Journal of Visualized Experiments

Conducting Hyperscanning Experiments with Functional Near-Infrared Spectroscopy

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58807R1
Full Title:	Conducting Hyperscanning Experiments with Functional Near-Infrared Spectroscopy
Keywords:	neuroscience, functional near-infrared spectroscopy, fNIRS, hyperscanning, brain-to-brain synchrony, parent-child interaction, cooperation
Corresponding Author:	V Dr. Reindl
Corresponding Author's Institution:	
Corresponding Author E-Mail:	vreindl@ukaachen.de
Order of Authors:	Vanessa Reindl
	Kerstin Konrad
	Christian Gerloff
	Jana A. Kruppa
	Laura Bell
	Wolfgang Scharke
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

Conducting Hyperscanning Experiments with Functional Near-Infrared Spectroscopy

AUTHORS AND AFFILIATIONS:

Vanessa Reindl[1,2], Kerstin Konrad[1,2], Christian Gerloff[2,3], Jana A. Kruppa[1,2,4], Laura Bell[1], Wolfgang Scharke[1]

[1]Child Neuropsychology Section, Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, Medical Faculty, RWTH Aachen University, Germany
[2]JARA-Brain Institute II, Molecular Neuroscience and Neuroimaging, RWTH Aachen & Research Centre Juelich, Germany
[3]Lehrstuhl II für Mathematik, RWTH Aachen University, Germany
[4]Translational Brain Research in Psychiatry and Neurology, Department of Child and Adolescent Psychiatry, Psychosomatics, and Psychotherapy, University Hospital Aachen, Germany

Wolfgang Scharke (wscharke@ukaachen.de)
Christian Gerloff (gerloff@math2.rwth-aachen.de)
Jana A. Kruppa (jkruppa@ukaachen.de)
Laura Bell (lbell@ukaachen.de)
Kerstin Konrad (kkonrad@ukaachen.de)

Corresponding Author:
Vanessa Reindl
vreindl@ukaachen.de

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SUMMARY:

The present protocol describes how to conduct fNIRS hyperscanning experiments and analyze brain-to-brain synchrony, to discuss challenges and possible solutions.

ABSTRACT:

Concurrent brain recordings of two or more interacting persons, an approach termed hyperscanning, are gaining increasing importance for our understanding of the neurobiological underpinnings of social interactions, and possibly interpersonal relationships. Functional near-infrared spectroscopy (fNIRS) is well suited for conducting hyperscanning experiments because it measures local hemodynamic effects with a high sampling rate and, importantly, it can be applied in natural settings, not requiring strict motion restrictions. In this article, we present a protocol for conducting fNIRS hyperscanning experiments with parent-child dyads and for analyzing brain-to-brain synchrony. Furthermore, we discuss critical issues and future directions, regarding the experimental design, spatial registration of the fNIRS channels, physiological influences and data analysis methods. The described protocol is not specific to

parent-child dyads, but can be applied to a variety of different dyadic constellations, such as adult strangers, romantic partners or siblings. To conclude, fNIRS hyperscanning has the potential to yield new insights into the dynamics of the ongoing social interaction, which possibly go beyond what can be studied by examining the activities of individual brains.

INTRODUCTION:

In recent years, neuroscientists have started to study social interactions by recording the brain activities of two or more persons simultaneously, an approach termed hyperscanning[1]. This technique opens new opportunities to elucidate the neurobiological mechanisms underlying these interactions. To fully understand social interactions, it may not be sufficient to study single brains in isolation but rather the joint activities of brains of interacting persons[2]. Using different neuroimaging techniques, hyperscanning studies have shown that brain activities of interacting persons or groups synchronize, *e.g.*, while they coordinate their actions[3], make music[4], communicate[5], engage in classroom activities[6] or cooperate[7].

The article presents a protocol for conducting simultaneous recordings with functional near-infrared spectroscopy (fNIRS). Similar to functional magnetic resonance imaging (fMRI), fNIRS measures the hemodynamic response to brain activation. Changes in oxygenated and deoxygenated hemoglobin (oxy-Hb and deoxy-Hb) are calculated based on the amount of diffusively transmitted near-infrared light through tissue[8]. fNIRS is well suited for conducting hyperscanning experiments, especially with children, because it can be applied in less constrained and more natural settings than fMRI. Moreover, it is less prone to movement artifacts than both, fMRI and EEG[9]. In addition, fNIRS data can be acquired at high sampling frequencies (*e.g.*, 10 Hz), thus it highly oversamples the relatively slow hemodynamic response and thereby potentially provides a more complete temporal picture of the brain hemodynamics[10].

This protocol was developed within the study of Reindl *et al.*[11] and has been slightly modified (in particular with respect to the channel placement and bad channel identification) more recently. The aim of the study was to examine synchronized brain activity of parent-child dyads as well as its relationship to emotion regulation. Using fNIRS hyperscanning, we assessed brain-to-brain synchrony in prefrontal brain areas of children (aged five to nine years) and their parents, mostly mothers, during a cooperative and a competitive computer task. Prefrontal brain regions were targeted as they had been identified as important regions for social interactive processes in previous hyperscanning studies[12]. The cooperative and competitive task were originally developed by Cui *et al.* [13] and recently employed by several previous studies[14-16]. For the study of Reindl *et al.*[11], the tasks were modified to be suitable for children. Participants were instructed to either respond jointly *via* button presses in response to a target (cooperation) or to respond faster than the other player (competition). Each child performed each task once with the parent and once with an adult stranger of the same sex as the parent. Within each child-adult dyad, wavelet coherence was calculated for the oxy-Hb signals of corresponding channels as a measure of brain-to-brain synchrony.

