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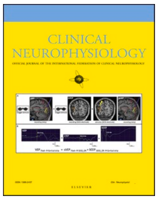

Estimating sensor-space EEG connectivity PART 1: Identifying best performing methods for functional connectivity in simulated data

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ABSTRACT

Objective: Functional brain connectivity (FC) can be estimated using electroencephalography (EEG). However, there is considerable variability across studies in the FC measures used and in data (pre-)processing methods, leading to difficulties comparing and amalgamating results between studies. Thus, standardisation of EEG (pre-)processing for the measurement and reporting of FC is needed. We aimed to assess differences in FC estimates produced by different settings across multiple EEG pre-processing steps, (including re-referencing and epoching) to validate a reliable methodological pipeline for assessing EEG-FC in simulated EEG data.

Methods: We simulated EEG-FC data where the 'ground truth' of the connections is known and compared estimates of FC from this ground truth data across multiple FC measures and variations in multiple pre-processing steps.

Results: Our results indicated that pre-processing steps that included segmenting the data into 40 or more epochs that were 6 s or more in length provided the most accurate estimation of the simulated FC. With regards to the data re-referencing, the Reference Electrode Standardization Technique or the common average re-referencing appeared best when used in conjunction with imaginary coherence and weighted phase lag index metrics. However, the magnitude-squared coherence FC measure performed best with the Current Source Density reference free techniques.

Conclusions & Significance: Our paper provides an evidence-base for the influence of referencing, epoch length and number, controls for volume conduction, and different FC metrics on EEG-FC measurement. Using this evidence, we present an initial and promising account of the best performing (pre-)processing choices for robust EEG-FC assessment.

1. Introduction

Electroencephalography (EEG) is a method through which we can measure electrical activity generated by the brain with millisecond precision (Cohen, 2014; Jackson & Bolger, 2014). EEG data contains both voltage oscillations and non-oscillatory voltage shifts and is thought to reflect the activation of various functionally connected neuronal populations, primarily from the brain's cortex, but with input from subcortical structures (Bustzaki, 2012). This functional connectivity (FC) of the brain can be measured using EEG data. Within EEG research, FC is defined as statistically significant synchronisation between the signals obtained from two or more EEG electrodes (or sources of activity when source localisation approaches are used). FC is assumed to indicate the existence of an interaction between the underlying brain regions (Cohen, 2014; Jackson & Bolger, 2014).

EEG FC has proved useful in distinguishing various mental states, for example while participants are at rest compared to while participants perform various goal-orientated tasks (Cohen, 2014). This offers the opportunity to investigate relationships between aspects of FC and specific cognitive functions. Further, studies have demonstrated differences between various psychiatric populations in EEG FC. For example, studies assessing EEG FC have been able to characteriae the brains of individuals with depression, differentiating their connectivity pattern from individuals who do not have depression (Park et al., 2021; McLoughlin et al., 2014; Miljevic et al., 2022; Zhang et al., 2021). However, while EEG assessments of FC have proved useful in various settings, there is considerable variability in the methods used to pre-process EEG data and, in the methods, used to quantify FC. These methodological differences have been shown to exert a confounding influence on the outcome of FC analyses and to limit the potential comparability of FC outcomes across studies (Miljevic et al., 2023).

It is particularly critical to control for confounds in the estimation of EEG FC due to the influence of volume conduction. Volume conduction refers to the spread of electrical activity from one source in the brain to a potentially large number of (or even all) scalp EEG electrodes with a near-instantaneous delay due to the conductibility of brain tissue (van

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Diessen et al., 2015). EEG electrodes record activity generated by multiple sources as a combination of the activity generated by those sources, with the contribution from each source to each electrode depending on the distance from each source to the electrode (Iriarte et al., 2003; Stone, 2002). This poses an issue for FC estimation, as volume conduction can cause two separate electrodes to be influenced by activity from a single common source, inflating FC estimates without the higher FC estimate being indicative that that two separate brain regions have synchronised. As such, if volume conduction is not controlled for, then FC estimates can be inflated by this zero-delay lag connectivity without the increased FC estimate reflecting physiologically meaningful FC between different brain regions.

Having noted the potential confound of volume conduction on zero-delay lag connectivity, it is also worth noting that research suggests signal transmission between neurons can take approximately 0.1 to 100 ms (ms), which encompasses the initiation of an action potential, its journey through the cell and down the axon to the release of neurotransmitters at the synaptic cleft and the action potential reaching the next neuron (Sabatini & Regehr, 1999). However, while there are some major monosynaptic connections in the brain, FC at the near-instantaneous time-lag is indistinguishable from volume conduction in EEG when using currently available methods. Thus, for the purposes of this paper and its methods, we assume the position that it is impossible to ascertain whether near-instantaneous connectivity indicates physiologically meaningful communication between two brain regions or simply the volume conduction of a common generator of neural activity.

Other issues that can adversely affect FC estimation include the use of brief epochs of EEG data to estimate FC, and very large numbers of electrode pairs, both of which can inflate the false positive rate due to the number of multiple comparisons (Miljevic et al. 2022). In our previous work (Miljevic et al. 2022), we identified six of the most influential EEG analysis steps on EEG FC estimates. These included: (1) the re-referencing system applied to the data; (2) the epoch length of EEG data segments used to estimate FC; (3) the number of epochs used to estimate FC; (4) the approaches used to reduce artifacts in the EEG data; (5) the methods used to control for volume conduction; and (6) the implementation of multiple comparison statistical controls. We recommended best practices for each of these seven analysis steps, and proposed a checklist that may be used to analyse the quality of studies employing EEG FC analyses.

Subsequently we utilised this checklist to assess study quality in a systematic review of differences in EEG FC in individuals with depression compared to healthy controls (see Miljevic et al., 2023). Within this review, our checklist showed that although some consistent differences in alpha connectivity were observed, there was considerable inconsistency in EEG pre-processing methods across studies, as well as common use of methods that have been demonstrated to provide non-optimal control against confounds for the estimation of EEG FC. In the context of this methodological inconsistency, results across studies were also inconsistent, highlighting the need for further evidence to establish high performing approaches to estimate EEG FC, enabling improved standardisation across studies.

Although previous research has examined single methodological aspects of FC estimation in isolation, to date there appear to be no studies that have attempted to present a validated pipeline that includes examination of multiple FC estimation steps and parameters in combination. A better understanding of how different methodological steps affect FC estimation could allow for the development of a standardised best-practice approach which would facilitate improved comparability across studies, and a more reliable foundation for estimating changes in EEG FC in different cognitive or disease states.

To provide a sound validation of an EEG methodology, the identification of the underlying dynamics of the EEG signal(s) of interest (i.e., ground truth) is required. Unfortunately, the ground truth cannot be known in real EEG data as we cannot yet accurately identify the causal relationship between the underlying workings of the human brain's 100 trillion connections and the scalp EEG data (Cohen, 2014). Simulating complex EEG datasets is a powerful method to overcome this limitation as it allows for the creation of EEG data in which the ground truth is known. Several toolboxes exist which specifically enable the simulation of synchronous (FC) brain signals (i.e., the EEGSourceSim toolbox [Barzegaran et al., 2019]).

