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Inter-Brain Synchrony in Open-ended Collaborative Learning:An fNIRS-hyperscanning Study

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TITLE:

Inter-Brain Synchrony in Open-Ended Collaborative Learning: An fNIRS-Hyperscanning Study

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KEYWORDS:

Inter-Brain Synchrony; fNIRS hyperscanning; Wavelet Transform Coherence; open-science

SUMMARY:

The protocol for conducting fNIRS hyperscanning experiments on collaborative learning dyads in a naturalistic learning environment is outlined. Further, a pipeline to analyze the Inter-Brain Synchrony (IBS) of oxygenated hemoglobin (Oxy-Hb) signals is presented.

ABSTRACT:

fNIRS hyperscanning is widely used to detect the neurobiological underpinnings of social interaction. With this technique, researchers qualify the concurrent brain activity of two or more interactive individuals with a novel index called inter-brain synchrony (IBS) (i.e., phase and/or amplitude alignment of the neuronal or hemodynamic signals across time). A protocol for conducting fNIRS hyperscanning experiments on collaborative learning dyads in a naturalistic learning environment is presented here. Further, a pipeline of analyzing IBS of oxygenated hemoglobin (Oxy-Hb) signal is explained. Specifically, the experimental design, the process of NIRS data recording, data analysis methods, and future directions are all discussed. Overall, implementing a standardized fNIRS hyperscanning pipeline is a fundamental part of second-person neuroscience. Also, this is in line with the call for open-science to aid the reproducibility of research.

INTRODUCTION:

Recently, to reveal the concurrent brain activity across the interactive dyads or members of a group, researchers employ the hyperscanning approach[1,2]. Specifically, electroencephalogram (EEG), functional magnetic resonance imaging (fMRI), and functional near-infrared spectroscopy (fNIRS) are used to record the neural and brain activities from two or more

subjects simultaneously[3,4,5]. Researchers extract a neural index entailing concurrent brain coupling based on this technique, which refers to inter-brain synchrony (IBS) (i.e., phase and/or amplitude alignment of the neuronal or hemodynamic signals across time). A large variety of hyperscanning research found IBS during social interaction between multiple individuals (e.g., player-audience, instructor-learner, and leader-follower)[6,7,8]. Furthermore, IBS holds specific implications of effective learning and instruction[9–14]. With the surging of hyperscanning research in naturalistic learning scenarios, establishing a standard protocol of hyperscanning experiments and the pipeline of data analysis in this field is necessary.

Thus, this paper provides a protocol for conducting fNIRS-based hyperscanning of collaborative learning dyads and a pipeline for analyzing IBS. fNIRS is an optical imaging tool, which radiates near-infrared light to assess the spectral absorption of hemoglobin indirectly, and then hemodynamic/oxygenation activity is measured[15–17]. Compared with fMRI, fNIRS is less prone to motion artifacts, allowing measurements from subjects who are doing real-life experiments (e.g., imitation, talking, and non-verbal communication)[18,7,19]. In comparison with EEG, fNIRS holds higher spatial resolution, allowing researchers to detect the location of brain activity[20]. Thus, these advantages in spatial resolution, logistics, and feasibility qualify fNIRS to conduct hyperscanning measurement[1]. Using this technology, an emerging research body detects an index term as IBS—the neural alignment of two (or more) people's brain activity—in different forms of naturalistic social settings[9–14]. In this research, various methods (i.e., Correlation analysis and Wavelet Transform Coherence (WTC) analysis) are applied to calculate this index; a standard pipeline on such analysis is essential but lacking. A protocol for conducting fNIRS-based hyperscanning and a pipeline using WTC analysis to identify IBS is presented in this work.

This study aims to evaluate IBS in collaborative learning dyads using the fNIRS hyperscanning technique. First, a hemodynamic response is recorded simultaneously in each dyads' prefrontal and left temporoparietal regions during a collaborative learning task. These regions have been identified as associated with interactive teaching and learning[9–14]. Second, the IBS is calculated on each corresponding channel. The fNIRS data recording process consists of two parts: resting-state session and collaborative session. The resting-state session lasts for 5 min, during which both the participants (sitting face-to-face, apart from one another by a table (0.8 m)) are required to remain still and relax. This resting-state session is served as the baseline. Then, in the collaborative session, the participants are told to study the entire learning materials together, eliciting understanding, summarizing the rules, and making sure all learning materials are mastered. Here, the specific steps of conducting the experiment and fNIRS data analysis are presented.

PROTOCOL:

All recruited participants (40 dyads, mean age 22.1 ± 1.2 years; 100% right-handed; normal or corrected-to-normal vision) were healthy. Before the experiment, participants gave informed consent. Participants were financially compensated for their participation. The study was approved by the University Committee of Human Research Protection (HR-0053-2021), East China Normal University.

1. Preparation steps before adopting data

1.1. Homemade NIRS caps

1.1.1 Adopt elastic swimming cap to place optode holder grid.

NOTE: Considering that the head sizes of the participants are different, two sizes of caps are used. Small caps are prepared for participants with a head circumference of 55.4 ± 1.1 cm, and large caps are for the participants with a head circumference of 57.9 ± 1.2 cm.

1.1.2. Anchor the location of the EEG electrodes (inion, Cz, T3, T4, Fpz, and P5) as reference optodes according to the standard international 10–10 system on elastic swimming caps (see **Table of Materials**).

1.1.2.1 First, place the standard 10-10 EEG cap (see **Table of Materials**) on the head mold, and put the elastic swimming cap on the EEG cap. Second, mark reference optodes (inion, Cz, T3, T4, Fpz, and P5) with chalk on each cap. Finally, cut two holes about 15 mm in diameter to place the two reference optodes (i.e., Fpz and P5, **Figure 1**).

NOTE: Specifically, a 3 x 5 optode probe set and a 4 x 4 optode probe set are separately placed over the prefrontal area (reference optode is placed at Fpz, **Figure 1B**) and left temporoparietal regions (reference optode is placed at P5, **Figure 1B**).

1.1.3. Cut holes to place the other optodes. Arrange a swimming cap with two grid holders directly on the head mold. Then, mark the location of other optodes with chalk. After that, cut the rest holes to make sure the grid holder fits in.

1.1.4. Mount two probe sets (i.e., 3 x 5 and 4 x 4) to the swimming caps (see **Table of Materials**).

NOTE: The NIRS measurement system (see **Table of Materials**) provides these standard probe sets (i.e., 3 x 5 and 4 x 4) with standard holder sockets ensuring the 30 mm optode separation.

1.1.5. Open the probe set monitor window at the NIRS measurement system and select four probe sets arranged in 3 x 5 and 4 x 4 for each person, separately.

NOTE: The probe arrangements of the two caps should correspond to the structures in the probe set window (i.e., the exact location of the receiver probe numbers and the respective emitter).

1.2. Preparation of the experiment

1.2.1. Before recording data, ensure the NIRS system is keeping a stable operating

temperature by starting the system for at least 30 min.

NOTE: The stable operating temperature ranged from 5 °C to 35 °C.

1.2.2. Set the measurement mode to event-related measurement. Ensure the triggers receiver is active (i.e., the RS232 serial input).

NOTE: The experiment is programmed in commercially available psychology software (see **Table of Materials**). The absorption of near-infrared light (two wavelengths: 695 and 830 nm) is measured with a sampling rate of 10 Hz.

1.2.3. Prepare the lighted fiber optic probe, which can be used to move hair aside.

1.2.4. Set the experiment environment with one table with two chairs to keep participants' seats face-to-face.

2. Adopting data by instructing participants

2.1. Prepare the participants

2.1.1. Instruct the participants, including the details of NIRS measurement methods.

NOTE: All the participants were healthy and were financially compensated for participation. No participants withdrew from the experiment halfway through. The laser beam of the NIRS may be harmful to the participants' eyes, and they were instructed not to look directly into those laser beams.

2.1.2. Make the participants sit face-to-face (apart from a table (0.8 m)) to make sure they can see each other directly. Adjust the chair-to-table distance (i.e., nearly 0.3 m) to make the participants sit comfortably.

2.1.3. Turn on the laser button, and place the caps with the probe sets on the participants' heads.

NOTE: The 3 x 5 probe sets cover the forehead of the participants (middle probe of the bottom row is placed on Fpz); the 4 x 4 probe sets cover the left temporoparietal cortex (the third probe of the third row is placed on P5).

2.1.4. Put the four optical fiber bundles loosely on the holder's arms without contact with the participants or chairs.

NOTE: Here, the NIRS measurement system has four bundles of optical fibers. Additionally, ensure that the participants do not feel too heavy to pull off the caps.

2.1.5. Let the probe tips touch participants' scalp by carefully pushing each spring load probe further into its socket.

2.1.6. Perform signal calibration.

2.1.6.1. First, check the quality of the signal by clicking the **Auto Gain** in the probe set monitor window of the fNIRS machine. Then, a channel's poor signal and sufficient signal are marked in yellow and green in the probe set monitor window, respectively.

NOTE: For a channel with insufficient signals, lighted fiber optic probes are used to move the hair under the probe's tip to one side.

2.1.6.2. Then, push the probes further into their sockets to get sufficient signals. Repeat this process until all the channels are marked in green in the probe set monitor window of the NIRS measurement system, indicating that the quality of signals is accessible.