This protocol describes the procedures to collect fNIRS hyperscanning data of parent and child during the cooperative and competitive game. The overall procedure, however, is not specific to this research design but is appropriate for different populations (*e.g.*, adult strangers, romantic partners, siblings, *etc.*) and can be adapted for a number of different experimental tasks. This protocol also outlines one possible analytical procedure, which covers necessary and optional data analysis steps, including fNIRS data preprocessing, bad channel detection, wavelet coherence analysis and validation by random pair analysis.

PROTOCOL:

Prior to participation, all parents / children provided informed consent / assent. The study was approved by the ethics committee of the Medical Faculty of RWTH Aachen University.

1. Preparation before the Participant Arrives

1.1. Prepare NIRS caps.

1.1.1. Choose the cap sizes approximately 1-2 cm larger than the participant's head circumference.

1.1.2. Cut 15 holes with a diameter of approximately 15 mm each, arranged in a horizontal 3x5 grid, into the forehead area of each of 2 raw EEG caps (see **Table of Materials**). Make sure that the holes are spaced 30 mm from each other in any direction, that the middle column of holes is located in the center of the forehead, *i.e.,* above the nose, and that the bottom row is located above the eyebrows.

1.1.3. Mount an empty 3x5 probe holder grid (see **Table of Materials**) to each of the modified EEG caps such that the holder grid itself is placed on the inside of the cap and the holder sockets stick in the holes.

1.1.4. In order to make the caps more comfortable and minimize pressure marks, attach soft foam material (*e.g.*, adhesive window sealing tape) at the inner side of the holder grid between the probe sockets and at the edges. Use double-faced adhesive tape if necessary.

NOTE: The NIRS measurement system (see **Table of Materials**) has two separate probe sets, use one probe set for each participant.

1.1.5. Gently insert the probes into the appropriate holder sockets on the grids such that only the first ridge of each probe is mounted in the socket, which results in one clicking sound.

1.1.6. Open the probe set monitor window at the NIRS measurement system and select 2 probe sets arranged in a 3x5 grid each, one for the participating child and one for the adult. Ensure that the probe arrangements of the two caps corresponds to the arrangements in the probe set window (*i.e.,* same location of the respective emitter and receiver probe numbers).

1.2. Prepare the experiment.

1.2.1. Start the NIRS measurement system with laser diodes switched on 30 min before measuring, such that the system reaches a stable operating temperature.

1.2.2. Set all necessary options at the NIRS measurement system. Make sure that the device is set to event-related measurement and that the RS232 serial input, necessary for receiving triggers from the experimental paradigm, is active.

Note: The experiment is an adapted version by a paradigm devised by Cui *et al.*[13], programmed in the non-commercial Psychophysics Toolbox extensions, version 3.0.11[17].

1.2.3. Prepare the experimental paradigm by starting the technical computing software (see **Table of Materials**) that serves as base for the Psychophysics Toolbox extensions and setting the current directory to the folder that the paradigm is saved in.

1.2.4. Place 2 chin rests in front of the computer screen to prevent head movements during the experiment.

2. Participant Arrival in the Laboratory

2.1. Prepare the participants.

2.1.1. Show and explain the experimental setup including the NIRS measurement system to the participants. Always make sure that the participants do not look directly into the laser beam of the NIRS measurement system as this may be harmful to the eye.

2.1.2. Seat the participants next to each other in front of the computer screen. Adjust the height of the chin rests such that both participants sit comfortably.

2.1.3. Instruct the participants and administer practice trials of both the cooperative and the competitive game. Give additional instructions during the practice trials if necessary.

2.1.4. Measure and mark the Fpz point according to the 10-20 system, which is 10% of the distance between nasion and inion, on each participant's head.

2.1.5. Place the caps with the probes carefully on the participants' heads, with the laser turned off. Place the front of the cap, including the probe grid, on the participant's forehead first and then pull down the back of the cap towards the neck. Make sure that the middle probe of the bottom row is placed on Fpz and the middle probe column is aligned along the sagittal reference curve.

2.1.6. Place the fiber strings on the holder arm attached to the NIRS measurement system so that they hang loosely without contact with the participant or chair and that they do not pull on the caps. Use an additional holder (*e.g.*, modified microphone stand or similar) for the second participant if necessary.

2.1.7. Push each probe further into its socket until the small white nose in the center of the top of the probe casing is visible.

Note: The nose is pushed upwards by a coil spring mechanism as soon as the probe tip touches the participant's scalp.

2.1.8. Turn the laser on again and test the signal quality by clicking on the **Auto Gain** button in the probe set monitor window of the NIRS measurement system.

2.1.9. If a channel does not have a sufficient signal (*i.e.,* if it is marked in yellow), gently put the hair underneath the surrounding probe tip aside. If necessary, push the probes further into their sockets but ensure the comfort of the participant. Check whether the signal quality has improved (*i.e.,* the channel is now marked in green) by clicking on the auto gain button again.

2.1.10. If step 2.1.9. does not lead to a signal improvement, adjust the signal intensity. If there is too much signal (*i.e.,* if the channel is marked in red), change the signal intensity to low signal intensity by repeatedly clicking on the respective probe's symbol in the probe set monitor window of the NIRS measurement system. If there is not enough signal (*i.e.,* if the channel is marked in yellow), change the signal intensity to high signal intensity, again by repeatedly clicking on the respective probe's symbol.

2.2. Run the experiment

2.2.1. When there are no questions after the practice trials and a good signal quality is ensured, start the experimental paradigm.

2.2.2. Place a towel over the participants' hands so that they cannot see the hand movements of their respective game partner.

2.2.3. After the experiment, export the raw light intensity data as a text file by clicking on the text file out button and save the data. Do not apply any filters in the NIRS measurement system.

2.2.4. Clean all necessary materials (probes, probe holders, chin rests) with ethanol. Wash the caps regularly in a gentle cycle with mild detergent.