The present study used the EEGSourceSim toolbox to generate complex simulated EEG FC data, to enable simple testing and comparisons of performance across multiple (pre-)processing steps in the analysis of various time-delayed EEG FC signals. Using these datasets, we assessed how FC metrics were impacted by different choices across four methodological steps: (1) the choice of reference montage (including the common average reference [CAR], the Reference Electrode Standardization Technique [REST], and the Current Source Density [CSD] approach); (2) the duration of the EEG epochs used to assess FC (2-, 4-, 6-, and 8- second (s) epochs, henceforth referred to as "epoch length"), (3) the number of epochs used to assess FC (20 epochs, 40 epochs, and the maximum number of epochs that could be generated within each of the settings of different epoch lengths – explained in more detail in our methods), and (4) the connectivity metric used to assess FC (real magnitude squared coherence [rMSC], imaginary coherence [iCOH], and the debiased weighted phase lag index [wPLI]). For a detailed description of each of the techniques specified above, please see the Methods section and the Supplementary Materials. Variations in these pre-processing steps were chosen for our analysis as they appear to exert the greatest impact on FC outcomes and their implementation varies widely in the literature. The individual parameters for each step were selected as they reflect common choices within previous literature.

This study focused specifically on assessing FC in the alpha frequency, which is the most prominent and the most studied frequency in the human brain. We compared the performance of each pre-processing pipeline on its ability to detect FC from simulated data containing two connections, including a simulated connectivity signal with a delayed transmission (similar to true FC in the human brain), a signal with instantaneous transmission (replicating a volume conduction signal), as well as simulated data consisting only of 'noise' and no connectivity. Based on previous literature (summarised in Miljevic et al. (2022)), we expected that the following pre-processing steps would provide the most accurate identification of the delayed FC signal (*delay_FC*), while also producing low estimates for the instantaneous volume conducted signal (*instant_VC*), and in the noise signals (*noise1*, *noise2*) where no connectivity was simulated: (1) for re-referencing, we expected that the CSD referencing approach would perform the best, followed by REST, then CAR; (2) for epoch length, we expected that $\geq$ 6 s epochs will show the best performance; (3) for epoch number, we expected that $\geq$40 epochs will show the best performance, however, we anticipated that the use of more epochs and shorter time windows (i.e., 2 s × 100 epochs) would produce similar results to the use of longer time windows and fewer epochs (i.e., 6 s × 40 epochs); (4) for the connectivity metrics, we expected that FC estimates obtained using the wPLI metric would produce the most accurate FC results as this method was specifically designed to control for spurious connectivity produced by volume conduction (Stam et al., 2007; Vinck et al., 2011). We also expected iCOH to perform similarly to wPLI, and that both wPLI and iCOH would perform better than rMSC (which does not control for spurious connectivity produced by volume conduction).

2. Methods

2.1. Data simulation

The EEGSourceSim toolbox (Barzegaran et al., 2019) was used to generate 10 individual simulated EEG datasets, each with a 128-channel array based on the EGI system, with a 300 Hz sampling frequency, and a network-specific configuration of connected source nodes. For simplicity, we generated EEG data using a validated example script provided by Barzegaran et al., (2019) with slight amendments to epoch lengths and times (the script can be found at: https://github.com/svndl/EEGSourceSim).

For full specifics of the methods and processes involved in the toolbox, we refer the reader to Barzegaran et al. (2019). Briefly, source connectivity signals were generated using a multivariate auto-regressive (MAR) model. There were three source nodes including T01, V1d, and V3d. These ROIs were modelled based on known occipital lobe networks of activity but instead of being projected to three occipital EEG electrodes, were projected to three electrodes in the left hemisphere located over the frontal cortex based on the EGI Sensor Net array and the 10–20 system. Within this system, T01 (electrode 29) corresponds to the 10–20 system electrode FC3, V3d (electrode 41) corresponds to C5, and V1d (electrode 25) corresponds to FTT7h.

The signal simulated in FTT7h contained internal dynamics comprised of lower frequency oscillatory activity (8 Hz, alpha frequency) and higher frequency oscillatory activity (20 Hz, beta frequency). These oscillations were propagated to the other ROIs (FC3 and C5) using three separate computations: (1) a high pass Finite Impulse Response (FIR) filter (with a cut-off frequency of 13 Hz) projected this activity instantaneously from FTT7h to FC3 so that only the 20 Hz oscillation was transferred to FC3 (this signal was not assessed in this study); and (2) a connection projected from FTT7h to C5 via a low-pass FIR filter (with a cut-off frequency of 13 Hz), so that only the 8 Hz oscillation was transferred to C5 (this produced instantaneous connectivity between these nodes, which is characteristic of volume conduction rather than real FC—signal named '*instant_VC*'); (3) next, FC with a 2-millisecond delay was simulated to match 'physiologically meaningful' FC (named '*delay_FC*'). To achieve this, a low-pass filter was used to produce a connection from C5 to FC3, simulated after the delay. Fig. 1 depicts the interactions between the three ROIs, the resulting amplitude spectrum at each node, and the naming conventions for the resulting simulated connectivity signals used in the rest of this article. Following simulation of activity within these connected source nodes, activity was projected to the electrode space.

In addition to the ground truth EEG FC dynamics described above, the EEGSourceSim toolbox also simulates biological and artificial noise, including activity with a 1/f log-frequency log-power distribution (typical of non-oscillatory EEG data), alpha activity (without connectivity); and sensor noise (or white noise, see Barzegaran et al., 2019 for further details on how each of these are simulated). This noise data was combined with the *instant_VC* and *delay_FC* signals to make the 10 EEG-like datasets (see Fig. 1 for detailing of all FC pairs sitmulated). We also saved the Noise only (no FC signal) data for each of the signal connections (named '*noise1*' and '*noise2*') to assess the effects of different processing steps in the absence of FC.

This same data was then simulated four times using different combinations of epoch numbers and epoch lengths: (1) 150 epochs × 2 s epoch lengths; (2) 80 epochs × 4 s epoch lengths; (3) 50 epochs × 6 s epoch lengths; and (4) 40 epochs × 8 s epoch lengths. Each of these conditions equate to approximately 5-minute EEG recordings. Data were simulated to provide EEG data lengths that matched the maximum possible number of epochs for each epoch length condition, enabling us to match the total data length across all epoch number and length combinations; however, it was only possible to simulate EEG data of approximately 5-minutes in length with the processing power we had available.

2.2. *EEG data pre-processing*

To enable efficient computation of our FC metrics, the raw simulated EEG data were first down sampled to 256 Hz and reduced to 64-channels. Next, data were re-referenced according to the specific referencing condition being tested, then organised according to the various epoch numbers and lengths under investigation, and finally, EEG FC was computed using one of the three FC estimation methods. Fig. 2 illustrates the process of data pre-processing and analysis.

As described in the introduction, for each level of the pre-processing steps under investigation (i.e., reference choice, epoch length, epoch number, and connectivity metric), 3–4 different options were tested to determine the best parameter setting for that methodological step. The different options for each of the pre-processing steps are detailed below.