2.2. Run the experiment

2.2.1. Start the experiment with a 5 min rest state, which serves as the baseline. Then, two participants are required to co-learn the learning materials.

2.2.2. After the experiment, click on **Text File Out** to export the raw light intensity data and save the data as a text file.

NOTE: No filters are applied in the NIRS measurement system.

2.2.3. Use the three-dimensional (3D) digitizer (see **Table of Materials**) to determine the locations of emitters, receivers, and other references (i.e., inion, nasion, Cz, and left and right ears) for each participant.

2.2.3.1. Obtain the MNI coordinates for the recording channels using the commercially available numeric computing platform[21] (see **Table of Materials**). **Supplementary Table S1** shows the corresponding anatomical locations of each channel.

2.2.4. Clean probes and probe holders with ethanol. Wash caps with mild detergent and let the caps air dry.

3. Data analysis

3.1. Data preprocessing

NOTE: Previous research has adopted variable non-commercial software packages (e.g., Homer2[22], AnalyzIR[23], or nirs LAB[24]) with numeric computing platforms (see **Table of Materials**) on fNIRS data analysis are all available on the website. Here Homer2 was used to do the

preprocessing of the NIRS data. Additionally, both fNIRS recording data collected in the rest and collaborative learning phases share the same preprocessing and analysis pipeline.

3.1.1. Copy the dataset from the fNIRS machine. Convert the original data formation to the proper formation (i.e., convert cvs file to nirs file).

3.1.2. Convert raw data to optical density (OD) data with hmrIntensity2OD function provided in the Numeric computing platform (see **Table of Materials**).

3.1.3. Delete the bad channels. Then average the OD value for each participant on each channel and full sample points, respectively.

NOTE: Here, 46 averaged OD values are obtained.

3.1.3.1. Calculate the standard deviation (SD) for each participant.

3.2.3.2 Mark as unusable and remove the channels with very low or high OD (which exceeded 5 SDs) from the analysis for each participant.

NOTE: This step can be performed before and/or after fNIRS data preprocessing. In this data analyses pipeline, the bad channels are detected before the fNIRS data preprocessing.

3.1.4. Convert the OD time data into Oxy-Hb, DeOxy-Hb, and combined signal based on the modified Beer-Lambert Law[25].

NOTE: Reference[25] says, "All data analysis steps are conducted on Oxy-Hb data, which is an indicator of the change in regional cerebral blood flow having higher signal-to-noise ratio[26]. Additionally, previous research employed fNIRS hyperscanning in teaching and learning scenarios mainly focused on Oxy-Hb concentration[11–14]."

3.1.5. Calibrate Oxy-Hb time series from motion artifacts by the channel-by-channel wavelet-based method.

NOTE: Specifically, the Daubechies 5 (db5) wavelet with tuning parameter at 0.1 (see details in Homer2 manual)[27,28] is adopted in removing motion artifacts.

3.1.6. Apply the band-pass filter (i.e., 0.01–1 Hz) on the calibrated Oxy-Hb data to reduce the high-frequency noise and slow drift.

3.1.7. Conduct principal components analysis (PCA) on the OxyHb signal to remove non-neural global components (e.g., blood pressure, respiration, and blood flow variation)[29].

NOTE: The PCA analysis proposed by Zhang and colleagues[29] is adopted here.

3.1.7.1. First, decompose the signal.

NOTE: The specific formula of decomposition of fNIRS signal is: $\mathrm{H} = \mathrm{U}\Sigma V^T$. Here, temporal and spatial patterns of fNIRS data are presented in two matrices (i.e., U and V). U is a 2D (sample point x principal component) matrix. V is also a 2D (principal component x principal component) matrix. The column in V indicates one principal component (PC), and the strength of that PC for a particular channel is estimated in each entry of the column. The relative importance of each PC is represented by the value of the diagonal matrix Σ.

3.1.7.2. Second, conduct spatial smoothing.

NOTE: Gaussian kernel convolution is employed to remove localized signals and get the global component.

3.1.7.3. Third, reconstruct the signal.

NOTE: To calculate the global component of the fNIRS data, the smoothed spatial pattern matrix V^* is plugged back into the decomposition formula: $H_{Global} = \mathrm{U}\Sigma(V^*)^T$. Then, localized derived neuronal signal can be obtained using original data H to subtract H_{Global}: $H_{Neuronal} = H - H_{Global}$.

3.2. Inter-brain synchrony

NOTE: To reveal brain coupling in second-person neuroscience, wavelet transform coherence (WTC) is adopted here. Briefly, WTC measures the correlation between two-time series as a function of frequency and time. The specific formula of wavelet coherence of two-time series x and y is:

$$\mathrm{WTC(t,s)} = \frac{\left|\langle s^{-1} w^{ij}(t,s)\rangle\right|^2}{|\langle s^{-1} w^{i}(t,s)\rangle|^2 |\langle s^{-1} w^{j}(t,s)\rangle|^2}$$

T and s denote the time and wavelet scale separately, ⟨·⟩ indicates a smoothing operation in scale and time. W represents the continuous wavelet transform. Then, a 2D (time x frequency) WTC matrix is generated[30]. Several toolboxes are used to calculate the WTC value. Here the toolbox created by Grinsted and colleagues was used[30].

3.2.1. Adopt the WTC function of the numeric computing platform (see **Table of Materials**).

NOTE: Here, the default setting of the mother wavelet (i.e., Generalized Morse Wavelet with its parameters beta and gamma) is used. Mother wavelet converts each time series into the frequency and time domain.

3.2.2. Set the default setting on the other parameters (i.e., MonteCarloCount, representing the number of surrogate data sets in the significance calculation).

3.2.3. Calculate the WTC value for two corresponding channels (the same channel in two participants) in a numeric computing platform (see **Table of Materials**). Following the same procedure, 46 WTC matrices are generated from 46 channels.

3.2.4. Determine the frequency band of interest (FOI), which is sensitive to collaborative learning.

NOTE: Here, a cluster-based permutation approach is adopted to detect such FOI[31]., multi-channel and multi-frequency data offers a solution to multiple comparisons being a non-parametric statistical test.

3.2.4.1. Perform Time-average of the WTC values in the resting and collaborative learning phases, respectively, for each channel combination. Then, conduct paired sample *t*-tests along with the entire frequency (frequency range: 0.01–1Hz[32]) on these time-averaged WTC values (collaborative learning vs. rest). Next, identify frequency bins at which the task effect is significant (collaborative learning > rest, $p < 0.05$).

3.2.4.2. Obtain significant frequency neighboring points (≥2) as observed clusters and corresponding T values.

3.2.4.3. Conduct a series of paired sample *t*-tests on permuted data to generate the T values for each cluster qualified in step 3.2.4.2 for 1000 times.

NOTE: For forming permuted data, participants are randomly assigned to form new two-member pairs. As the length of datasets varied across dyads for each random pair, the longer dataset is trimmed to the same length as the shorter one[33].

3.2.4.4. Compare the averaged cluster-based T values from original pairs with the T values of 1000 permutations.

NOTE: The p values evaluated by this formula[34]:

$\mathrm{erfc}((\frac{|s_o-\mu_p|}{\sigma_p}/\sqrt{2})$, where S_0 denotes observed averaged-cluster t-value, μ p and σ p indicate the mean and standard deviation of permutation values.

3.2.5. Average WTC values in the identified FOI in each channel in each dyad. Then, apply fisher z transformation to the WTC values to get a normal distribution of WTC values. Use this value to index the IBS for further statistical analysis.

REPRESENTATIVE RESULTS:

Figure 1 illustrates the experimental protocol and probe location. The fNIRS data recording process consists of two parts: resting-state session (5 min) and collaborative session (15–20 min). The collaborative learning dyads are required to relax and to keep still in the resting-state session. After that, participants are told to co-learning the learning material (**Figure 1A**).

Their prefrontal and left temporoparietal regions are covered by the corresponding probe set (**Figure 1B**).

Figure 2 illustrates the fNIRS data analysis pipeline. The fNIRS data analysis is applied to all fNIRS data recorded from each participant and each channel. First, optode density in channel 33 for a certain dyad is visualized in **Figure 2A**. Optode density is recorded in 46 channels (CHs) of each collaborative learning dyad by the fNIRS measurement system. Second, With the operation clarified in steps 3.1.5 and 3.1.7, viable data are prepared for WTC analysis. Here, the red curve represents the data extracted by the wavelet-based motion artifacts removing method; the blue curve represents the data extracted by both the Wavelet-based motion artifacts removing method and PCA. Visualized difference between two curves suggests PCA is efficient in removing non-neural signals (**Figure 2B**). Third, with the operation stated in steps 3.2.4, the comparison between the observed T value and the distribution of random T value (i.e.,1000 times) shows significant results ($t(38) = 3.31$, FDR corrected $p < 0.05$, Cohen's $d = 1.05$) in identified FOI (0.015 Hz–0.021 Hz) (**Figure 2C**). Finally, the WTC matrix is visualized in **Figure 2D**. The color map varies from blue to yellow, representing the value of IBS raged from 0 to 1 (correlation coefficients as a function of time and frequency). Here, 1 denotes the largest coherence between two fNIRS signals, and 0 denotes no coherence is detected. A red rectangle in the plot marks significant coefficients. Additionally, results show a strong coherence around 1 Hz, representing the dyad's cardiac rhythm coherence.

Figure 3 presents the critical steps of the cluster-based permutation approach used to detect the collaborative learning relevant frequency band.