3. Data Analysis

3.1. Data Preprocessing

Note: There are several non-commercial software packages available for fNIRS data analysis, *e.g.*, HomER[18], NIRS Brain AnalyzIR[19] or SPM for fNIRS[20]. The latter was used for the following preprocessing steps. For more information on how to perform these steps, please see the toolbox manual.

3.1.1. Convert the data files to the SPM for fNIRS data format.

3.1.2. Calculate oxy-Hb and deoxy-Hb concentration changes using the modified Beer-Lambert law by pressing the convert button in the main window. Enter the age of the subject and the distance between source and detector (*e.g.*, 3 cm). Accept the default values for the molar absorption coefficients of oxy-Hb and deoxy-Hb at wavelength (λ) 1 and λ 2 as well as the default values for the differential pathlength factor (DPF) at λ 1 and λ 2.

3.1.2. Preprocess the time series of hemodynamic changes to reduce motion artifacts by selecting the MARA button (for more information on the MARA algorithm see Scholkman *et al.*[21]).

3.1.3. Preprocess the time series to reduce slow drifts by selecting the **DCT** button.

3.2. Bad channel detection

Note: Bad channel detection can be performed before and/or after fNIRS data preprocessing. In this protocol, different objective criteria for detecting bad channels and visual inspection are combined. Please note that the proposed list of objective criteria is not exhaustive. For bad channel detection, self-written scripts were used (for the technical computing software see **Table of Materials**).

3.2.1. Exclude channels in which there is no signal change for several continuous samples, which is indicated by a flat line when plotting the time series.

3.2.2. Calculate the coefficient of variation CV = SD/mean*100 for the raw attenuation data. Exclude channels in which the CV is above a predefined percentage (*e.g.*, 10%; see for instance van der Kant *et al.*[22]).

3.2.3. If in doubt whether to exclude a channel or not, plot the power spectrum of the signal. If there is no heartbeat visible in the signal spectrum around 1 Hz, as indicated by an increased power in this frequency band, exclude the channel from the analysis.

3.2.4. Visually inspect all data before and/or after preprocessing. Decide whether to include the channel based on the objective criteria, described in 3.2.1 – 3.2.3, as well as on subjective visual detection of noisy channels.

3.3. Brain-to-brain connectivity

Note: Two different estimate types of brain connectivity can be distinguished: non-directed estimates, which quantify the strength of the connectivity, and directed estimates, which seek to establish statistical evidence for causation from the data[23]. Here the focus was on the wavelet transform coherence (WTC), a widely applied non-directed estimate for fNIRS brain-to-brain connectivity. Several non-commercial software solutions for the computation of the WTC are available, *e.g.*, one by Grinsted and colleagues[24] or the ASToolbox[25], which was used in this protocol for the following steps.

3.3.1. In the AWCO function of the ASToolbox, specify the mother wavelet (*e.g.*, Generalized Morse Wavelet with its parameters beta and gamma), which is used to transform each time-series into the time and frequency domain by the continuous wavelet transformation.

3.3.2. Specify the smoothing window type (*e.g.,* Hanning window) and the smoothing window size for the time and scale domain in the AWCO function.

3.3.3. To examine the significance of the WTC coefficients and to calculate their *p*-values, specify the number of surrogate time series (n≥300) and the ARMA model (*e.g.*, AR (1)) in the AWCO function.

3.3.4. With the parameters specified in steps 3.3.1 to 3.3.3, calculate the wavelet coherence of two corresponding channels (the same channel in two participants).

3.3.5. Choose a frequency band of interest in which the task-related brain-to-brain synchrony is expected to occur based on previous studies and visual inspection of the data (for an alternative approach see Nozawa *et al.* [26]).

3.3.6. Calculate the percentage of significant WTC coefficients in the task-related frequency band for each task block in each channel and for each participant. Use this value as an outcome measure of brain-to-brain synchrony for further statistical analysis (for more information see Reindl *et al.*[11]).

3.4. Comparison to Random Pairs

Note: To validate the results, we recommend comparing the WTC of the actual dyads to the WTC of random adult-child pairings, who did not play with each other but performed the same experimental task.

3.4.1. Calculate the WTC, as described in 3.3., for participant pairs who did not play together but performed the same experimental task (*i.e.,* random pairs). Choose the number of random pairs (e.g., 300 for each condition) and calculate the WTC for each random pair.

3.4.2. Compare the coherence of the random and actual pairs to avoid the detection of spurious synchronicity.

REPRESENTATIVE RESULTS:

Representative data of one parent-child dyad during the cooperative condition are shown in **Figure 1**. The cooperative task consists of three 30 s rest blocks and two task blocks, with 20 trials each, presented in alternating order. In each trial, participants have to react as simultaneously as possible to a signal to earn a point[11].

[insert **Figure** 1 about here]

The results are exemplified for the fNIRS data of channel 8 of both participants of a parent-child dyad. Before preprocessing, raw light attenuation data, received from the fNIRS device, are converted to changes in oxy-Hb and deoxy-Hb for both participants. Next, fNIRS time series are preprocessed to reduce motion artifacts and drifts. Finally, the significant WTC is calculated from the preprocessed oxy-Hb signals of both participants.

Figure 1 illustrates a real valued WTC matrix, which is composed of the coherence coefficients in time and frequency domain (here in period length). The coefficients can range between 0 and 1, with 1 indicating a perfect relationship at a specific time and frequency between both signals[25]. The coefficients are visualized using a color map ranging from blue (little or no coherence) to red (strong or maximum coherence). Significant coherence values are marked by solid black lines surrounding the respective areas in the plot. The beginning and end of each task block are indicated by vertical dashed lines.

Results show a strong coherence throughout the experiment in a high frequency band, until a period length of ~ 1 s (1 Hz). This results from the cardiac rhythms of parent and child. Additionally, results show a strong coherence in a lower frequency band between ~ 2 s and 8 s period length (0.5 - 0.125 Hz). Trial lengths differed due to pseudo-randomized variable cue durations (600 - 1500 ms) and participants' individual reaction times but were around 7 s on average, assuming reaction times of about 1 s. Therefore, coherence in this low frequency range likely reflects a synchronization of brain activities of both subjects during the task.