2.2.1. *Re-reference choice*

Research has indicated that re-referencing techniques for FC analyses should ideally be electrically neutral to avoid contamination from the other signals of interest (Chella et al., 2016; Cohen, 2014; van Diessen et al., 2015). Typically, this is achieved by re-referencing the EEG data to an alternative reference scheme offline, by computing a virtual reference electrode (for example, the common average reference from all other electrodes) then subtracting the signal in the virtual reference electrode from all other electrodes. However, several re-referencing

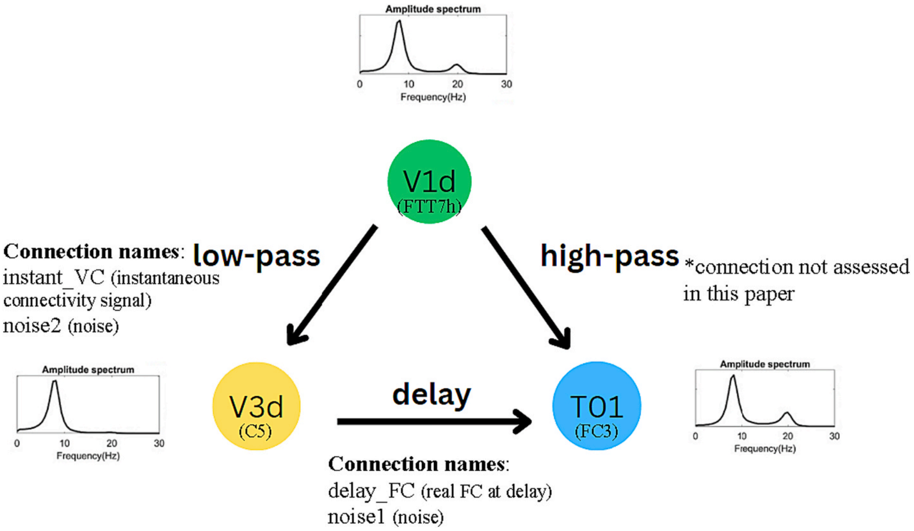

Fig. 1. Visual depiction of ROIs generated for analysis. Note. This figure demonstrates the simulated interaction between the three ROIs and includes the simulated amplitude spectrum at each node, as well as the corresponding electrodes in the EGI Sensor Net array and the 10–20 system electrode, and the names allocated to each connectivity node. Both noise only data and an FC signal (to be combined with the noise data) were generated at each node. Amplitude spectrum diagrams were adapted from the original EEGSourceSim toolbox paper (Barzegaran et al., 2019; J Neurosci Methods).

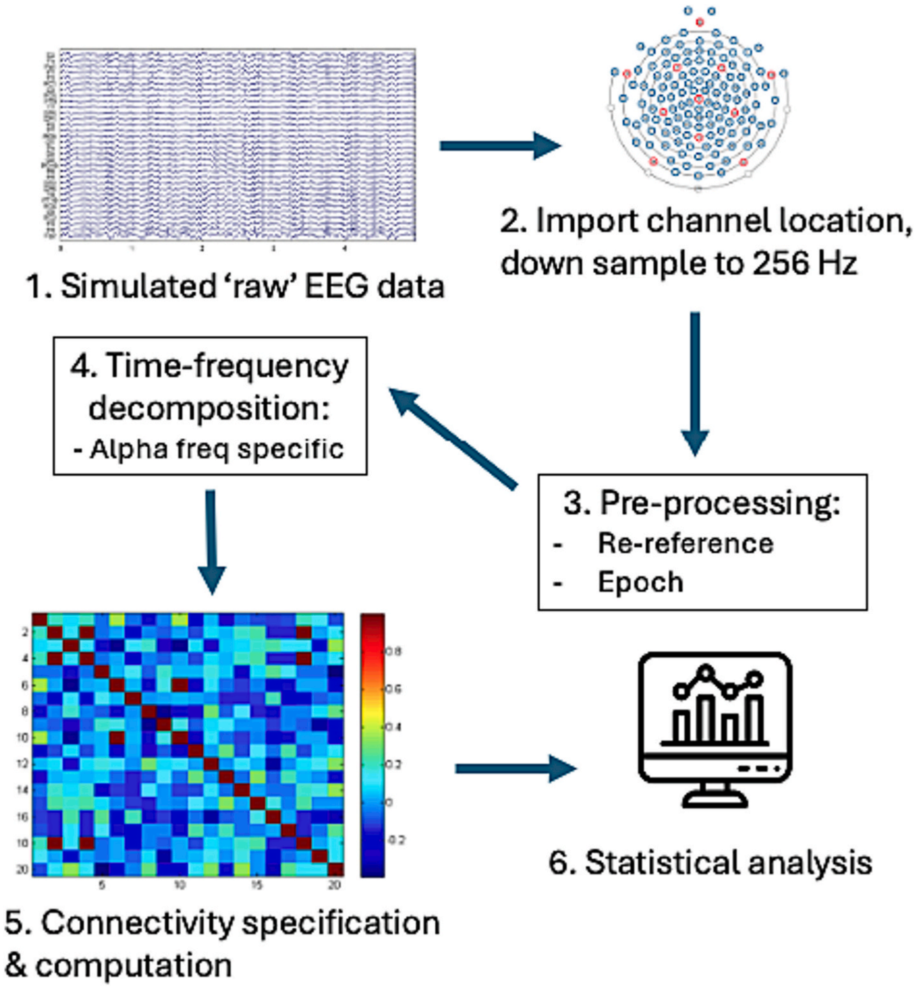

Fig. 2. Illustration of EEG data pre-processing to analysis process. *Note.* Freq = frequency. Figure above depicts the EEG FC analysis pipeline where recorded raw data is first opened and EEG channel locations for the number of channels recorded are imported. Next, the data is down-sampled to 256 Hz for further analysis to reduce size and computational load. This is followed by the implementation of several pre-processing steps including re-referencing and segmenting the data from the continuous recording into epochs. Data were not assessed for or cleaned of artifacts because the data were simulated. Next, the data was subject to a time–frequency decomposition and alpha frequency oscillations. Connectivity analyses were performed to extract alpha frequency connectivity (8–12 Hz) and a connectivity map for 128 × 128 electrodes was generated, followed by the extraction of electrode pairs of interest, which were then subject to statistical analysis.

techniques exist which achieve varying levels of electrical neutrality based on the effects they have on the data (Chella et al., 2016; van Diessen et al., 2015; Lei & Liao, 2017; Lepage et al., 2014; Qin et al., 2010; Yao, 2001). In this study, we investigated the effects of three different re-referencing techniques on the EEG FC metric: (1) CAR; (2) REST; and (3) CSD. See Supplementary Materials for a detailed description of each re-referencing technique.

2.2.2. *Epoching: Length and number of trials*

The present study tested a range of epoch lengths, epoch numbers, and epoch length-number interactions to discern which parameter settings produce the most accurate and reliable connectivity estimates for the alpha EEG frequencies. Epoch lengths included: 2 s, 4 s, 6 s, and 8 s. Epoch numbers included: 20, 40, and *'max*'. Max epoch number was different for every epoch length, as the maximum number of epochs that could be produced from simulated data of a fixed length depended on the epoch length (2 s = 150 epochs, 4 s = 80 epochs, 6 s = 50 epochs, and for 8 s the max was 40 epochs). A fixed total length of data was used (320 s), and this total length was epoched so that all epochs for comparisons were extracted from the same data, ensuring that no random bias could be introduced from the selection of epochs. However, since the conditions with lower epoch numbers required selection of a sub-set of the total EEG data, a random selection of epochs was obtained from the fixed data length for comparison per epoch length (i.e., 20 epochs were randomly chosen from the 150 available 2 s epochs, and then a new set of 40 epochs was randomly chosen from the 150 available 2 s epochs).

2.2.3. *EEG FC metrics*

All FC metrics used in this study were estimates of functional, *non-directed* connectivity and the metrics included in our study were: rMSC, iCOH, and wPLI. These metrics were chosen as they appear to be the most commonly used metrics in the EEG literature assessing FC changes in depression (Miljevic et al., 2023). Note, all metrics were calculated based on specific frequencies, which required the EEG signal to be first transformed into the time–frequency domain using a Fast Fourier Transform. This allowed for the estimation of the power spectra of the signals at each time and frequency point, and provided the characterisation of between signal relationships required for FC estimation. As such, prior to the FC computation, a Fast Fourier Transformation (FFT) was used to transform the data into the frequency-domain and allow for the extraction and analysis of signals at the alpha frequency (8 Hz). This was performed individually for each data type. For more detailed definitions of each of the FC metrics and their techniques, please refer to Supplementary Materials.

All FC metrics provide a measure of the degree of FC between two

EEG signals, with values ranging from 0 to 1. A value closer to 1 indicates higher FC, while a value closer to 0 indicates lower (or no) FC.