Taken together, following the data analysis pipeline, the frequency band (raged from 0.015 Hz to 0.021 Hz), which sensitive to collaborative learning, is identified by cluster-based permutation approach. Further, for each channel, the time-averaged IBS value is compared between the rest and the collaborative learning phases using a series of paired sample *t*-tests. For solving the multiple comparison problem, all the observed p-values in 46 channels are corrected by FDR methods[35,36]. The results show that the IBS at channel 33 reaches significance during collaborative learning (FDR corrected $p < 0.05$). No other corresponding channels indicated significant effects ($p > 0.05$).

FIGURE LEGENDS:

Figure 1: Experimental protocol and probe location. (**A**) Experimental procedure. Brain activity from dyads is acquired simultaneously using fNIRS. The resting-state session lasts for 5 min, in which dyads are required to relax and keep still. After that, participants are told to co-learn the learning material (15–20 min). (**B**) Optodes probe set. Two probe sets cover the prefrontal and left temporoparietal regions.

Figure 2: Overview of the fNIRS data analysis. (**A**) Optode density in channel 33 for one exemplary dyad. Optode density is recorded in 46 channels (CHs) of each collaborative learning dyad. i, j, Optode density of two participants of a collaborative learning dyad; t, time. (**B**) Data preprocess procedure. Wavelet-based motion artifacts removing method and PCA

are applied on Oxy-Hb data in sequence. Here, the red curve represents the data extracted by the wavelet-based motion artifacts removing method; the blue curve represents the data extracted by both the Wavelet-based motion artifacts removing method and PCA. $k_{\text{wavelet-based method}}$, data extracted by the Wavelet-based motion artifacts eliminating process. $k_{\text{wavelet-based method + PCA}}$, data extracted by both Wavelet-based motion artifacts removing method and PCA. (**C**) Cluster-based permutation approach. Compare the observed T value with the distribution of random T values in identified FOI (0.015 Hz–0.021 Hz). (**D**) WTC plot in channel 33 for one exemplary dyad. The color map varies from blue to yellow, representing the value of IBS ranged from 0 to 1 (correlations coefficients as a function of time and frequency). Here, 1 denotes the largest coherence between two fNIRS signals, and 0 indicates that no coherence is detected. A red rectangle in the plot marks significant coefficients. WTC estimates IBS on two clean Oxy-Hb time series.

Figure 3: Flowchart of identifying the collaborative learning-related FOI.

DISCUSSION:

First, in the present protocol, the specific steps of conducting fNIRS hyperscanning experiments in a collaborative learning scenario are stated. Second, the data analysis pipeline that assesses the IBS of hemodynamic signals in collaborative learning dyads is also presented. The detailed operation on conducting fNIRS hyperscanning experiments would promote the development of open-science. Furthermore, the analysis pipeline is provided here to increase the reproducibility of hyperscanning research. In the following, the critical issues of experiment design, conducting an experiment, data analysis in (fNIRS) hyperscanning experiments are all highlighted. Additionally, possible solutions to present limitations are also discussed.

Experimental design

The experimental design for the fNIRS hyperscanning study is flexible. Here, the fNIRS hyperscanning technique is applied in the collaborative learning scenario. Two participants were asked to learn specific rules of the figure matrix together, and their brain activities were recorded by fNIRS simultaneously. This approach allows researchers to explore real-time concurrent neural dynamics (i.e., IBS) in collaborative learning dyads. According to previous research, IBS has been detected in teaching and learning scenarios and tracks the effective teaching mode[11]. Neural alignment detected in collaborative learning dyads may serve as a potential neural mechanism underpinning successful learning and provides implications for designing effective collaborative learning patterns. Meanwhile, critical issues on experimental design need to be addressed: the experiment time is limited to 30 min in this experiment. Two reasons account for this setting: First, wearing caps with fNIRS optodes on the head is not comfortable, participants cannot stand for a long time. Second, it's hard to ask participants to keep still during co-learning for a long time. The limited experiment time would allow good-quality signals to be obtained.

Conducting the experiment

The most challenging part of doing fNIRS hyperscanning in a collaborative learning scenario is

getting high-quality brain signals. Based on the present protocol, three critical steps are highlighted: making appropriate caps, placing optodes, and conducting spatial registration of corresponding channels. First, since head circumference varies across participants, making caps that fit different individuals is essential. Second, when placing an appropriate cap on the participants' heads, ensure the tips of optodes can directly contact scalp skin. To achieve this goal, practicing this operation before the experiment is needed. Third, conducting spatial registration with a 3D digitizer can identify the corresponding anatomical locations of NIRS channels (CHs) on the cerebral cortex[37–39]. This protocol suggests completing spatial registration for all participants to get averaged and robust results. Along this line, previous research asked participants to conduct a pre-test to ensure accurate hemodynamic signals can be obtained. Specifically, participants performed a classical finger-thumb tapping task with their right hand, during which fNIRS recorded hemodynamic dynamics. Participants who detected a significant fNIRS signal ($p < 0.05$) in the left motor cortex are qualified to participate in the study. This technique ensures recorded signals are usable on all participants[40].

Data analysis

The data analysis process in this protocol consists of two parts: preprocess and WTC analysis. Three critical data analysis steps should be highlighted here: First, conducting the principal component spatial filter algorithm (PCA) on the neural data. Zhang and couleage[29] proposed this approach for the separation of the global and local effects. Although fNIRS allows relatively free movement and communication, PCA is necessary to extract accurate signals from systemic changes (e.g., breathing rate, blood pressure, heart rate, breathing rate, and autonomic nervous system activity). The protocol here suggests PCA is efficient in removing the global effects. This method is widely used in fNIRS hyperscanning studies[13]. Altogether, non-neural components can be removed successfully using spatial filtering. Second, WTC is adopted to identify the IBS of collaborative learning dyads. WTC is an approach of assessing the correlation coefficients between two-time series as a function of time and frequency[41]. This method can reveal locally phase-locked behavior that might not be detected with a traditional approach such as Fourier analysis[30]. And this method is widely used to estimate IBS in fNIRS hyperscanning with varied paradigms, such as cooperative and competitive behaviors[4,42], studying action monitoring[43], imitation[44], verbal communication[8], non-verbal communication[19], teaching and learning activity[11–14] and mother-child social interaction[45].

Meanwhile, other techniques, such as Granger Causality Analyze (GCA), correlation analysis, and phase synchrony analysis, are used in hyperscanning research. GCA is a method for revealing directed (causal) information between two time-series data[46]. This method has once been used to test the direction of information flow between instructor and learner[12]. Correlation analysis is also adopted in the fNIRS-based hyperscanning field to estimate IBS in dyads who conduct cooperative or competitive tasks[47,48]. Compared to WTC analysis, this method only characterizes the covaried features of two fNIRS time series along time stream and missed potential information in frequency.

Additionally, other approaches that quantified phase synchrony with Phase locking value (PLV) were used in EEG hyperscanning studies. PLV estimates the consistency of the phase

difference between two signals[49]. However, Burgess suggested PLV shows bias on detecting hyperconnectivity that doesn't exist, especially when small samples are employed[50]. Third, adopting a non-parametric statistical test to detect the collaborative learning-related frequency is essential. At first, task-related FOI is selected by either following suggestions in previous research or according to specific experiment design (i.e., how long for one task trial in an experiment). Recently, to obtain robust and reproductive results in the FOI selecting process, non-parametric statistical test approaches are adopted. Here, this technique operated efficiently. The collaborative learning-related FOI (0.015–0.021 Hz) is identified, and similar frequency bands have been identified in fNIRS hyperscanning research in teaching scenario[13] and in verbal communication paradigms[8]. It is necessary to apply this technique in the multi-brain data analysis pipeline. All in all, establishing suitable algorithms and methods for the analysis of hyperscanning data will be a prominent field.

Limitation and future direction

Several limitations can be improved in the future to obtain reproductive and robust IBS within a realistic social interaction context from a multi-brain. First, the weight of the fiber is too heavy and uncomfortable to wear for a long time; thus, the time of the experiment is limited to 30 min. In the future, if recording the multi-brain activity in the classroom, it is hard to ask students to wear fNIRS caps during one school period (i.e., 50 min). Thus, wearable fNIRS settings are required in actual lecturing and learning scenario. Second, although the fNIRS shows higher tolerance to head motion than fMRI, this technique can only detect the brain activity of the surface cortex[15]. Thus, fNIRS hyperscanning cannot be used in the reward-related neural mechanism exploring paradigm, in which the amygdala plays a crucial role[51]. Meanwhile, the limited number of sources and detectors in the fNIRS setup suggests not the whole brain cortex would be measured. That means researchers have to select the region of interest (ROI) to measure. Third, PCA is adopted to eliminate the system contaminants. While this technique is efficient, in the future, adding short-channels that account for extra-cerebellar blood flow, which may contaminate fNIRS signals, is also an efficient approach[29,39]. Fourth, the data analysis procedure in this protocol can be applied in other naturalistic fNIRS hyperscanning studies. The next step is to develop fNIRS-specific data analysis packages with the standard guideline. Fifth, in this protocol, WTC is employed to identify the concurrent brain activity (i.e., IBS). With the development of a technique for calculating covaried neural activity, other methods such as graph theory and GCA also can be used. Sixth, it is necessary to recruit control conditions, such as talking conditions that require dyads to talk on specific topics to exclude confounding effects. Meanwhile, to reveal which learning activity in collaborative learning (i.e., knowledge co-construction[52]) would lead to the IBS. And whether these detected IBS can be used to track the learning performance of collaborative learning dyads are also important. Finally, it is also urgent to provide a framework to explain the mechanism of IBS. Researchers try to discern whether this is only the epiphenomenon or a neural mechanism of social interaction by Hamilton[53]. To achieve this goal, on the one hand, Hamilton proposed a xGLM approach that models brain activity, behavior data, and physiological data together to explore the reliable explanation of brain coupling[53]. On the other hand, Novembre and Lannetti suggested conducting multi-brain stimulation (MBS) to reveal the mechanism of concurrent brain activity[54].