FIGURE AND TABLE LEGENDS:

Figure 1: Hyperscanning data analysis and representative results. Light intensity data is collected in 22 channels (CHs) of two participants. First, bad channels are detected and excluded from further analyses. Afterwards, light intensity data is converted to changes in oxy-hemoglobin (Δ Oxy-Hb) and deoxy-hemoglobin (Δ Deoxy-Hb). Signals are shown for one exemplary parent-child dyad in CH 8 during the cooperative condition. Data is preprocessed by reducing motion artifacts and slow drifts. Afterwards, the wavelet coherence is calculated from the preprocessed oxy-Hb signals. To estimate the significance of each wavelet coherence value, 300 surrogate time series are generated. If the observed wavelet coherence value is higher than 95% of the wavelet coherence values obtained from the surrogate time series at the same point in time and scale, it is regarded as significant. Significant wavelet coherence values are marked by solid lines surrounding the respective areas in the plot. Coherence in the task-related frequency band is depicted within the black box. Please note that the analysis steps and the

parametrization depicted in the figure should be understood as an example. The optimal parameterization depends on the data, *e.g.*, different parameters of the MARA algorithm might work best for different types of artifacts[21], and there is no gold standard for any of the analysis steps yet.

DISCUSSION:

In this protocol, we show how to conduct fNIRS hyperscanning experiments and one possible way to analyze brain-to-brain synchrony, measuring concentration changes of oxy-Hb and deoxy-Hb at frontal brain regions of two subjects simultaneously. FNIRS hyperscanning is relatively easy to apply: a single NIRS device is sufficient to measure brain activities of both subjects by splitting the optodes between them. Thus, no synchronization between different devices is necessary[1]. Moreover, since fNIRS does not require strict motion restriction, it is well suited for conducting hyperscanning experiments in a natural environment and in children. In the following, we highlight some critical issues when designing, analyzing and interpreting (fNIRS) hyperscanning experiments, discuss challenges as well as possible solutions.

Experimental Design. One important issue of hyperscanning studies concerns the experimental design. Two participants who complete the same experimental task independently of each other might show similar brain activities, which then might be detected as brain-to-brain synchrony[27]. To differentiate between brain-to-brain synchrony induced by the experimental task and by the social interaction, appropriate experimental control conditions are necessary. On the one hand, the cooperative and competitive tasks are very well suited because they differ only in the cooperative task component and not in the stimulus material and the participant's motor behavior. On the other hand, less standardized and more natural interactions (*e.g.*, making a puzzle together) might induce more variance in social interactive behavior and might have a greater ecological validity.

Spatial registration of channels. One challenge in fNIRS hyperscanning is measuring hemodynamic activity in corresponding channels. Attaching emitter and detector probes at corresponding locations of two participants' heads does not warrant that activity in two corresponding cortical regions is tapped, as individual brain anatomy is liable to differ across participants. Simultaneously measuring an adult and a child exacerbates this problem by introducing developmental differences on top of anatomical ones. Moreover, with an increasing number of channels, the placement of the channels is less reproducible across subjects because of variability in head shape and size[28]. One optional accessory to the ETG-4000 is a probe positioning unit which creates probe positions relative to fiducial points on the head in three-dimensional space. These data can then be co-registered to the structural MR image of the participant's brain[28]. Acquiring MR images and using the positioning unit will enable the experimenter to better control whether activity is actually measured in corresponding brain regions across two participants. Additionally, researchers could partly circumvent this problem by calculating an all-to-all connectivity model, measuring the connection between any two channels of the two participants.

Influence of the systemic physiology. Another important issue is that hemodynamic changes are known to be influenced not only by the effect of the neurovascular coupling, thus neuronal activity, but also by systemic changes, such as changes in heart rate, blood pressure, breathing rate, and autonomic nervous system activity[29]. Therefore, any synchrony detected in the hemodynamic changes of two cooperating participants may also be attributable to a synchrony of those factors. Previous studies have shown that two interacting partners do indeed synchronize their physiological activities[30]. Note, however, that in tasks with different experimental conditions which are directly compared to each other, this is only a confounder if physiological coupling is more prominent in one but not the other condition. Nevertheless, it can be helpful to acquire physiological data in hyperscanning studies to enable experimental control of these parameters. Another option, as demonstrated recently by Nozawa *et al.*[26], is to add measurement channels with a short source-detector (S-D) separation (e.g., 1 cm), which are sensitive to the superficial skin blood-flow signal. The corresponding component can then be removed from the fNIRS signal obtained from measurement channels with a regular S-D separation (*e.g.*, 3 cm), thus reducing the influence of physiological confounders. Such a dual or multi-distance approach has been shown to improve the sensitivity to task-enhanced (here: communication-enhanced) brain-to-brain synchrony.

Data Analysis. Hyperscanning results depend on an estimator to quantify brain-to-brain synchrony. In the current study, we calculated the WTC of oxy-Hb signals of corresponding channels as a measure of brain-to-brain synchrony. Wavelet-based methods have the advantage that they consider the non-linear dynamics of time series in the time-frequency space. The WTC is a non-directed measure calculated from wavelet transformed time series, representing the strength of the relationship between two time series. In future studies, it would be interesting to additionally include directed measures, such as Granger causality, in order to examine which participant "leads" the activity (see for instance Pan *et al.*[16]). Furthermore, while many previous fNIRS-based hyperscanning studies examine brain-to-brain synchrony in only one signal (*e.g.,* oxy-Hb), it is advisable to consider both oxy-Hb and deoxy-Hb (and possibly total-Hb) in order to take full advantage of the fNIRS technique[16].