2.3. Data analysis

We tested a total of 108 different combinations of FC estimation pipelines (per electrode pair and data type). Fig. 3 depicts the different combinations of parameters at each of the pre-processing steps. As described in our methods, there were three primary ROIs: FC3, C5, and FTT7h, resulting in four FC pairings for comparison at the alpha frequency: *delay_FC* (which included FC connection at a delay), *instant_VC* (which included an instantaneous FC connection), *noise1* (which included only the noise component of the data from which the delayed connection was generated in the *delay_FC* conditions) and *noise2* (which included only the noise component of the data from which the instantaneous connection was generated).

A total of four repeated-measures ANOVAs (rmANOVA) were conducted per signal testing all possible combinations of EEG (pre-)processing steps. Multiple comparison controls were implemented by way of Holm's post hoc test for any significant interactions identified (Holm, 1979). All statistical analyses were conducted using JASP software version 0.17.1.0 (https://jasp-stats.org/). Controls were not applied for experiment-wise multiple comparisons, because it was more important to provide sensitivity to differences in pipelines than to protect against false positives, an approach supported by the literature when exploring a wide range of EEG methods to determine the best approach (Bender & Lange, 2001).

3. Results

A total of 576 comparisons were conducted in the present study, resulting from 144 different combinations of FC estimation pipelines (see Fig. 3) across the four comparison signals. All simulated pairs and data types were evaluated to obtain an accurate understanding of the FC estimation pipelines and pre-processing steps, and how they measure real FC and control for volume conduction FC. 'High performing' pipelines or steps are those that provide high FC estimate values for the simulated data with a delayed connection (*delay_FC*) but low FC for the instantaneous connection (*instant_VC*) and the two noise signals (i.e., *noise1* and *noise2*). We have also presented the two *instant_VC* signals to demonstrate how the steps/pipelines perform in the absence of any connections (i.e., a true 'control' condition).

The following sections (Sections 3.1–3.4) report the best performing parameters at each of the individual pre-processing steps (i.e., best re-referencing methods, or best epoch length, etc.). The final section in our results (Section 3.5) provides a summary of the overall, combined findings, when considering both the results observed for each of the individual pre-processing steps and the results of the interaction effects (for example, the best combination of re-refencing methods and epoch lengths, or the best combination of epoch length and epoch number).

3.1. Re-referencing choice

Significant differences between the re-referencing conditions (averaged across other conditions) were observed in the tests of the *instant_VC* signal, and the two noise signals (all $p < 0.001$). All re-referencing techniques provided higher FC values for conditions where connectivity signals were simulated as compared to conditions where only noise was simulated. However, no main effect differences were observed between the instantaneous volume conducted signals and the *delay FC* signal for any of the re-refencing techniques, suggesting that across the re-referencing montages were not able to differentiate between FC with a delay and volume conduction. Thus, re-referencing montages needs to be considered in combination with the choices made in other pre-processing steps. Fig. 4 depicts condition means and details the post-hoc test results for between condition comparisons.

The use of the CAR and REST re-referencing approaches appeared to lead to consistently higher FC values in signals where both instantaneous connections (instant_VC) and the physiologically plausible delayed FC was present (delay_FC), and lower FC in the noise only signals. Specifically, these differences between the CAR versus CSD referencing approaches, and REST versus CAR referencing approaches were statistically significant at $p < 0.001$ in the *delay_FC* signal (where the CAR re-referencing approach provided higher FC values than the REST re-referencing approach), the *noise1* (where the CSD re-referencing approach provided higher FC values than the CAR re-referencing approach), and *noise2* signals (where the REST re-referencing approach provided higher FC values than the CSD re-referencing approach). Overall, CAR and REST appeared to perform equally to each other, but better than rMSC.

3.2. Epoch length

Significant differences between the different epoch length conditions

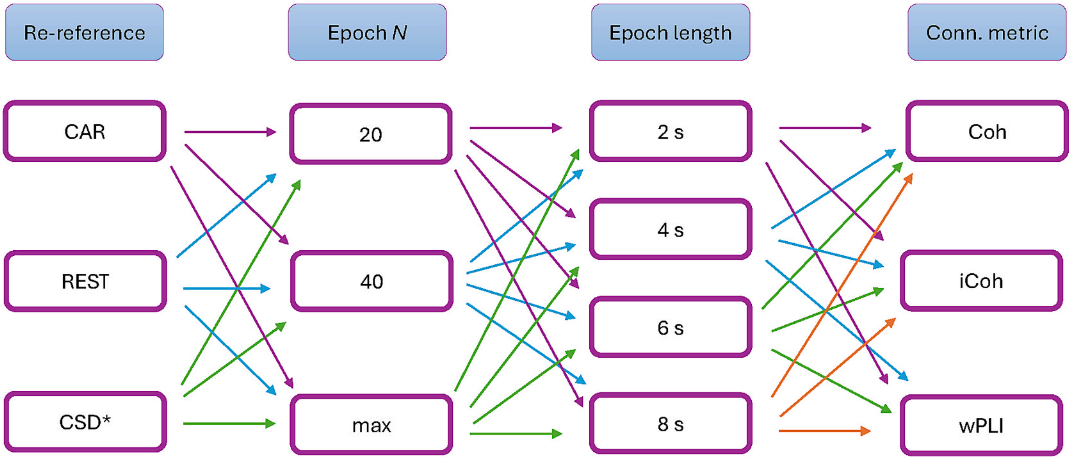

Fig. 3. *Depiction of pipeline processing steps used in the current study.*Note. *Technically, the surface Laplacian is a spatial filter and a reference free method of assessing EEG. For simplicity it has been included in the "re-referencing" step in this figure (and included in comparisons between the different re-referencing approaches). When all individual pre-processing pathways are accounted for, there were 144 comparisons to be run across the different combinations of processing steps. Acronyms: CAR = Common Average Reference; CSD = Current Source Density; Coh = Coherence; conn. = connectivity; iCoh = imaginary Coh; Epoch N = number of epochs; REST = Reference Electrode Standardisation Technique; max = the maximum number of epochs that could be generated for the given interval; s = seconds; and wPLI = weighted phase lag index.

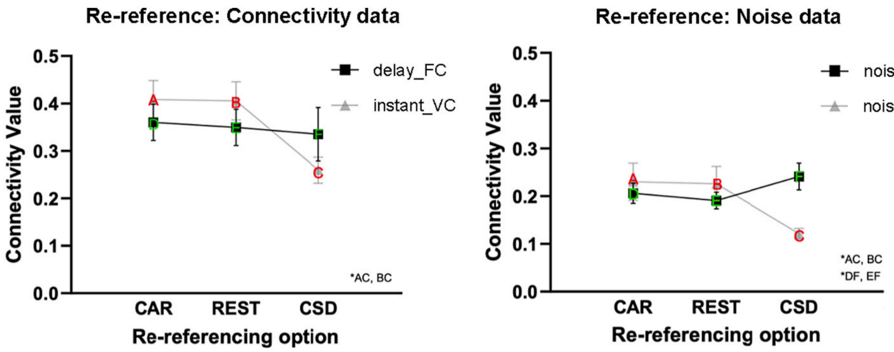

Fig. 4. *Depiction of mean alpha frequency FC (y-axis) obtained using each of the three re-referencing options (x-axis), averaged across all parameter settings for non-re-referencing steps.Note.* Threshold for significance was set at $p < 0.05$. All points in the graph have been labelled with letters (A to F) to enable the use of the * symbol to identify conditions that show significant differences in our post-hoc tests (i.e., for noise1, points B and C significantly differed). These plots demonstrate how the use of the CSD re-referencing approach improves FC estimation, enabling FC values that are higher in the case of FC with a delay, and lower for instantaneous volume conducted connectivity (simulated data with instantaneous connectivity between the nodes that is indicative of volume conduction). However, it is worth noting that these results are averaged across other conditions, and so are not indicative of potential interactions between the re-referencing approach and the FC estimation algorithm (where CAR and REST show good performance when the wPLI and iCOH algorithms are used). *Abbreviations:* CAR = common average reference; REST = Reference Electrode Standardisation Technique; CSD = current source density.