Conclusion

fNIRS hyperscanning lead to a paradigm shift from traditional experiment design to realistic social interaction scenarios in social neuroscience. The IBS extracted by this method provides a new view to explain the neurobiological mechanism of social interactions. Finally, the established standardized pipeline of collecting and analyzing data would be the milestone for generating valid results and advancing the recent hyperscanning experiment.

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The authors have nothing to disclose.

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Figure 1

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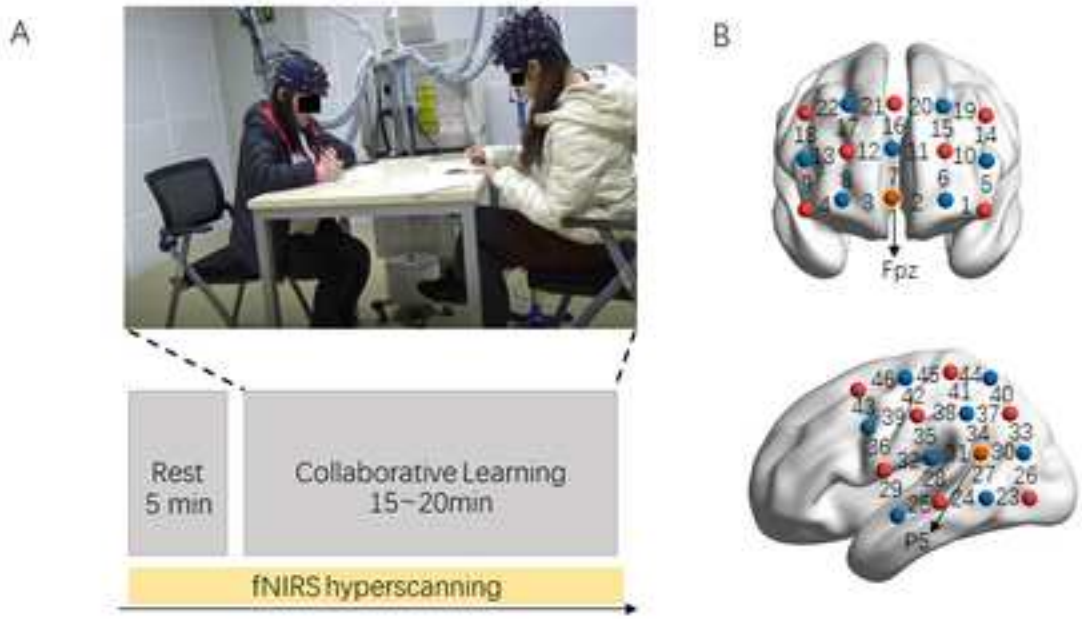


Figure 2

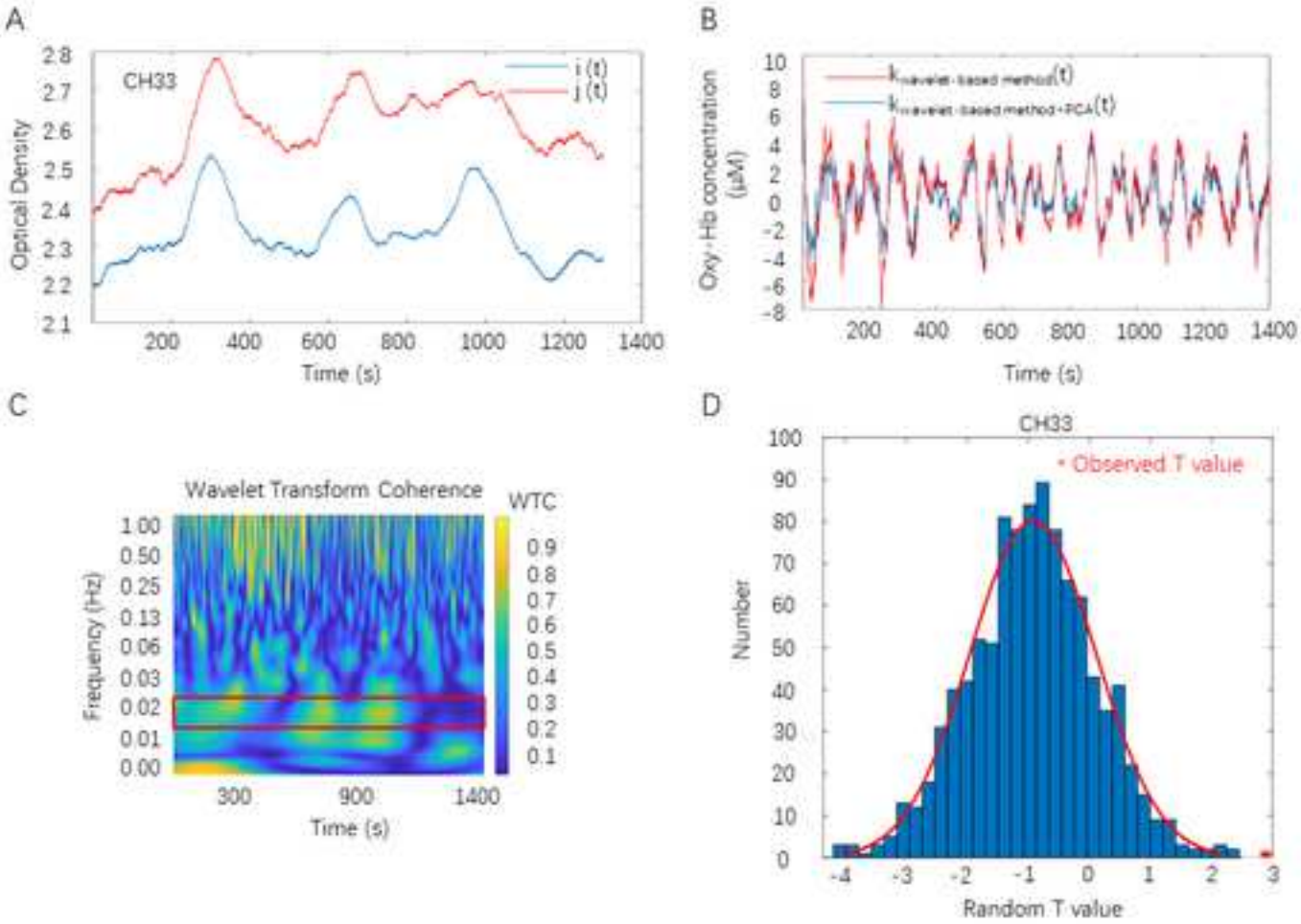
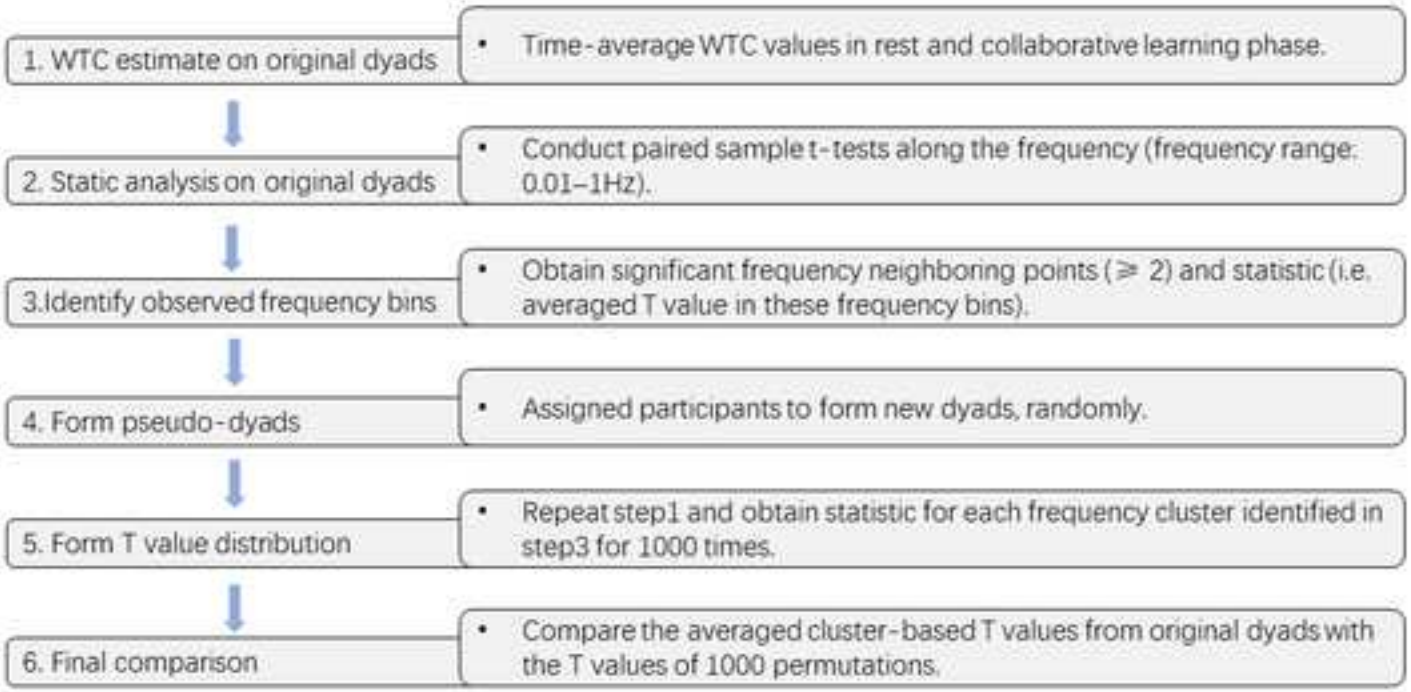