Limitations. Although fNIRS offers a promising, rapidly growing neuroimaging technique, some technical limitations associated with the device need to be considered when planning such a study (for a recent review see Pinti *et al.*[31]). In comparison to EEG and fMRI, fNIRS is more resistant to motion artifacts, yet, it still requires sufficient motion artifact control and detection. There are several potential causes of artifacts. First, some participants tend to move their head abruptly, in particular infants and children, and thereby might pull on the fiber tracts, affecting the optode contact. Developments of new fiberless devices are more robust to movement and even allow investigations of active tasks[31]. The use of a chin-rest can serve as an additional motion artifact control; however, it limits the ability to record brain activities in natural interactions. Second, acquiring an adequate optode contact can be hindered by dark, curly and/ or thick hair of the participant. Placing the optodes can thus be time-consuming and a perfect signal is not always guaranteed. Third, depending on the fNIRS system, wearing optodes for a longer period of time can put pressure on the participant's head, which can be experienced as unpleasant. This does not only limit the recording time of the experiment but might also lead to

more movement and artifacts (*e.g.,* smaller children might pull on the cap). In addition to motion artifacts, it is noteworthy that fNIRS provides measures of the cortical surface only. Finally, there are no standardized data analysis guidelines yet. Several toolboxes were developed over the past years and first attempts were made to analyze the effectiveness of various preprocessing techniques (*e.g.,* Brigadoi *et al.*[32] and Cooper *et al.*[33]). Moreover, the analytical protocol presented in this article shows one way to analyze fNIRS hyperscanning data. Importantly, the selected parameters of the analysis should be understood as one possible option and not as a standard guideline. Several other analytical protocols for fNIRS hyperscanning have been developed in the last years by different research groups (see for instance Cui *et al.*[13]; Hirsch *et al.*[34]).

Conclusion. fNIRS hyperscanning is a promising technique to gain further insights into the neurobiological underpinnings of social interactions[35]. In the future, portable and fiberless NIRS devices may be particularly important when examining brain-to-brain synchrony in natural interactions and moving from the dyad towards larger groups of subjects. Finally, combining different neuroimaging techniques, *e.g.,* EEG-fNIRS, may provide new insights, broadening our understanding of brain-to-brain synchrony.

ACKNOWLEDGMENTS:
This work was funded by the Excellence Initiative of the German federal state and governments. The Hitachi NIRS system was supported by a funding of the German Research Foundation DFG (INST 948/18-1 FUGG).

DISCLOSURES:
The authors have nothing to disclose.

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Figure

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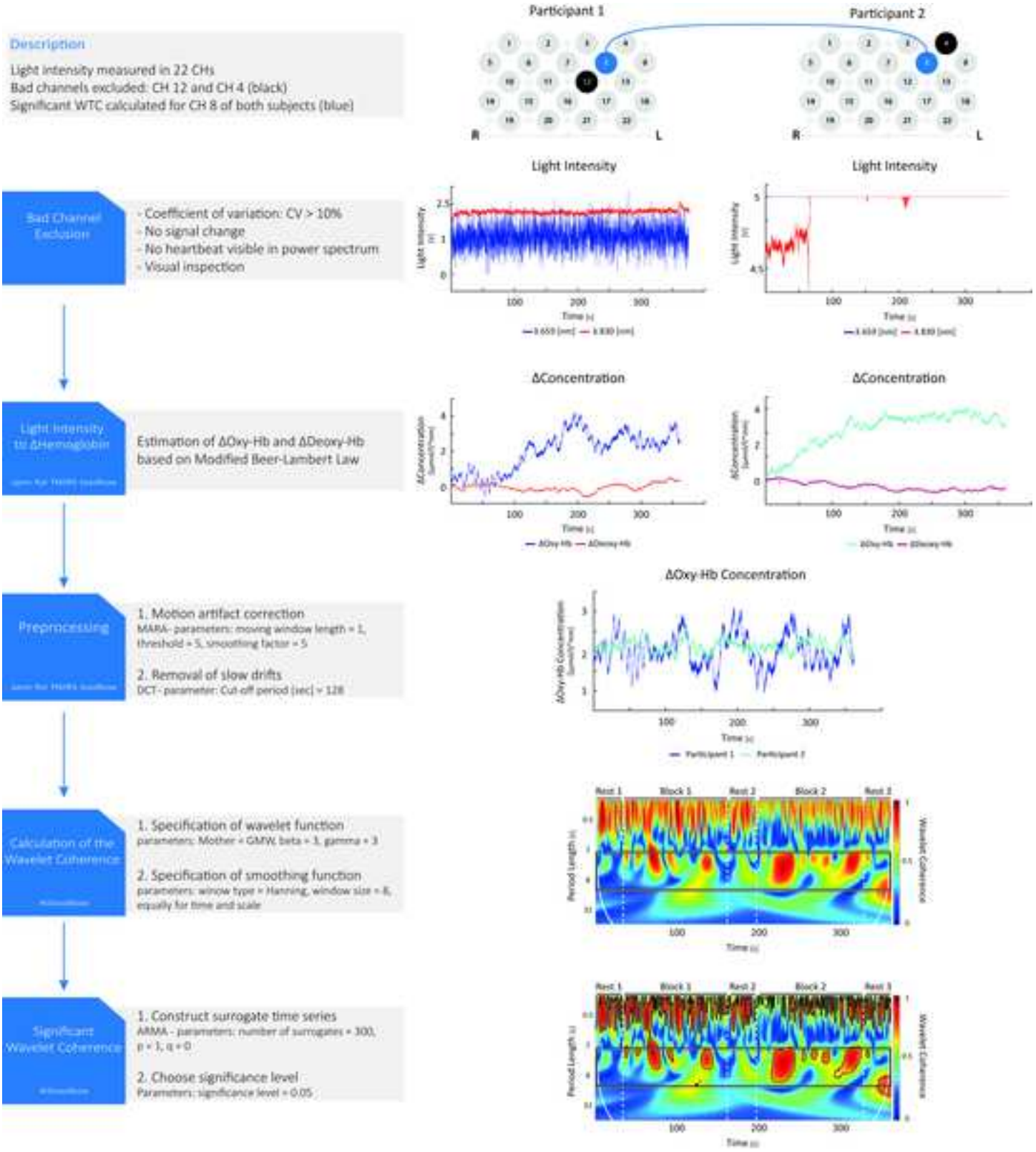