(averaged across all other parameter settings) were observed only in the *delay_FC* signal at a $p < 0.001$. There were no statistically significant differences in any of the volume conducted signals or the *instant_VC* signal. Fig. 5 depicts the means for each of the three re-referencing options used and details the post-hoc test results for between condition comparisons.

Overall, 8 s epochs showed significantly higher FC estimates for the delay_FC signal compared to 2–6 s epochs (at $p < 0.01$), and thus 8 s epochs were found to perform the best at identifying delayed FC while simultaneously providing low estimates for conditions where no physiologically plausible FC was simulated (i.e., in the volume conduction conditions).

3.3. *Epoch number*

Statistically significant differences were observed when comparing the effects of different choices of epoch numbers on FC across all four rmANOVAs and the signals assessed (all $p < 0.05$). See Fig. 6 for a visual depiction of the means for each of the three re-referencing conditions and for details of the post-hoc test results for between condition comparisons.

There were statistically significant differences in the FC estimates provided by the different epoch number condition (all $p < 0.05$). However, while the 20 epoch condition appeared to provide the highest FC for conditions where delay_FC connections were simulated, the 20 epoch condition also provided the highest FC estimates in the volume conduction conditions. As such, we would recommend the use of 40 or more epochs to robustly estimate EEG-FC at the alpha frequency, as our results showed that 40 or more epochs provided sufficient detection of plausible FC, while also protecting against the inflation of FC estimates by volume conduction.

3.4. *EEG FC metric*

All rmANOVAs for all data types showed statistically significant differences between the FC metric used to estimate FC for all signal pairs (all $p < 0.001$). See Fig. 7 for a visual depiction of the means for each of the three re-referencing options used and for details of the post-hoc test results for between condition comparisons.

When comparing the various FC metrics in isolation (averaged across the other epoch length, epoch number, and re-referencing conditions), rMSC appears to be the worst performing method for estimating FC, as it provided high FC estimates for both delay (FC) and instantaneous signals, as well as providing high FC estimates in the two noise only conditions. However, we note that the selection of FC algorithm interacted with the re-referencing condition (reported in a following section), so that rMSC can still provide utility when combined with the correct re-referencing montage. Next, iCOH provided low FC values from the two noise signals, provided the highest FC values out of all the algorithms when identifying delayed FC and did not identify high FC in the instantaneous volume conducted signal. This robustness against volume conducted FC is likely due to iCOH begin designed specifically to ignore

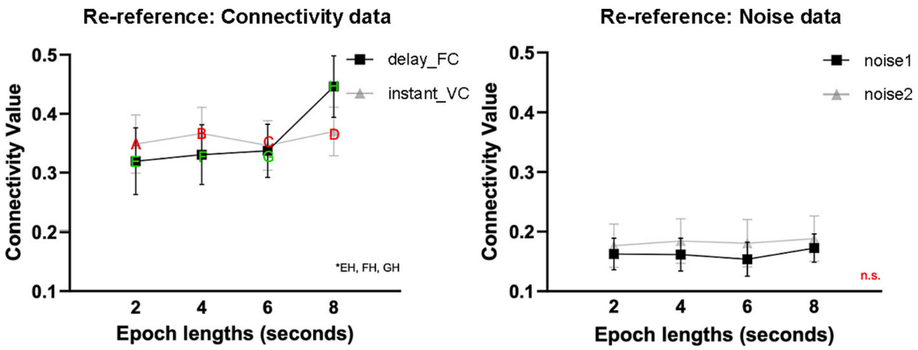

Fig. 5. *Depiction of mean FC (y-axis) for each of the four epoch length options (x-axis) in the alpha frequency for all data types, averaged across all parameter settings for non-epoching steps.Note.* The threshold for significance was set at $p < 0.05$. All points in the graph have been labelled with letters (A–H) to enable the use of the * symbol to identify conditions that show significant differences in our post-hoc tests. The plot on the left indicates that 8 s epochs provide higher FC values for conditions where delay_FC was stimulated (a connection that represents physiologically plausible FC that is not influenced by VC). *Abbreviations:* n.s. = non-significant.

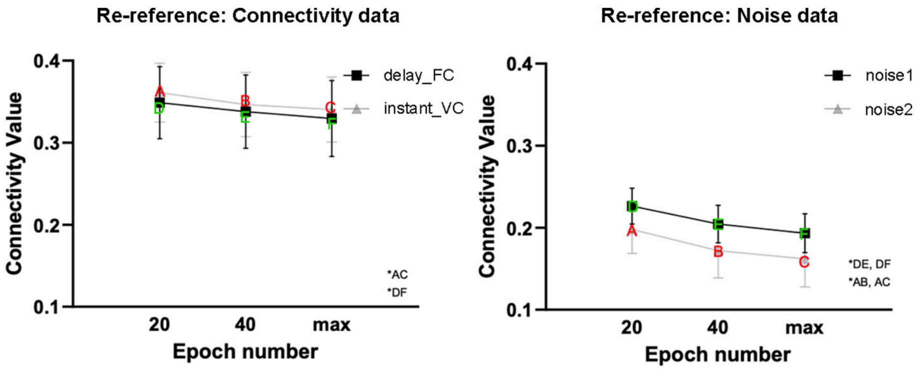

Fig. 6. *Depiction of mean alpha frequency FC (y-axis) for each of the three epoch trial number options (x-axis) for each of the data types, averaged across all parameter settings for non-epoching steps. Note.* The threshold for significance was set at $p < 0.05$. All points in the graph have been labelled with letters (A to F) to enable the use of the * symbol to identify conditions that show significant differences in our post-hoc tests. These results indicate that conditions including 20 epochs provide higher FC values, but this is the case for both delay_FC and instant_VC, as well as when only noise was simulated. As such, it is not clear that 20 epochs provides better FC estimation performance. However, we note that these findings are averaged across other conditions (e.g. re-referencing approach and FC algorithm) and that the number of epochs seemed to interact with other conditions such that recommendations could be made about an optimal number of epochs (see Fig. 8). *Abbreviations:* E20 = 20 epochs, E40 = 40 epochs, max = maximum number of epochs that were generated. “Max” epochs for 2 s = 150, “Max” epochs for 4 s = 80, “Max” epochs for 6 s = 50, and “Max” epochs for 8 s = 45.