Figure 3



Response to Editor's and Reviewers' Comments

We thank the Editor for handling the review process for our submission and allowing us to resubmit a revised copy of the manuscript. We also would like to thank for receiving insightful comments and constructive suggestions concerning this manuscript from the editor and reviewers. We have fully addressed all the review concerns and significantly improved the quality of the manuscript. All revised sentences are printed in BLUE color in the revised version of the manuscript to facilitate the re-review. Below we highlight the point-by-point response (in BLACK) to the all comments.

Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you for your suggestion. We have proofread the manuscript to correct grammar/language errors carefully throughout the text. Revised sentences are printed in BLUE color in text.

2. Please revise the following lines to avoid previously published work: 77-78, 82-84, 101-108, 124-125, 128-132, 137-138, 141-142, 147-148, 150-152, 191-193,197-199, 205-207, 219-218, 235-237, 262-267, 324-325, 338-339, 353-356.

Response: We have revised these lines carefully to avoid previously published work. They all printed in BLUE color in text. For example, (p. 4, line 85–87, p. 5, line 90–91)

3. Please ensure that abbreviations are defined at first usage.

Response: In revised manuscript any abbreviations exist at first usage have been carefully defined to avoid confusing. For example, (p. 2, line 27–28)

> "Further, a pipeline of analyzing Inter-Brain Synchrony (IBS) of oxygenated hemoglobin (Oxy-Hb) signals is presented."

4.Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have thoroughly revised the sentences which use personal pronouns. For example, (p. 2, line 25–28)

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: We are sorry about not including the ethics statement in previous manuscript. We have included the ethics statement in this revised manuscript on (p. 5, line 93–96). The manuscript now reads:

> "All recruited participants were healthy. Prior to the experiment, participants gave informed consent. Participants were financially compensated for their participation. The study was approved by the University Committee of Human Research Protection (HR-0053-2021), East China Normal University."

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: Thanks for your suggestion. We have added more details to clarify "how" question.

Step 1.1.1: Please provide details regarding the swimming cap used

"1.1.1. Adopt elastic swimming cap to place optode holder grid. Considering the head sizes of participants are different, two sizes of caps are used. Small caps are prepared for participants with head circumference of 55.4 ± 1.1 cm, and large caps are prepared for participants with head circumference of 57.9 ± 1.2 cm."

Step 1.2.2: Please specify the laser diodes used

"The absorption of near-infrared light (two wavelengths: 695 and 830 nm) is measured with a sampling rate of 10 Hz."

Step 2: Please include any participant inclusion/exclusion criteria.

"All participants were healthy and were financially compensated for participation. No participants withdrew from the experiment halfway through."

Step 2.1.2: Please specify the distance between the chairs

"Seat the participants face-to-face (apart by a table (0.8 m)) to make sure they can see each other directly. Adjust the chair-to-table distance (i.e. nearly 0.3 m) to make participants sit comfortably."

Step 2.1.6: Please provide details on the procedure of signal calibration. What are the measurement criteria for the sufficient and insufficient signal?

"2.1.6. Perform signal calibration. First, check the quality of signal by clicking on the Auto Gain button. Then, insufficient signal and sufficient signal of a channel is marked in yellow and green in the probe set monitor window, respectively. For channel with insufficient signals, lighted fiber optic probes are used to move the hair under the tip of probe to one side. Additionally, push the probes further into their sockets is also allowed to get sufficient signals. Repeat this process until all the channels are marked in green in the probe set monitor window of the NIRS measurement system, indicating the quality of signals is accessible."

Step 2.2.4: Please specify the cleaning and the washing procedure

"2.2.4. Clean probes and probe holders with ethanol. Wash caps with mild detergent and let the caps air dry."

Step 3.1.7: Please elaborate on how the PCA analysis was performed

"The PCA analysis which proposed by Zhang et al., is adopted here. First, decompose the signal. The specific formula of decomposition of fNIRS signal is: $H = U\Sigma V^T$ Here, temporal and spatial patterns of fNIRS data are presented in two matrices (i.e. U and V). U is an 2D (sample point × principal component) matrix. V is also an 2D (principal component × principal component) matrix. The column

in V indicates one principal component (PC), and the strength of that PC for certain channel is estimated in each entry of the column. The relative importance of each PC is represented by the value of the diagonal matrix Σ. Second, conduct spatial smoothing. Gaussian kernel convolution is employed to remove localized signals and to get the global component, Third, reconstruct the signal. To calculate the global component of the fNIRS data, the smoothed spatial pattern matrix V^* is plugged back into the decomposition formula: $H_{Global} = \mathrm{U}\Sigma(V^*)^T$. Then, localized derived neuronal signal can be obtained by using original data H to subtract H_{Global} : $H_{Neuronal} = H - H_{Global}$."

7. Line 221: Please remove the reference description

Response: Reference description has been removed in the revised manuscript. The text now reads:

"Compare the averaged cluster-based T values from original pairs with the T values of 1000 permutations. The p values evaluated by this formula[34] "

8. In the software, please ensure that all button clicks and user inputs are provided throughout.

Response: We have double checked the revised manuscript to ensure that all button clicks and user inputs are provided on (p. 8, line 159; p. 8, line 172; p. 9, line 185).

9. Please include a one-line space between individual protocol steps and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: First, revised manuscript has included a one-line space between individual protocol steps. Second, the essential steps of the protocol for the video have been highlighted with GREY background in the revised manuscript. For example, (p. 5, line 100).

10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response: The critical steps of the protocol have been highlighted in the revised manuscript with a logical flow. Specifically, the complete sentences have been highlighted, and highlighted action is written in imperative tense. For example, (p. 9, line 182).

11. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently

referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material.

Response: First, all commercial language has been removed from revised manuscript. For example, (p. 9, line 192). Second, all commercial products have been referenced in the Table of Materials, which have been sorted by the name of the material.

12. Please remove the titles and Figure Legends from the uploaded figures. Please include all the Figure / Table Legends together at the end of the Representative Results in the manuscript text.

Response: First, the new version of uploaded figures has been removed the titles and Figure legends. Second, the Figure / Table Legends have been presented at the end of the Representative Results in the manuscript text.

13. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results.

Response: First, for each figure legend, the title and the short description of the data have been presented in the Figure and relevant symbols. The discussion of the Figure has been placed in the Representative Results in revised text on (p. 15–16, line 318–337)

> "Figure 2 illustrates the fNIRS data analysis pipeline. The fNIRS data analysis is applied on all fNIRS data recorded from each participant and each channel. First, optode density in channel 33 for certain dyad is visualized in Figure 2A. Optode density is recorded in 46 channels (CHs) of each collaborative learning dyad by fNIRS measure system. Second, With the operation clarified in steps 3.1.5 and 3.1.7, viable data are prepared for WTC analysis. Here, the red curve represents the data extracted by the wavelet-based motion artifacts removing method; the blue curve represents the data extracted by both Wavelet-based motion artifacts removing method and PCA. Visualized difference between two curves suggest PCA is efficient in removing non-neural signals (see Figure 2B). Third, WTC matrix is visualized in the Figure 2D. The color map varying from blue to yellow, which represents the value of IBS raged from 0 to 1 (correlations coefficients as a function of time and frequency). Here, 1 denotes the largest coherence between two fNIRS signals, and 0 denotes no coherence is detected. Finally, with the operation stated in steps 3.2.4., the comparison between the observed T value and the distribution of random T value (i.e.1000 times) show significant results ($t(38) = 3.31$, FDR corrected $p < 0.05$, Cohen's $d = 1.05$) in identified FOI (0.015 Hz-0.021 Hz) (see Figure 2D). Significant coefficients are marked by red rectangle in the plot. Additionally, results show a strong coherence around 1 Hz, which represent cardiac rhythm coherence of dyad. "

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: Thank you for your suggestion. We have revised the Discussion carefully to cover the critical points you mentioned above, the revised Discussion as follows: (p. 19, line 374–380; p. 20, line 386–400; p. 21–23, line 422–465; p. 23–24, line 469–495)

Figure 2: Please mark the different panels of the Figure as A, B, C, etc and provide the description accordingly. Please also provide an X/Y axis description and use SI abbreviations for time: s instead of seconds.
Response: First, we have marked the different panels of the Figure1 as A, B and Figure 2 as A, B, C, D. Second, the corresponding description has also been added in the revised manuscript. Third, X/Y axis description has also been added in the revised manuscript. Finally, in the revised manuscript, SI abbreviations for time (i.e. s instead of seconds) has been used. (p. 17; p. 18)

15. Please spell out journal titles in all the references.
Response: Thanks for your suggestion. We have gone through all the references to ensure all the journal titles have been spelled out. (p. 26–p. 33)

Reviewer #1:
This article described a co-learning task protocol design and associated data analysis pipeline for fNIRS-based hyperscanning study. Hyperscanning is an important topic in fNIRS field and it could be the future direction for this imaging technique. However, this article had quite a few issues that need to be addressed before it can be considered for publication. Also, the English in the article needs to be proofread. Please see my detailed comments below.
Response: We thank the Reviewer for his/her comments on this protocol. The revised text has been proofread. In what follows, we fully address all the comments and suggestions.

