blood flow and leads to an increase in HbR levels. This phenomenon suggests that changes in systemic physiology have a larger impact on HbO signals compared to HbR signals (Franceschini et al., 2003; Tachtsidis and Scholkmann, 2016).

We chose to focus this study on the sensorimotor, auditory, and FPC networks (**Figure 3 and Figure 4**) for two primary reasons. Firstly, these networks are known to be robust and have been frequently reported across different populations and neuroimaging techniques (Biswal et al., 1995; Beckmann et al., 2005). Secondly, these networks are clinically significant and offer crucial insights into distinct aspects of brain function. For example, the FPC plays a critical role in maintaining normal consciousness, and disruptions in this network could indicate a potential marker for patients at risk of developing disorders of consciousness (Demertzi et al., 2014; Kazazian et al., 2021). Therefore, extracting reproducible rsFC networks for these brain regions at the single-subject level is crucial to demonstrate the potential of fNIRS to be used as a tool for assessing awareness at the bedside longitudinally.

Based on previous work, we than focused on the rest of the analysis on HbT, generating the rsFC networks (**Figure 3**) and calculating intra-subject variability (**Figure 4**) using the correlation coefficients for HbT (Sheth et al., 2004; Culver et al., 2005; Novi et al., 2016). The findings in **Figure 3** show removal of systemic physiology localizes the rsFC networks for the sensorimotor and FPC regions but not for the auditory region. Similarly, the findings in **Figure 4** show removal of systemic physiology decreased the intra-subject variability within the sensorimotor and FPC regions but not in the auditory region. The networks for the auditory region may not show change after regressing systemic physiology because it is less susceptible to systemic physiological noise compared to other networks. The auditory cortex receives its blood supply from the middle cerebral artery, which has a more direct and less variable path to the brain compared to other cerebral arteries, making it less vulnerable to systemic physiological noise (Mangold and M Das, 2022). Furthermore, the auditory cortex is more optically accessible and less likely to be contaminated by systemic physiology because of the reduced thickness of the extracerebral components (scalp, skull, and cerebrospinal fluid) between the source/detectors and the auditory cortex compared to other brain regions (Brigadoi and Cooper, 2015).

The findings in **Figure 4**, indicate that removal of systemic physiology from the fNIRS signals improves reproducibility of fNIRS-based rsFC networks by decreasing intra-subject variability. Interestingly, we found that SC + physiology regression did not enhance intra-subject reproducibility when compared to SC-only regression. This suggests that SC regression alone may suffice for longitudinal studies at the intra-subject level. However, it is possible that the physiological regressors used in our analysis did not fully capture the complexity of systemic physiology, and more physiological regressors, such as heart rate, may be needed to further decrease the intra-subject variability (Caldwell *et al.,* 2016). Thus, future studies, should focus on including more physiological regressors when comparing SC only regression and SC + Phys regression. Nevertheless, our findings emphasize the importance of removing systemic physiology to extract reproducible rsFC networks.

Previous research has shown that a seed-based approach, as used in this study has been reliable in extracting rsFC networks. However, a challenge in using a seed-based approach to extract rsFC networks, particularly for standard fNIRS systems, is choosing the appropriate seed location for each network, as it involves selecting which channel best represents which region of the brain (White et al., 2009; Eggebrecht et al., 2014). To address this issue, the seed locations were chosen based off previous studies (Eggebrecht *et al.,* 2014), and the rsFC networks were computed for all possible seeds to investigate the effect of alternate seed locations on the extracted networks using the SC + Phys regression method. Interestingly, the highest correlations were observed for inter-hemispheric connections with contralateral-homotopic brain regions for HbO, HbR, and HbT in most seeds.

When regressing MAP and end-tidal CO_2, an important methodological consideration is the temporal shift between the peripheral physiological signals and the fNIRS measurements on the head due to the transit time of blood circulation. Low-frequency oscillations, such as those observed in MAP, measured in the periphery (I.e., finger and toe) have been shown to be strongly correlated to the rsFC measured with the BOLD contrast in fMRI with varying time delays (Tong *et al.,* 2013). To improve the regression's performance, we allowed a maximum shift of ±20 s because these oscillations can arrive at the brain earlier or later than the periphery

in relation to the measurement sampling. This shift was necessary to account for the difference in arrival times of oscillations due to vessel size, pathlength, and flow rate of blood to different sites. Our observation that allowing this shift resulted in higher removal of spurious correlations compared to zero-lag regressions suggests the importance of considering temporal delays when performing physiological signal regression in fNIRS studies.

Although our study investigates different approaches to mitigate the impact of systemic physiology on fNIRS signals to improve its clinical and research application, it also has some notable limitations. First, we had a limited sample size of 15 participants and our analysis focused on only two measurements for 12 out of the 15 participants. We believe that although a larger sample size could increase the statistical power of our findings, the observed reproducible effect of removing systemic physiology across participants is reassuring and indicates that the observed results were not random. Second, motion artifacts (MA) were not removed in our preprocessing procedure. MA refer to changes in the fNIRS signal caused by movement or motion during data acquisition. Previous studies have shown that MA can severely degrade temporal correlations across fNIRS channels and can reflect movement-related changes in the amount of light reaching the scalp or changes in the position of the sources and detectors (Scholkmann et al., 2010; Novi et al., 2020). Therefore, it is important to remove MA as they can influence our interpretation of the results, decreasing the sensitivity and specificity of the measurement. Finally, the fNIRS cap was placed on the participant's head using a standard procedure based on the 10-20 system, used in electroencephalography (EEG). This system involves identifying specific locations on the scalp based on their distance from anatomical landmarks like the nasion (bridge of the nose) and inion (back of the head). However, using this method could lead to variability in cap placement, which may impact the sensitivity of the probes to certain brain regions. To minimize this variability and increase the accuracy of our results, a real-time neuro-navigation system which helps us place the caps on the participants head in a more precise way can be used (Khoshnevisan and Sistany, 2012). This would help decrease the variability and increase the average correlation within each brain network, improving our results (Novi *et al.,* 2020).

In conclusion, this study investigated the impact of systemic physiological on fNIRS-based rsFC networks and evaluated the effectiveness of various regression techniques on the reproducibility of rsFC networks. The study found that systemic physiology can lead to false positives, overestimating rsFC, and hinder the detection of changes in rsFC. The study showed that SC regression and SC + Phys regression techniques localized rsFC networks, allowing for more precise mapping of brain regions involved in specific cognitive functions. Furthermore, removal of systemic physiology from the fNIRS signals improves reproducibility of fNIRS-based rsFC networks by decreasing intra-subject variability. Overall, our findings underscore the potential for fNIRS as a clinical tool for assessing awareness at the bedside longitudinally.

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