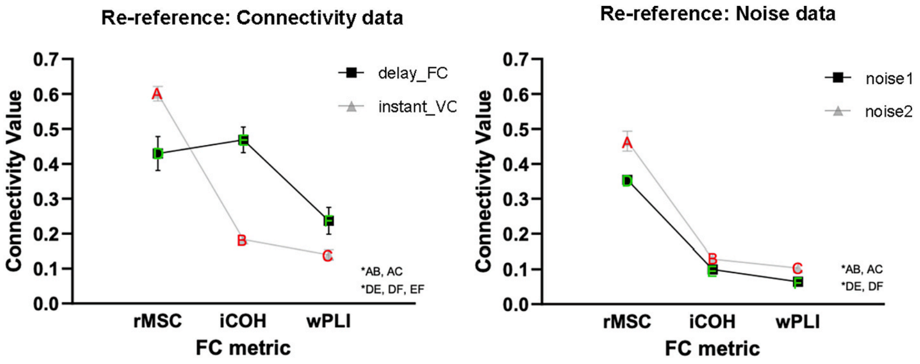

Fig. 7. *Depiction of mean alpha frequency FC (y-axis) for each of the three EEG-FC metrics (x-axis) for each of the data types, averaged across all parameter settings for non-FC metric steps.Note.* Threshold for significance was set at $p < 0.05$. All points in the graph have been labelled with letters (A to F) to enable the use of the * symbol to identify conditions that show significant differences in our post-hoc tests. Overall, these figures indicate that iCOH provides the best separation of FC estimates between physiologically plausible simulated FC (delay_FC) and conditions where only noise is simulated or only volume conducted FC is simulated. However, we note that these results are averaged across the other conditions, and interactions between the different analytic choices are worth accounting for (see Fig. 8). *Abbreviations:* FC = functional connectivity; rMSC = real magnetic squared coherence (or coherence); iCOH = imaginary coherence; wPLI = weighted phase lag index.

this instantaneous FC. Interestingly, while wPLI provided low FC values in the two volume conducted signals (lower than iCOH), it provided similar FC values across both the delayed and instantaneous FC signals, but higher FC values from the delayed signal. Overall, iCOH and wPLI appeared to provide the highest performance at identifying real FC when data were averaged across the other pre-processing conditions.

3.5. Interaction effects and the highest performing overall pipeline

There were several significant interaction effects that were observed across the four rmANOVAs. For the sake of brevity, a summary of the best performing parameter settings for each of the many interactions is provided in Table 1, and the full report of the key findings can be found in the supporting Supplementary Materials.

Overall, the results of our explorations of interactions between different EEG (pre-)processing parameters suggested that epoch lengths of ≥6 s with ≥40 epochs and application of the CAR or REST reference technique with the iCOH FC estimation method provided the best performance at identifying delayed alpha EEG FC while also providing low FC estimates when only volume conduction or noise connections were simulated. Alternatively, use of the rMSC FC estimation method is acceptable, though we note that it is critical to use the CSD referencing approach when using the rMSC metric. These steps in an overall pipeline were also less likely to estimate high FC values from simulations that only contained noise and no FC. Figs. 8A and 8B further presents the means for the top 20 performing combination pipelines for both the *delay_FC* and the *instant_VC* signals (see Supplementary Materials 2 for full graph).

4. Discussion

The aim of this paper was to assess the difference in connectivity estimates produced by multiple EEG pre-processing steps (as identified in Miljevic et al., 2022) to validate a pipeline for the assessment of EEG FC, primarily for the alpha frequency. We utilised the EEGSourceSim toolbox to generate complex EEG FC data where the ground truth of the connections is known, with four simulated connectivity (or non-connectivity) signals: (1) where physiologically plausible alpha FC was generated with a delay, (2) where instantaneous alpha frequency signals were generated to reflect volume conduction, (3) a noise only condition (extracted from the signal generated with a delayed transmission but without the delayed FC signal being included) FC, (4) a noise only condition (extracted from the signal with the instantaneous volume conducted signal transmission, but without the instantaneous signal

Table 1

Summary table depicting the top performing* combinations at each of the rmANOVA interactions.

INTERACTION	TOP RESULTS FOR EACH INTERACTION
Epoch length * epoch number	8 s * any epoch number
Re-reference * Epoch length	CAR * 4 or 6 s
Re-reference * Epoch number	40 + epochs * REST
Epoch length * Metric	2–8 s * iCOH
Metric * Epoch number	Any epoch number * wPLI
Re-reference * Metric	CSD * rMSC, andCAR or REST * iCOH
Epoch length * Re-reference * Epoch number	8 s * any re-refence * 40 + epochs
Epoch length * epoch number * metric	8 or 6 s * 40 + epochs * wPLI or iCOH
Epoch length * Re-reference * Metric	4–8 s * REST * iCOH or wPLI, and 4–8 s * CSD * rMSC
Epoch number * re-reference * metric	40 + epochs * REST * iCOH, and 40 + epochs * CSD * rMSC

Note: * "Top performing" pipelines were determined as the combination of each parameter across the different pre-processing steps that consistently demonstrated highest FC in the delay_FC node and lowest FC in the insta_FC node, and the two VC nodes within each of the interaction tests. *Abbreviations:* * = and (i.e., CSD and rMSC); s = seconds; CAR = common average reference; REST = Reference Electrode Standardisation Technique; iCOH = imaginary coherence; wPLI = weighted phase lag index; CSD = current source density; rMSC = magnitude squared coherence.

being included).

Using these simulated data, our findings indicated that the following combination of pre-processing decisions produced the best outcomes in terms of correctly identifying true FC and minimising the detection of spurious FC in the alpha frequency: (1) use of the iCOH FC estimation metric after applying CAR re-referencing with 40 or more epochs of 6 s duration; (2) use of the wPLI metric after applying CAR re-referencing with 40 or more epochs of 8 s duration; (3) use of the rMSC metric after applying CSD re-referencing with 40 or more epochs of 6–8 s duration.

4.1. High performing re-referencing techniques

The results of this study indicate that all three referencing options (CAR, REST, and CSD) performed equally when assessing a realistic FC connection with a time delay. However, when assessing simulations that included instantaneous FC connection without a time delay (a condition that should result in a low FC estimate if researchers aim for robustness against volume conduction), CAR and REST appeared to outperform CSD. When combined with other pre-processing steps (i.e., epoch number, length, and FC metric), these results varied further. The largest differences between the approaches appeared to be driven by the combination of different re-referencing techniques with different FC metrics. For example, conditions that applied REST and CAR re-referencing were found to perform equally in alpha FC identification when combined with iCOH and wPLI FC metrics. However, the CSD re-referencing approach performed best when combined with rMSC.

Past literature suggest that CAR and REST generate less EEG signal distortion (which can lead to false FC identification), compared to other methods such as mastoid or nose re-referencing (Cohen, 2015). Indeed, REST has been most consistently found to outperform other referencing techniques (except the robust CAR re-referencing approach; Qin et al., 2010). CSD is believed to mitigate the confounds of volume conduction by localising sources. Here, CSD most substantially improved the performance of rMSC (with rMSC only able to distinguish between FC and spurious connectivity when using CSD) and in some cases, the iCOH metric but not wPLI. This is aligned with previous research, where studies have noted improvements in rMSC and phase-based connectivity estimates where CSD has been applied beforehand (Cohen et al., 2015; Dominguez et al., 2007). Overall, our findings highlight the importance of considering the interaction effects of pre-processing steps such as re-referencing and FC metric use.

It should be noted that we only used one variation of the CSD re-reference technique. However, the algorithm can vary substantially based on user inputs, which can alter study outcomes. We used CSD parameters that are commonly applied in existing literature (and set as the defaults within EEGLAB) as a demonstration of the typical utility of CSD in FC analysis. However, investigations into the optimal CSD parameter specifications for estimation of functional connectivity are needed.

4.2. High performing epoch length and number

As per the results, when assessed alone, the 8 s epoch condition appeared to provide the best performance at identifying alpha FC.