1. Line 26, please consider replacing the IBS as inter-brain synchrony.
Response: We have replaced the IBS as inter-brain synchrony. We also ensure that abbreviations are defined at first usage. The text now reads:

> "Further, a pipeline of analyzing Inter-Brain Synchrony (IBS) of oxygenated hemoglobin (Oxy-Hb) signals is presented."

2. Line 74, the phrasing of "IBS of each participant's prefrontal and left temporoparietal regions" is not proper. Since IBS stands for inter-brain synchrony, so it should be an index representing the two brains' association.

Response: We have revised these lines to:

> "First, hemodynamic response is recorded simultaneously in each dyads' prefrontal and left temporoparietal regions during a collaborative learning task."

3. Line 142, Please specify if there is a quantitative threshold for the statement "Repeat this process until the quality of the signal is accessible."

Response: In this protocol, when all the channels are marked in green in the probe set monitor window of the NIRS measurement system, it represents the signal is sufficient. The specific details can be found in revised text. (p. 8, line 172–179).

4. When validating the localization with the 3D digitization, have the authors considered different head sizes?

Response: Yes, considering the head sizes of participants are different, two sizes of caps are used. The statement can be found in revised text:

> "Considering the head sizes of participants are different, two sizes of caps are used. Small caps are prepared for participants with head circumference of 55.4 ± 1.1 cm, and large caps are prepared for participants with head circumference of 57.9 ± 1.2 cm." (p. 5, line100-103)

5. Please list references that support 5 min is long enough for acquiring sufficient data for functional connectivity analysis.

Response: Functional connectivity analysis was not conducted on 5 min rest phase. Here, rest phase is served as a baseline which prepared to be compare with collaborative learning phase. The whole fNIRS recording process last for 20-25 min. This process includes 5 min rest-state, which serves as baseline, and also includes collaborative learning phase, which lasts for 15-20 min. We did not conduct wavelet transform coherence (WTC) analysis on two part of data separately. For each dyad, the whole data (include rest phase and collaborative learning phase) entered into the WTC analysis. Specifically, we conduct WTC analysis on two fNIRS time serious in each channel of specific collaborative learning dyads, and then for each channel, these data generated a 2-D matrix of the WTC value. In the matrix, each column represents a specific time point; each line represents a specific frequency point (ranged from ~0.01 Hz in the low-frequency part to 1 Hz in the high-frequency part); Next, the WTC values were time-averaged across the entire resting-state session and collaborative learning session. More details can be checked in revised text (p. 4, line 74–76; p. 13, line 276–278; p. 13, line 285–288)

6. Line 145, how long was the co-learning phase in the protocol.

Response: The whole time contain 5 min rest-state, which serves as baseline, and then each dyad conduct collaborative learning for 15-20 min, so the whole fNIRS recording time lasts 20-25 min.

Specific description can be checked in revised text on (p. 15, line 310–316)

7. Line 166, please consider spelling out SD as standard deviation. In addition, please

provide details on how the SD was calculated for each channel/participant/group?
Response: We have spelled out SD as standard deviation. We also ensure that abbreviations are defined at first usage. The details are included in revised manuscript on (p. 10, line 214–218)

> "Delete the bad channels. For each participant, average OD value on each channel along full sample points, respectively, and 46 averaged OD values are obtained. Then calculate standard deviation (SD) for each participant. Finally, for each participant, channels with very low or high OD, which exceeded 5 SDs, are marked as unusable and removed from the analysis.".

8. Were the RS data sharing the same preprocessing and analysis pipeline with the co-learning phase data? If so, was the co-learning phase data also filtered using the "band-pass filter with cut-off frequencies of 0.01-1 Hz"?
Response: Both RS data and co-learning phase data shared the same preprocessing and analysis pipeline. The band-pass filter was applied on collaborative learning phase and rest phase as well. The details are included in revised manuscript on (p. 10, line 204–206)

> "Additionally, both fNIRS recording data collected in rest phase and collaborative learning phase share the same preprocessing and analysis operation."

9. Please state the reason why the deoxy-Hb data was excluded from the analysis.
Response: First, previous research suggested that oxy-Hb is a sensitive indicator of the change in regional cerebral blood flow and has a high signal-to-noise ratio (Hoshi, 2007). Second, previous researches which employed fNIRS hypersacnning in teaching and learning scenario mainly focus on oxy-Hb concentration (Zheng et al., 2018,2020; Pan et al., 2019,2020). As such, we followed previous research to conduct analysis on oxy-Hb data. We have added the statement in revised text on (p. 11, line 224–229).

> "In this protocol, all data analysis steps are conducted on Oxy-Hb data, which is considered as an indicator of the change in regional cerebral blood flow with higher signal-to-noise ratio[28]. Additionally, previous research which employed fNIRS hypersacnning in teaching and learning scenario mainly focus on Oxy-Hb concentration[11-14]."

10. The current study used two artifact correction techniques, the Wavelet and PCA. It would be very helpful if the authors could show the effects of the two correction techniques respectively by maybe presenting example curves from a participant/channel?
Response: The example plot has been showed in revised Figure 2B. We also added the statement in revised text on (p. 15, line 322–327)

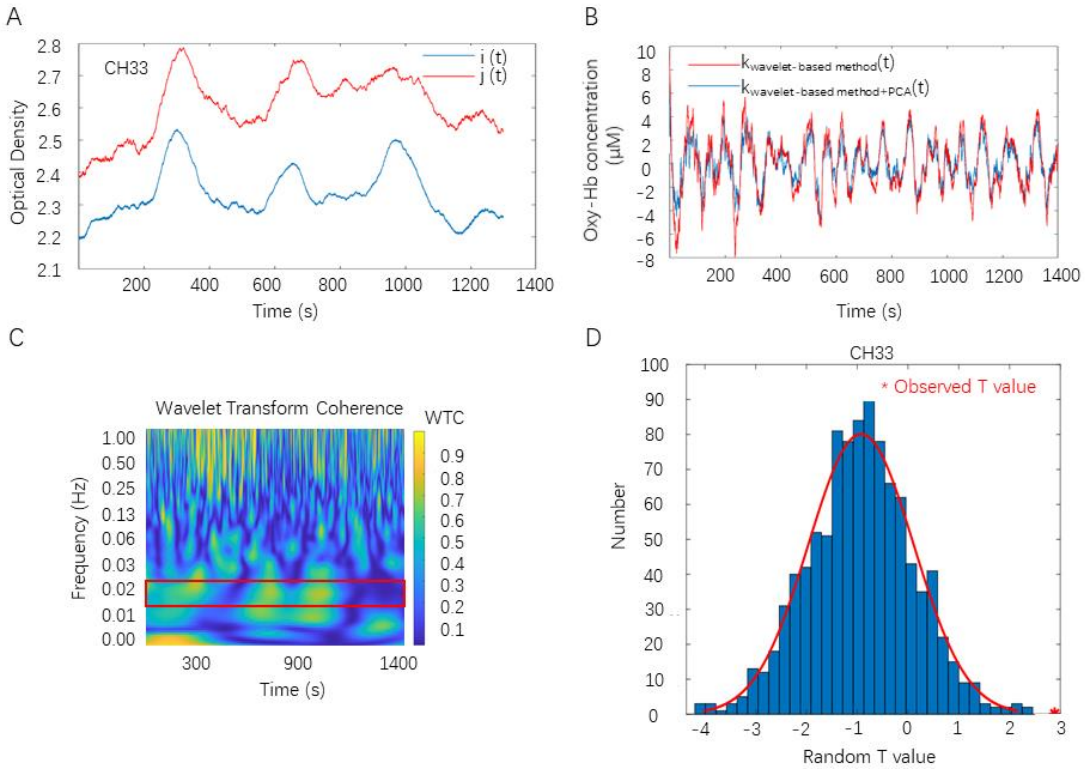


Figure 2. Overview of the fNIRS data analysis. (A) Optode density in channel 33 for one exemplary dyad. Optode density is recorded in 46 channels (CHs) of each collaborative learning dyad. i, j, Optode density of two participants of a collaborative learning dyad; t, time. (B) Data preprocess procedure. Wavelet-based motion artifacts removing method and PCA are applied on Oxy-Hb data in sequence. $k_{\text{wavelet-based method}}$, data extracted by the Wavelet-based motion artifacts removing method. $k_{\text{wavelet-based method+PCA}}$, data extracted by both Wavelet-based motion artifacts removing method and PCA. (C) Cluster-based permutation approach. Compare the observed T value with the distribution of random T values in identified FOI (0.015 Hz-0.021 Hz). (D) WTC plot in channel 33 for one exemplary dyad. IBS is estimated by WTC on two clean Oxy-Hb time series.