Table of Materials

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

Name of Material/ Equipment	Company
NIRS measurement system with probe	Hitachi Medical Corpo
raw EEG caps	EASYCAP GmbH, Herr
Technical computing software	The MathWorks, Inc.,

	Catalog Number	Comments/Description
ETG-4000 Optical Topography System		The current study protocol requires an o
C-SCMS-56; C-SCMS-58		Caps must be provided with holes for NIF
MATLAB R2014a (or later versions)		Serves as base for Psychophysics Toolbo

ptional second adult probe set for 52 channels of measurement in total as well as two 3x5 probe holder grids.

RS probes by the experimenter. Choose cap size approx. 1-2 cm larger than participant's head circumference.

‹ extensions (stimulus presentation), SPM for fNIRS toolbox (fNIRS data analysis), and ASToolbox (WTC computa

ation).

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Title of Article: Conducting Hyperscanning Experiments with Functional Near-Infrared Spectroscopy

Author(s): Vanessa Reindl, Wolfgang Schaeke, Christian Gerloff, Jana A. Krupp, Dawa Bell & Kerstin Konrad

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CORRESPONDING AUTHOR

Name:	Vanessa Rendl
Department:	Department of child and adolescent Psychiatry, Psychosomatics & Psychotherapy
Institution:	University Hospital RWTH Aachen
Title:	
Signature:	V. Rendl
Date:	20.07.2018

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September 11th, 2018

Submission of the revised manuscript: "Conducting Hyperscanning Experiments with Functional Near-Infrared Spectroscopy"

Dear Dr. Steindel,

We would like to thank you and both reviewers for the careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which helped to improve its quality. Please find attached our point-by-point responses to each of the comments as well as the revised manuscript (with tracked changes).

Thank you for considering our manuscript for publication!

Kind regards,

Vanessa Reindl

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**
2. **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 and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Easycap GmbH, Hitachi ETG-4000, Matlab, Wavelet Toolbox™, etc.**

   We have proofread the manuscript and removed all commercial names.

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

   We have now avoided all personal pronouns in the protocol text.

4. **Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.**

   We have revised the text as suggested and have used imperative tense throughout the protocol text (except for the "Notes").

5. **The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.**

   Each step now contains a maximum of 4 sentences (mostly 2-3 sentences).

6. **Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.**

   We have added more details to many of the protocol steps and added references where appropriate.

7. **1.1.2: How to attach the soft foam material? Is glue needed?**

We have added the information on how to attach the soft foam material to the protocol: "In order to make the caps more comfortable and minimize pressure marks, attach soft foam material (e.g., adhesive window sealing tape) at the inner side of the holder grid between the probe sockets and at the edges. Use double-faced adhesive tape if necessary." (step 1.1.4.)

8. **1.2.3: Please describe how this is actually done.**

   The step is now described in more detail:

   "1.2.2. Set all necessary options at the NIRS measurement system. Make sure that the device is set to event-related measurement and that the RS232 serial input, necessary for receiving triggers from the experimental paradigm, is active.

   NOTE: The experiment is an adapted version by a paradigm devised by Cui et al.[13], programmed in the non-commercial Psychophysics Toolbox extensions, version 3.0.11[17].

   1.2.3. Prepare the experimental paradigm by starting the technical computing software (see table of materials) that serves as base for the Psychophysics Toolbox extensions and setting the current directory to the folder that the paradigm is saved in."

9. **2.1.8: How to adjust the signal intensity?**

   It is now described how to adjust the signal intensity: "[…] by repeatedly clicking on the respective probe's symbol in the probe set monitor window of the NIRS measurement system." (step 2.1.10.)

10. **Step 3 and sub-steps: Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

    We have added more details, such as button clicks, to the protocol, e.g. "Calculate oxy-Hb and deoxy-Hb concentration changes using the modified Beer-Lambert law by pressing the convert button in the main window. Enter the age of the subject and the distance between source and detector (e.g., 3 cm). Accept the default values for the molar absorption coefficients of oxy-Hb and deoxy-Hb at wavelength (λ) 1 and λ 2 as well as the default values for the differential pathlength factor (DPF) at λ 1 and λ 2" (step 3.1.2.)

    Moreover, we have included information about the analysis parameters in Figure 1.

11. **Lines 189-195: Please remove the weblinks and use a superscripted numbered reference instead.**

    We have removed the weblinks and replaced them with references.

12. **Discussion: Please also discuss any limitations of the technique.**

    We have added a new paragraph "Limitations" to the discussion:

"**Limitations.** Although fNIRS offers a promising, rapidly growing neuroimaging technique, some technical limitations associated with the device need to be considered when planning such a study (for a recent review see Pinti *et al.*[31]). In comparison to EEG and fMRI, fNIRS is more resistant to motion artifacts, yet, it still requires sufficient motion artifact control and detection. There are several potential causes of artifacts. First, some participants tend to move their head abruptly, in particular infants and children, and thereby might pull on the fiber tracts, affecting the optode contact. Developments of new fiberless devices are more robust to movement and even allow investigations of active tasks[31]. The use of a chin-rest can serve as additional motion artifact control, however, it limits the ability to record brain activities in natural interactions. Second, acquiring an adequate optode contact can be hindered by dark, curly and/ or thick hair of the participant. Placing the optodes can thus be time-consuming and a perfect signal is not always guaranteed. Third, depending on the fNIRS system, wearing optodes for a longer period of time can put pressure on the participant's head, which can be experienced as unpleasant. This does not only limit the recording time of the experiment but might also lead to more movement and artifacts (e.g., smaller children might pull on the cap). In addition to motion artifacts, it is noteworthy that fNIRS provides measures of the cortical surface only. Finally, there are no standardized data analysis guidelines yet. Several toolboxes were developed over the past years and first attempts were made to analyze the effectiveness of various preprocessing techniques (e.g., Brigadoi *et al.*[32] and Cooper *et al.*[33]). Moreover, the analytical protocol presented in this article shows one way to analyze fNIRS hyperscanning data. Importantly, the selected parameters of the analysis should be understood as one possible option and not as a standard guideline. Several other analytical protocols for fNIRS hyperscanning have been developed in the last years by different research groups (see for instance Cui *et al.*[13]; Hirsch *et al.*[34])."

13. References: Please do not abbreviate journal titles.

We have done accordingly.

14. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have revised the table, as suggested.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript describes a protocol on a functional near-infrared spectroscopy (fNIRS) hyperscanning experiment between dyads (e.g., parent - child, spouses, etc.) to assess brain-to-brain synchrony. The authors describe in detail the setup and preparation and also outline the relevant analyses steps.

Major Concerns:

1. **Preprocessing: This part is somehow confusing as it is not clear which steps are conducted by which program. For example, the ETG-4000 automatically calculates the concentration changes, it also has specific built-in low-/highpass filters, moving average and detrending procedures. It may be that I have difficulties as I do not know the SPM for NIRS toolbox, but it should be more clearly stated. Did you export the raw data without any filters? Such questions came to my mind when reading the part.**

   We have modified Figure 1 and included information on the data analysis steps, the specific analysis parameters as well as the toolboxes. Additionally, we have added further information in the text to clarify which steps were performed with which toolbox.

   We exported the raw data from the ETG-4000 without any filters. We have added this step to the protocol: "After the experiment, export the raw light intensity data as a text file by clicking on the text file out button and save the data. Do not apply any filters." (step 2.2.3.)

2. **What filters do you recommend and which parameters (e.g., frequency thresholds) should be used?**

   We have now included the parameters in Figure 1 (see above). We did not filter the data.

3. **Bad Channels: It would be advisable to present a figure on some of these artifacts. Are there any criteria for the percentage of CV allowed?**

   We have now included two examples of bad channels in Figure 1. The percentage of allowed CV differs between studies, often 10% is used. As an example for specific criteria, we have now referred the reader to the publication of van der Kant:

   "Calculate the coefficient of variation CV = SD/mean*100 for the raw attenuation data. Exclude channels in which the CV is above a predefined percentage (e.g., 10 %; see for instance van der Kant *et al* [22])." (step 3.2.2.).

4. **I am not in expert in wavelet analysis, so I do not know all the terms. Here, other reviewer should give comments.**
   Shortly, disadvantages of NIRS should be stated.

   We have included a new paragraph on the disadvantages of fNIRS in the discussion, focusing especially on practical issues (please see comment 12 to the editor).

5. **It could be mentioned which parameters to use further on. In the manuscript only one exemplary channel is shown with some coherence within the dyad.**

   In step 3.3.6. we describe the outcome measures used for statistical analysis. We have modified the step, which hopefully makes it more clear:

   "Calculate the percentage of significant WTC coefficients in the task-related frequency band for each task block in each channel and for each participant. Use this value as an outcome measure of brain-to-brain synchrony for further statistical analysis (for more information see Reindl et al.[11])."

   Minor Concerns:

6. **Abstract:**
   In the Abstract the abbreviation fNIRS should be introduced one sentence above when it is first mentioned.

   The abbreviation fNIRS is now introduced one sentence above.

7. **Introduction:**
   Studies 4 and 5 are not really hyperscanning studies, but studies on synchronization. Regarding the definition of Hyperscanning that is presented, this issue should be clarified. It also seems that none of these studies measured groups of participants simultaneously as you introduced before. There was a study by Dikker et al. (2017) in Current Biology which measured EEG in all children in a small classroom simultaneously. I am not sure if this is suited, but at least a group was hyperscanned. It is essential to measure simultaneously to get insights into social interaction or dependence processes (>1 brain) and not only social cognition, where one brain measure suffices.

   Thank you for the suggestion. We have changed it accordingly.

8. **It is true that NIRS has a much better temporal resolution than fMRI, but that is somehow put into perspective by the sluggishness of the BOLD signal. Beyond, developments of scanning protocols (multiband protocols) increase fMRI temporal resolution.**

   We have modified the sentence:
   "In addition, fNIRS data can be acquired at high sampling frequencies (e.g., 10 Hz), thus it highly oversamples the relatively slow hemodynamic response and thereby potentially provides a more complete temporal picture of the brain hemodynamics[10]." (lines 71 & 72)

9. **On the one hand, you state that fNIRS enables more realistic, ecologically valid setting, being an advantage compared to fMRI or EEG, on the other hand you write that you are using chin rests. Given this, it is more similar to fMRI and EEG.**

   We have now included the use of chin rests as a limitation in the discussion: "The use of a chin-rest can serve as additional motion artifact control, however, it limits the ability to record brain activities in natural interactions." (lines 480 & 481)

We agree that fNIRS is similar to EEG with respect to the experimental setting. However, we think that the experimental setting is more natural compared to fMRI hyperscanning, even when chin rests are used: “fNIRS is well suited for conducting hyperscanning experiments, especially with children, because it can be applied in less constrained and more natural settings than fMRI. Moreover, it is less prone to movement artifacts than both, fMRI and EEG[10].”

10. **I would not use the term pediatric as this implies patients.**

    We have now changed it to “children”.

11. **2.1.6 Will this be shown in the video? I only understand it since I know the ETG-4000.**

    Yes, this will be shown in the video.