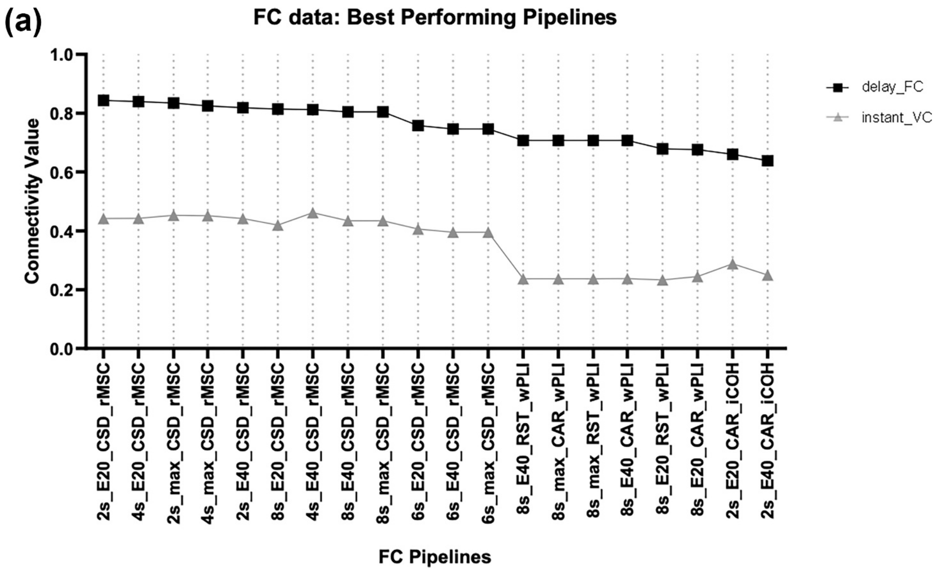

Fig. 8A. A visual summary of mean connectivity estimates (y-axis) for the top 20 performing pipeline (x-axis) in the alpha frequency for the delay_FC and instant_VC simulated data. *Note*. The pipelines are ordered from largest to smallest based on FC values from the delay_FC connection. Pipeline conditions are named using the format: epoch length (i.e., 2 s = two second epochs, 4 s = four second epochs etc.), epoch number (i.e., E20 = 20 epochs, max = maximum epochs that could be generated, "Max" epochs for 4 s = 80, "Max" epochs for 6 s = 50, and "Max" epochs for 8 s = 45).

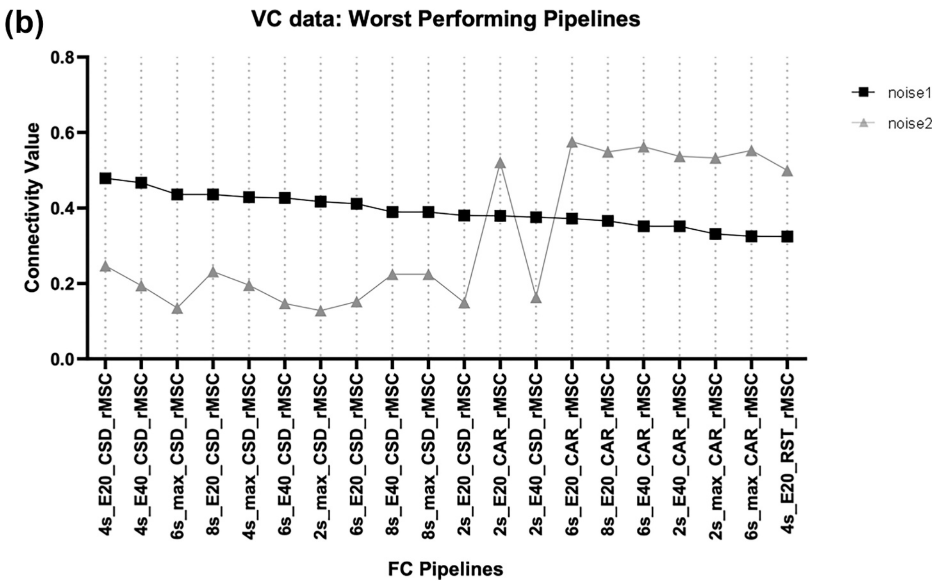

Fig. 8B. (Continued). *Note*. The pipelines are ordered from largest to smallest based on FC values from the noise1 connection. Pipeline conditions are named using the format: epoch length (i.e., 2 s = two second epochs, 4 s = four second epochs, etc.), epoch number (i.e., E20 = 20 epochs, max = maximum epochs that could be generated, “Max” epochs for 4 s = 80, “Max” epochs for 6 s = 50, and “Max” epochs for 8 s = 45).

Further, consistent with our predictions, epoch lengths of 8 and 6 s appeared to perform best at identifying FC when interacting with the other (pre-)processing steps including re-referencing and FC metrics. Meanwhile, the use of 40 or more epochs provided the most consistent identification of physiological plausible alpha FC, while concurrently providing low FC estimates for instantaneous and volume conducted (spurious) FC. When assessed in combination with different epoch number conditions, 8 s epochs with any number of epochs (i.e., 20, 40, and the maximum possible) provided the best performance at identifying time delayed FC. In contrast, the use of higher epoch numbers appeared to perform better when combined with other pre-processing steps (i.e., re-referencing techniques and FC metric). Therefore, the present findings suggest that 6–8 s epochs were best for FC identification, especially when combined with other high performing pre-processing steps including with the use of the wPLI or iCOH FC estimation metrics, with REST or CAR re-referencing approaches, and with the use of ≥ 40 epochs.

These findings are mostly consistent with the limited previous literature that has evaluated the effects of epoch length on FC estimation. For example, Fraschini et al. (2016) observed that 6 s epochs (as compared to 1, 2, 4, 6, 10, 12, 14 and 16 s) were best for FC estimation, especially using amplitude envelope correlation and PLI across alpha and theta frequencies. In contrast, FC estimates based on lower numbers of epochs have been found to bias the results toward false positives (Bastos & Schoffelen, 2016). Indeed, consistent with this observation, our results indicated that the use of 20 epochs provided higher FC estimates from the instantaneous signal and in the noise signals, whereas 40 or more epochs produced high estimates for true connections and low estimates where there were no simulated connections.

When assessing the combined effect of different epoch lengths and numbers of epochs, Haartsen et al. (2020) found that a larger number of shorter epochs (i.e., 2 s) provided more reliability in assessing connectivity in the alpha frequency. However, their study only assessed infant EEGs, where FC may be more complex and certainly more noisy (due to movement artifact) in comparison to adult EEG data. Interestingly, in our data, when examining the interaction between epoch numbers and epoch lengths, our results indicated that pipelines involving fewer 2 s epochs and more 8 s epochs generally identified more physiologically plausible FC and less spurious FC. More work is necessary to further delineate and understand the effects of epoch number and trial interactions on FC estimation.

Finally, it should be noted that epoch length, epoch number, and total data length are all related and inseparable, as such we cannot conclude that the effects detected in this study are not due to “total data length” effects rather than epoch length and epoch number interactions. In practice, a decision to estimate alpha frequency FC from ≥40 6 s epochs will necessitate longer total data lengths, which may not be feasible for a given study or study population. We recommendation further investigations into the interaction effects of epoch length and number, especially and frequencies other than alpha.

4.3. *High performing EEG FC metric*

It was expected that wPLI would be the best performing FC metric, as indicated by providing the highest alpha frequency FC estimate where FC was simulated and the lowest FC estimates where only instantaneous/volume conducted, and noise-like signals were simulated. However, when comparing the FC metrics alone, iCOH appeared to perform the best, followed closely by wPLI, while rMSC provided high FC estimates for the simulations that only contained volume conducted signals (i.e. did not contain physiologically plausible FC). As mentioned above, CSD re-referencing improved rMSC FC estimation, while both wPLI and iCOH performed the best when combined with REST (and sometimes CAR when combined with other parameter settings). When it came to the combination of all pre-processing steps, the following combinations performed the best at identifying realistic (time-lagged) FC while providing low FC estimates for conditions where FC was not physiologically plausible: (1) Use of the wPLI metric with more than 40 epochs of 8 s length and the CAR re-referencing approach, (2) Use of the iCOH metric with more than 40 epochs of 2 s length and the CAR re-referencing approach, and (3) Use of the rMSC metric with 40 epochs of 2 s length and CSD re-referencing approach.