11. Line 193, the authors stated that "Several toolboxes in Matlab established computation of the WTC", however, only the AST toolbox was mentioned later. Please make sure the statements are accurate.

Response: In this protocol, the toolbox which created by Grinsted was adopted. We have rephrased the statement in revised manuscript:

> "Several toolboxes are used to calculate the WTC value. Here we use the toolbox crated by Grinsted and colleagues.[30]"

12. Line 192, were the WTC matrices calculated from all channel pairs within the two participants?

Response: Yes, the WTC matrices calculated from all channel pairs in each dyad. For two corresponding channels (the same channel in one specific collaborative dyad), one 2-D matrix of the coherence value were generated. In the matrix, each column

represents a specific time point; each line represents a specific frequency point (ranged from ~0.01 Hz in the low-frequency part to 1 Hz in the high-frequency part). (p. 13, line 276–278)

> "3.2.3. Calculate the WTC value for two corresponding channels (the same channel in two participants) in numeric computing platform (see Table of Materials). Following the same procedure, 46 WTC matrix are generated from 46 channels."

13. Section 3.2.4.1, the paired sample t-tests described here are a bit confusing. Please consider rephrasing the paragraph. Specifically, what were the task effects tested here? Also, if I am understanding correctly, a difference in magnitude between the resting state and the co-learning phase is not necessarily a task effect. The authors will need at least a control task to validate this (e.g. two participants doing some other tasks that will not generate a "task effect").

Response: Following previous research, interpersonal interactions as opposed to resting state elicited significantly larger IBS (Cui et al., 2012; Jiang et al., 2012). The significant difference in WTC value between the rest phase and the collaborative learning phase indicate IBS exist during collaborative learning. This paragraph has been carefully revised on (p. 13–14, line 285–288).

> "Time-average WTC values in resting phase and collaborative learning phase respectively for each channel combination. Then, conduct paired sample t-tests along the full frequency (frequency range: 0.01–1Hz[32]) on these time-averaged WTC values (collaborative learning vs. rest)."

We agree with the reviewer that recruiting a control condition, such as talking condition in which require dyads to talk on certain topic, would be an ideal approach to validate the task effect. We have discussed this concern as a limitation in Discussion on (p. 24, line 489–494).

> "Sixth, it is necessary to recruit control condition, such as talking condition which requires dyads to talk on certain topic to exclude confound effects. Meanwhile, to reveal which learning activity in collaborative learning (i.e. knowledge co-construction[52]) would lead to the IBS. And whether these detected IBS can be used to track the learning performance of collaborative learning dyads are also important."

14. Moreover, it is interesting to know what frequency is relevant to the co-learning activity.

Response: In this protocol, the frequency band relevant to the collaborative learning activity is ranged from 0.015 Hz to 0.021 Hz. Similar frequency bands (0.017-0.024 Hz) was identified in previous fNIRS hyperscanning research which compared the elaborative feedback and simple feedback in teaching scenario (Zhu et al., 2021). Additionally, the identified frequency band is also overlapping with other frequency band identified by previous studies with communication paradigms (e.g., Jiang et al., 2012; 2015) and teaching paradigms (e.g. Zheng et al., 2018). We have state this results in revised text on (p. 16, line 331–335; p. 16, line 342–344; p. 23, line 459–463).

"Finally, with the operation stated in steps 3.2.4., the comparison between the observed T value and the distribution of random T value (i.e.1000 times) show significant results (t (38) = 3.31, FDR corrected $p < 0.05$, Cohen's d = 1.05) in identified FOI (0.015 Hz-0.021 Hz) (see Figure 2C)."

"Taken together, Following the data analysis pipeline, the frequency band (raged from 0.015 Hz to 0.021 Hz) which sensitive to collaborative learning is identified by cluster-based permutation approach."

"Here, this technique operated efficiently. The collaborative learning related FOI (0.015-0.021 Hz) is identified. and Similar frequency bands has been identified in fNIRS hyperscanning research in teaching scenario[13] and in verbal communication paradigms[8]."

15. It is suggested to provide a flow chart for step 3.2.4 to better representing the statistical process.

Response: Thank you for your suggestions, a flow chart (Figure 3) for step 3.2.4 is added as requested.

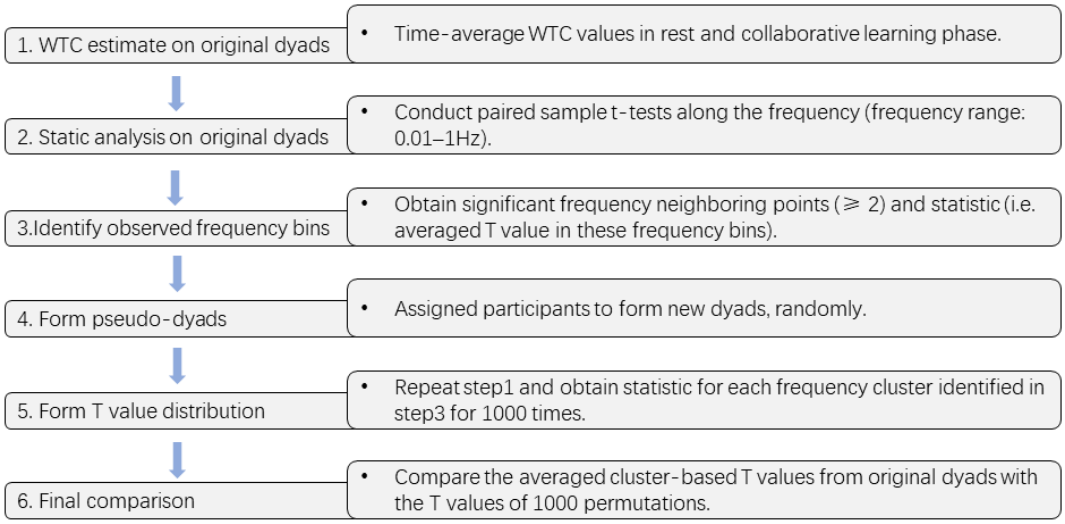


Figure 3. Flowchart of identifying the collaborative learning related FOI.

16. Line 240, the authors stated that they analyzed the data collected from CH 33. How about the data from other channels? Were they analyzed in the same way as to channel 33?

Response: Sorry for inaccurate statement. The data collected from all the channels were entered into the same data analysis pipeline. We have revised those unclear statement on (p. 13, line 276–278).

"3.2.3. Calculate the WTC value for two corresponding channels (the same channel in two participants) in numeric computing platform (see Table of Materials). Following the same procedure, 46 WTC matrix are generated from 46 channels."

17. In the result section, the authors need to show the relevant tests described in 3.2.4.

Response: We have carefully revised the result section on (p. 16, line 331–335).

> "Finally, with the operation stated in steps 3.2.4., the comparison between the observed T value and the distribution of random T value (i.e.1000 times) shows significant results ($t(38) = 3.31$, FDR corrected $p < 0.05$, Cohen's $d = 1.05$) in identified FOI (0.015 Hz-0.021 Hz) (see Figure 2D)."

18. The sentence in line 261 needs to be rewritten.

Response: We have rewritten the sentence in revised manuscript on (p. 19, line 374–377).

> "First, the specific steps of conducting fNIRS hyperscanning experiments in collaborative learning scenario is stated. Second, the data analyze pipeline which assesses the IBS of hemodynamic signals in collaborative learning dyads is also presented."

19. In the reviewer's opinion, the biggest limitation of fNIRS technique is the detection depth. The authors should discuss how this would limit the hyperscanning experimental design.

Response: We have added this limitation in discussion on (p. 23–24, line 473–480).

> "Second, although the fNIRS shows higher tolerance to head motion compared to fMRI, this technique can only detect the brain activity of surface cortex[15]. Thus, fNIRS hyperscanning cannot be used in reward-related neural mechanism exploring paradigm, in which amygdala play a crucial role but cannot be detected by fNIRS[51]. Meanwhile, the limited number of sources and detectors in the fNIRS setup suggests not whole brain cortex would be measured. That means, researchers have to select region of interest (ROI) to measure."

20. The authors should consider using the 3D digitizing, or photogrammetry registration methods to register fNIRS optodes at an individual level. Then consider integrating the individualized registration errors into the data analysis.

Response: 3D digitizing was used in this protocol to register fNIRS optodes at the individual level. Detailed description now reads on (p. 9, line 189–194).

> "2.2.3. A three-dimensional (3-D) digitizer (see Table of Materials) is used to determine the locations of emitters, receivers, and other references (i.e. inion, nasion, Cz and left and right ears) for each participant. For the recording channels, the MNI coordinates are obtained using the NIRS-SPM[21] with numeric computing platform (see Table of Materials), and corresponding anatomical locations of each channel are shown in Supplementary Table 1."

21. Though the authors mentioned that the GCA analysis was applied to the data in the current study, I didn't find the relevant descriptions in other sections, except at the end of the discussion section.

Response: Sorry for inaccurate statement. GCA analysis was not applied to the data.

At the end of the discussion section, this protocol compared this analysis with WTC, and suggested GCA can be a prominent method to reveal the direction information of brain-coupling. Especially in the experiment paradigm which assigned specific identity to each participant in a social interaction dyad (i.e. sender and receiver or teacher-student dyads). Statement can be found in revised manuscript on (p. 22, line 441–445).