12. **2.1.7 and 2.1.8 in both "yellow" channels are mentioned. Once you have to get the hair out of the way, once you should increase the signal.**

    First, the experimenter can try to brush the hair aside (step 2.1.9): “If a channel does not have a sufficient signal (i.e., if it is marked in yellow), gently put the hair underneath the surrounding probe tip aside. If necessary, push the probes further into their sockets but ensure the comfort of the participant. Check whether the signal quality has improved (i.e., the channel is now marked in green) by clicking on the auto gain button again.”

    If this is not successful, the experimenter can perform step 2.1.10: “If step 2.1.9. does not lead to a signal improvement, adjust the signal intensity. If there is too much signal (i.e., if the channel is marked in red), change the signal intensity to low signal intensity by repeatedly clicking on the respective probe’s symbol in the probe set monitor window of the NIRS measurement system. If there is not enough signal (i.e., if the channel is marked in yellow), change the signal intensity to high signal intensity, again by repeatedly clicking on the respective probe’s symbol”

13. **The figure legend should be more precise, for example by adding the details of processing, i.e. which parameters of preprocessing**

    Thank you for this suggestion. We have modified the figure legend and added the parameters in the figure:

    “Figure 1: Hyperscanning data analysis and representative results. Light intensity data is collected in 22 channels (CHs) of two participants. First, bad channels are detected and excluded from further analyses. Afterwards, light intensity data is converted to changes in oxy-hemoglobin (Δ Oxy-Hb) and deoxy-hemoglobin (Δ Deoxy-Hb). Signals are shown for one exemplary parent-child dyad in CH 8 during the cooperative condition. Data is preprocessed by reducing motion artifacts and slow drifts. Afterwards, the wavelet coherence is calculated from the preprocessed oxy-Hb signals. To estimate the significance of each wavelet coherence value, 300 surrogate time series are generated. If the observed wavelet coherence value is higher than 95% of the wavelet coherence values obtained from the surrogate time series at the same point in time and scale, it is regarded as significant. Significant wavelet coherence values are marked by solid lines surrounding the respective areas in the plot. Coherence in the task-related frequency band is depicted

within the black box. Please note that the analysis steps and the parametrization depicted in the figure should be understood as an example. The optimal parameterization depends on the data, e.g., different parameters of the MARA algorithm might work best for different types of artifacts[21], and there is no gold standard for any of the analysis steps yet."

Reviewers' comments:
Reviewer #2:

This interesting protocol, which was developed within the study of Reindl et al. (2018), described how to conduct fNIRS-based hyperscanning experiments and analyze interpersonal brain synchronization, and discussed several technical challenges and possible solutions. This protocol is novel and timely. The methods are sound. I believe that this protocol would be a nice addition to this field.

I just have a few minor concerns.

1. **line 75, it could be useful to also mention that this task was lately validated by Baker et al., 2016, Cheng et al., 2015, and Pan et al., 2017.**

   Baker, J. M., Liu, N., Cui, X., Vrticka, P., Saggar, M., Hosseini, S. H., & Reiss, A. L. (2016). Sex differences in neural and behavioral signatures of cooperation revealed by fNIRS hyperscanning. Scientific Reports, 6, 26492.

   Cheng, X., Li, X., & Hu, Y. (2015). Synchronous brain activity during cooperative exchange depends on gender of partner: A fNIRS-based hyperscanning study. Human Brain Mapping, 36(6), 2039-2048.

   Pan, Y., Cheng, X., Zhang, Z., Li, X., & Hu, Y. (2017). Cooperation in lovers: An fNIRS-based hyperscanning study. Human Brain Mapping, 38(2), 831-841.

   Thank you for this suggestion. We have added these studies.

2. **line 97 - line 103: Please mention that what your region of interest (ROI) was and why you chose it as the ROI.**

   We have now stated in the Introduction why we targeted prefrontal brain regions in the study: “Prefrontal brain regions were targeted as they had been identified as important regions for social interactive processes in previous hyperscanning studies[12].” (lines 81 - 83)

3. **line 232: WTC is the abbreviation of "wavelet transform coherence".**

   We have changed it accordingly.

4. **line 249: what is the meaning of "reasonable"?**

   We have changed the sentence to: “To examine the significance of the WTC coefficients and calculate their *p*-values, specify the number of surrogate time series (n>=300) and the ARMA model (e.g., AR (1)) in the AWCO function.” (step 3.3.3.)

5. **line 252: please simply explain how you chose the frequency of interest. These information (as well as the aforementioned region of interest) is helpful to readers.**

   We have now explained how to choose the frequency of interest: “Choose a frequency

band of interest in which the task-related brain-to-brain synchrony is expected to occur based on previous studies and visual inspection of the data (for an alternative approach see Nozawa *et al.*[26]).” (step 3.3.5.)

6. **I think it would be appropriate to add some reservations in the discussion. The current study did not provide the only way to analyze fNIRS hyperscanning data. It, however, provided a useful analytical protocol, which covered necessary and optional steps.**

   We absolutely agree that this is not the only possible way to analyze the data. We have added reservations to the protocol in the discussion:

   “Moreover, the analytical protocol presented in this article shows one possible way to analyze fNIRS hyperscanning data. Importantly, the selected parameters of the analysis should be understood as one possible option and not as a standard guideline. Several other analytical protocols for fNIRS hyperscanning have been developed in the last years by different research groups (see for instance Cui *et al.*[13]; Hirsch *et al.*[34]). (lines 491-495)

   Additionally, we have added some reservations to the figure legend:
   “Please note that the analysis steps and the parametrization depicted in the figure should be understood as an example. The optimal parameterization depends on the data, e.g., different parameters of the MARA algorithm might work best for different types of artifacts[21], and there is no gold standard for any of the analysis steps yet.” (lines 394 - 397)