The existing literature also suggests that the iCOH metric may demonstrate superior results to wPLI (Sanchez-Bornot et al., 2018). Specifically, Sanchez-Bornot et al. (2018) propose that, in some scenarios, iCOH outperforms other measures, including wPLI, in terms of capturing true interactions even when zero phase interactions are present, whereas wPLI relies solely on phase differences and the consistency of non-zero phase differences across epochs. However, the authors note that the important choice between the use of the iCOH and wPLI FC metrics depends on the specific characteristics of the data and the research questions being addressed. For example, if the primary interest is in capturing phase differences and the consistency of these differences across trials, wPLI might be a suitable choice. However, to avoid missing interactions between signals that are closer to zero or π-phase, iCOH may capture FC more accurately without biasing away from to zero or π-phase (Haufe et al., 2013; Vinck et al., 2011).

Further, while both wPLI and iCOH typically performed better than rMSC, when rMSC was combined with CSD it appeared to outperform the other pipelines. Indeed, CSD has been found to outperform the conventional EEG re-referencing measures, especially for phase-relationships between electrodes assessed in the scalp-space (Tenke & Kayser, 2015). Thus, the combination of rMSC with CSD re-referencing may provide performance that is equal to the combination of iCOH or wPLI with REST or CAR re-referencing.

Several previous works have aimed to compare the performance of different EEG FC metrics to identify the 'highest performing choice'. Our results suggest that that iCOH and wPLI are less confounded by noise and other extraneous factors that might inflate spurious FC estimation, but also that when rMSC is combined with CSD it provides robustness against the effects of volume conduction (Sanchez-Bornot et al., 2018; Stam et al., 2008; Vinck et al., 2011). It may be that particular FC metrics are more suitable in the context of specific data characteristics. For example, in situations where the data are particularly noisy, iCOH has been suggested to be more robust and less sensitive to certain types of noise compared to wPLI (Sanchez-Bornot et al., 2018). Preliminary research has suggested that iCOH is less sensitive to reference effects, while wPLI is more robust to zero-phase interactions (Nolte et al. 2004; Sanchez-Bornot et al., 2018; Stam et al., 2008; Vinck et al., 2011).

It is also worth noting that the variability in FC estimates is further complicated by decisions about whether to estimate FC within the sensor-space or source-space. In this study, we measure only the effects of FC metrics and pre-processing steps on the sensor-space and our results may not apply to the source-space, thus further investigation of the influence of pre-processing steps on source-space FC estimates is warranted.

Although our results have indicated which approaches should not be used due to the risk of spurious false positive connectivity (i.e., combining rMSC with CAR or REST re-referencing techniques), overall, there is unlikely to be a single "best" FC metric for analysing EEG data. The choice of metric likely depends on the specific research question, methodology, and data being analysed (as outlined above and in Miljevic et al. [2022]). It is important to carefully consider the advantages and limitations of different FC metrics and choose the metric(s) that are most appropriate for the specific research question, with the most appropriate combination of pre-processing steps. For example, studies that assess the nature of FC alterations related to specific diseases may prefer to use the iCOH metric, as it captures interactions between signals beyond the phase relationships and their consistency that are measured by wPLI. In diseases where abnormal directional connectivity plays a role, iCOH could provide more meaningful insights as it is more sensitive to near-but-non-zero or non-π phase differences and is able to provide directional information. In contrast, wPLI is less sensitive to near-zero-phase interactions and is designed to be less affected by volume conduction. Alternatively, when assessing EEG FC in relation to cognition, it may be that certain processes under investigation exhibit specific phase relationships for which wPLI and iCOH might be more suitable. In contrast, rMSC combined with CSD re-referencing might prove useful in situations where researchers are interested in measuring connectivity in power fluctuations, while still controlling for the effects of volume conduction.

4.4. Implications, and future directions

There are several implications that may result from the findings of this study, as well avenues for further investigation. First, due to limitations of the EEGSourceSim toolbox and computer processing capacity, we were unable to generate longer data segments (>5 min) and thus more epochs. As such, we do not present an exhaustive review of how variations in the number of epochs affect FC estimates, and how this interacts with other pre-processing parameters (e.g. epoch length) to influence outcomes. Future research should focus on further disentangling the effects of epoch length and number on FC assessments to provide a better understanding as to these parameters may influence results. Specifically, larger epoch numbers (i.e., longer EEG recording segments) may be ideal to investigate changes in FC across time and may provide more reliable and consistent estimates, so longer EEG recording lengths should be explored in future research. Additionally, researchers could further examine which FC metrics may work best for certain frequencies, conditions, questions, or data types (i.e., noisy versus clean data, source-space versus sensor-space data, child versus adult EEGs, typical versus neurological or psychiatric populations).

Indeed, more work is needed to consolidate the findings of all the identified 'highest performing' pre-processing steps and how they capture the complex interplay of differing brain dynamics. To enable this exploration, larger and more complex simulations of brain networks are needed, with more than just one true FC signal and more than one frequency of interest. Further the length of connections (short- or long-range) and how connection lengths relate to pre-processing steps and FC assessment needs more investigation, as well as potentially the influence of varying time delays.

We further acknowledge the fact that our results are based on FC within the alpha frequency, and investigation of other frequency bands is needed. We also acknowledge the limitations of the sensor-space EEG FC approach which reduces our ability to control signal leakage, signal mixing and other volume conduction effects. While signals for this study were simulated, the relationships between connected brain regions in real data are complex, and more investigation is needed. Future research of a similar nature (i.e., evaluation of the best pre-processing steps) could focus on optimising pre-processing steps for connectivity estimates in the source-space.

Nevertheless, sensor-space FC appears to demonstrate effects of interest in the literature relating to the study of normal and 'diseased' brain activity (Leuchter et al., 2012; Mumtaz et al., 2018). Thus, the improvement of techniques applied to sensor-space FC can only serve to better our understanding of its application.

5. Conclusion

This study aimed to assess the differences between multiple EEG pre-processing steps to validate the best performing steps for the assessment of alpha frequency FC. Pre-processing decisions have a significant impact on FC estimation and there is a need to establish best-practice guidelines for future EEG FC research. We employed the use of simulated EEG FC data where the ground truth of connectivity can be known. Our results highlight how decisions regarding pre-processing steps can alter the outcomes of analyses, specifically how assessment of alpha FC can be optimised by using specific combinations of pre-processing steps. Our results indicated that the following combinations of pre-processing steps appeared best for assessing alpha FC: 1) 40 or more epochs of 6 s epoch length, using the iCOH FC metric and CAR re-referencing montage, or 2) 8 s epochs using the wPLI FC metric and CAR re-referencing montage, or 3) 6–8 s epochs using the rMSC FC metric with the CSD re-referencing montage. We hope these findings will help to inform the development of pre-processing plans in future studies of EEG FC. We recommend more work to consolidate these findings and further our understand to eventually develop a standardised, best-practice pipeline for FC identification at all EEG frequencies. Overall, we present an initial and promising account of the highest performing re-referencing and epoching choices for robust EEG FC assessment.

6. Authorship confirmation & contribution statement

All who meet authorship criteria are listed as authors, and all certify that they have participated sufficiently in the work to take public responsibility for the content. With AM contributing to conceptualization, design, literature search and study inclusion/exclusion, writing – preparation, creation, writing – editing, and revision; OWM and PBF contribution to the writing – editing, and revision; and NWB to conceptualization, design, writing – editing, and revision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinph.2025.03.043.

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