> "Meanwhile, other techniques, such as Granger Causality Analyze (GCA), correlation analysis and phase synchrony analysis are used in hyperscanning research. GCA is a method for revealing directed ("causal") information between two time-series data[46]. This method once has been used to test the direction of information flow between instructor and learner[12]."

22. Please double-check the reference index in the text and the reference list at the end, some of them did not match.
Response: All references index has been double-checked in revised text on (p. 26–p. 33).

Reviewer #2:
It is of significance to understand the neural mechanism of social functions using hyperscanning. So, the current paper is in general interesting. However, the contribution was not clear at least to me. Some suggestions should be considered as follows.
Response: We thank the Reviewer for his/her comments on strengthen the advantages of technique we adopted in this protocol. In what follows, we fully address all suggestions.

1.The advantages of the index, inter-brain synchrony (IBS), should be discussed more clearly. Especially, it should be compared with correlation and phase synchrony (with wavelet transform analysis for instance).
Response: Thank you for your suggestion. We have discussed of IBS in comparison with correlation and phase synchrony on (p. 22–23, line 445–454):

> "correlation analysis is also adopted in fNIRS based hyperscanning field to estimate IBS in dyads who conduct cooperative or competitive tasks[47,48]. Compared to WTC analysis, this method only characterizes the covaried features of two fNIRS time series along time stream and missed potential information in frequency. Additionally, other approach which quantified phase synchrony with Phase locking value (PLV) has been used in EEG hyperscanning studies. PLV estimates the consistency of the phase difference between two signals[49]. While Burgess suggested PLV show bias on detecting hyperconnectivity that doesn't exist, especially when small samples are employed[50]."

2.The advantages of the proposed pipeline of analyzing IBS should be discussed more clearly.
Response: Thank you for your suggestions. We have added advantages of the proposed pipeline of analyzing IBS with more detailed description in (p. 21–23, line 422–465).

“The data analysis process in this protocol consists of two parts: preprocess and WTC analysis. Three critical data analysis steps should be highlighted here: First, conducting the principal component spatial filter algorithm (PCA) on the neural data. Zhang and couleage[29] proposed this approach for separation of the global and local effects. Although fNIRS allow relatively free movement and communication, PCA is necessary to apply in extracting real signals from systemic changes (e.g. breathing rate, blood pressure, heart rate, blood pressure, breathing rate, and autonomic nervous system activity). The protocol here suggests PCA are efficient to remove the global effects. This method is widely used in fNIRS hyperscanning studies[13]. Taken together, non-neural components can be removed successfully using spatial filtering. Second, WTC is adopted to identify the IBS of collaborative learning dyads. WTC is an approach of assessing the correlation coefficients between two time series as a function of frequency and time[41]. This method can be used to reveal locally phase-locked behavior that might not be detected with traditional approach like Fourier analysis[30]. And this method is widely used to estimate IBS in fNIRS hyperscanning with varied paradigms, such as cooperative and competitive behaviors[4,42], studying action monitoring[43], imitation[44], verbal communication[8], non-verbal communication[19], teaching and learning activity[11-14] and mother-child social interaction[45]. Meanwhile, other techniques, such as Granger Causality Analyze (GCA), correlation analysis and phase synchrony analysis are used in hyperscanning research. GCA is a method for revealing directed (“causal”) information between two time-series data[46]. This method once has been used to test the direction of information flow between instructor and learner[12]. correlation analysis is also adopted in fNIRS based hyperscanning field to estimate IBS in dyads who conduct cooperative or competitive tasks[47,48]. Compared to WTC analysis, this method only characterizes the covaried features of two fNIRS time series along time stream and missed potential information in frequency. Additionally, other approach which quantified phase synchrony with Phase locking value (PLV) has been used in EEG hyperscanning studies. PLV estimates the consistency of the phase difference between two signals[49]. While Burgess suggested PLV show bias on detecting hyperconnectivity that doesn't exist, especially when small samples are employed[50]. Third, adopting non-parametric statistical test to detect the collaborative learning related frequency is essential. At first, task-related FOI is selected by either following suggestions in previous research or according to specific experiment design (i.e. how long for one task trial in experiment). Recently, to obtain robust and reproductive results in FOI selecting process, non-parametric statistical test approach is adopted. Here, this technique operated efficiently. The collaborative learning related FOI (0.015-0.021 Hz) is identified. and Similar frequency bands has been identified in fNIRS hyperscanning research in teaching scenario[13] and in verbal communication paradigms[8]. It is necessary to apply this technique in the multi-brain data analysis pipeline. All in all, establishing suitable algorithms and techniques on analysis of hyperscanning data will be a prominent field.”

3. The results associated with collaborative learning should be discussed more deeply.
Response: Thank you for your suggestions, we have revised the text in results section on (p. 16, line 342–350; p. 23, line 454–464).

"Taken together, Following the data analysis pipeline, the frequency band (raged from 0.015 Hz to 0.021 Hz) which sensitive to collaborative learning is identified by cluster-based permutation approach. Further, for each channel, time-averaged IBS value is compared between the rest phase and the collaborative learning phase using a series of paired sample t-tests. To solve the multiple comparisons problem, all observed p-values in 46 channels are corrected by FDR methods[35,36]. The results show that the IBS at the channel 33 reaches significance during collaborative learning (FDR corrected $p < 0.05$). No other corresponding channels indicated significant results ($p > 0.05$)."

"Third, adopting non-parametric statistical test to detect the collaborative learning related frequency is essential. At first, task-related FOI is selected by either following suggestions in previous research or according to specific experiment design (i.e. how long for one task trial in experiment). Recently, to obtain robust and reproductive results in FOI selecting process, non-parametric statistical test approach is adopted. Here, this technique operated efficiently. The collaborative learning related FOI (0.015-0.021 Hz) is identified. and Similar frequency bands has been identified in fNIRS hyperscanning research in teaching scenario[13] and in verbal communication paradigms[8]. It is necessary to apply this technique in the multi-brain data analysis pipeline."

Table S1. MNI coordinates and corresponding maximum probability Anatomical Automatic Labeling for each channel used in this study.

Channels	MNI coordinates (mm)			Anatomical Automatic Labeling (AAL)
	x	*y*	*z*	
1	-35.863	62.6091	-7.2564	Left frontopolar cortex
2	-12.874	71.4609	3.9384	Left frontopolar cortex
3	15.0159	71.0702	-3.3197	Right frontopolar cortex
4	38.2233	63.0185	-7.0269	Right frontopolar cortex
5	-45.367	52.5914	1.1215	Left middle frontal cortex
6	-24.366	67.8844	8.4777	Left superior frontal cortex
7	2.1312	67.9781	9.0959	Superior/middle frontal cortex
8	26.8777	67.9781	8.452	Right superior frontal cortex
9	46.726	53.1491	1.7231	Right middle frontal cortex
10	-35.323	57.9453	18.7091	Left middle frontal cortex
11	-12.726	66.8	21.616	Left superior frontal cortex
12	14.4883	67.9538	22.7929	Right superior frontal cortex
13	37.6583	58.5391	18.4993	Right middle frontal cortex
14	-45.008	41.7017	27.3908	Left middle frontal cortex
15	-23.407	56.2073	32.6655	Left middle frontal cortex
16	1.5098	59.2197	34.0971	Superior/middle frontal cortex
17	25.7471	57.0163	32.9168	Right superior frontal cortex
18	47.0841	41.5836	27.8437	Right middle frontal cortex
19	-34.525	40.2455	41.748	Left middle frontal cortex
20	-11.211	50.3821	45.4175	Left superior frontal cortex
21	13.1334	50.4338	45.7225	Right superior/middle frontal cortex
22	36.1553	39.8036	42.1424	Right middle frontal cortex
23	-54	-78.333	-0.667	Left middle occipital cortex
24	-69	-52.333	0.333	Left middle temporal cortex
25	-73	-25.333	-3.667	Left middle temporal cortex
26	-45.667	-87.333	12.333	Left middle occipital cortex
27	-61	-65.333	16.333	Left middle temporal cortex
28	-71	-39.333	16.667	Left superior temporal cortex
29	-70	-10.667	14.667	Left primary motor cortex
30	-52.333	-74.333	31.333	Left angular
31	-66	-49.667	32.333	Left supramarginal gyrus
32	-70	-23.667	30.333	Left supramarginal gyrus
33	-39.667	-81.333	40.333	Left middle occipital cortex
34	-57	-59.667	45.667	Left inferior parietal cortex

35	-66	-36.667	45.667	Left inferior parietal cortex
36	-66	-9.333	39.333	Left postcentral gyrus
37	-46.333	-67.333	52.333	Left angular
38	-58.667	-45.333	53.667	Left inferior parietal cortex
39	-61.667	-21.667	51.333	Left inferior parietal cortex
40	-30.333	-71.333	59.333	Left superior parietal cortex
41	-43.667	-53.667	62	Left inferior parietal cortex
42	-53.667	-29.333	59.667	Left postcentral gyrus
43	-54.667	-4.667	54.333	Left postcentral gyrus
44	-30.667	-59.667	70	Left superior parietal cortex
45	-40.667	-36.333	69.667	Left postcentral gyrus
46	-46	-14	65	Left postcentral gyrus