

RS-FetMRI Semi-Automatic Pipeline

USER MANUAL



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Summary

Resting-state functional magnetic resonance imaging (rs-fMRI) has most recently proved to open a measureless window regarding functional neurodevelopment in utero. The development of the Fetal resting state functional MRI (**RS-FetMRI**) processing pipeline was focused on creating an effective, user-friendly and easy to use package that is completely integrated in SPM (Statistical Parametric Mapping). This processing pipeline is suitable for both single subject and group-based analyses and can be used for both 1.5T and 3T scanner acquisitions.

The RS-FetMRI is a semi-automatic and standardized pipeline composed of six (M1 to M6) modules tailored for processing fetal resting-state functional MRI (Fetal rs-fMRI) Nifti data. The first three modules, from M1 to M3, work *Within Session* (WS) while the last four, from M4 to M6, work *Between Session* (BS).

The RS-FetMR is capable of 1) detecting and correcting fetal-specific motion effects and signal intensity changes, especially through 2) the accuracy of time-series spatial normalization to a standardized gestational-week specific fetal template space via the synergetic action of each module. Furthermore, the whole processing procedure does not need a structural fetal scan, which can be difficult to acquire/process. This RS-FetMRI protocol is suitable for a large pool of users, from beginners to experts, although a basic technical knowledge of fetal functional image processing is required. In this manuscript, there will be a detailed explanation of how to deal with this pipeline.

For further information of the theoretical background, we strongly suggest reading the corresponding article (doi: [XXX](#)).

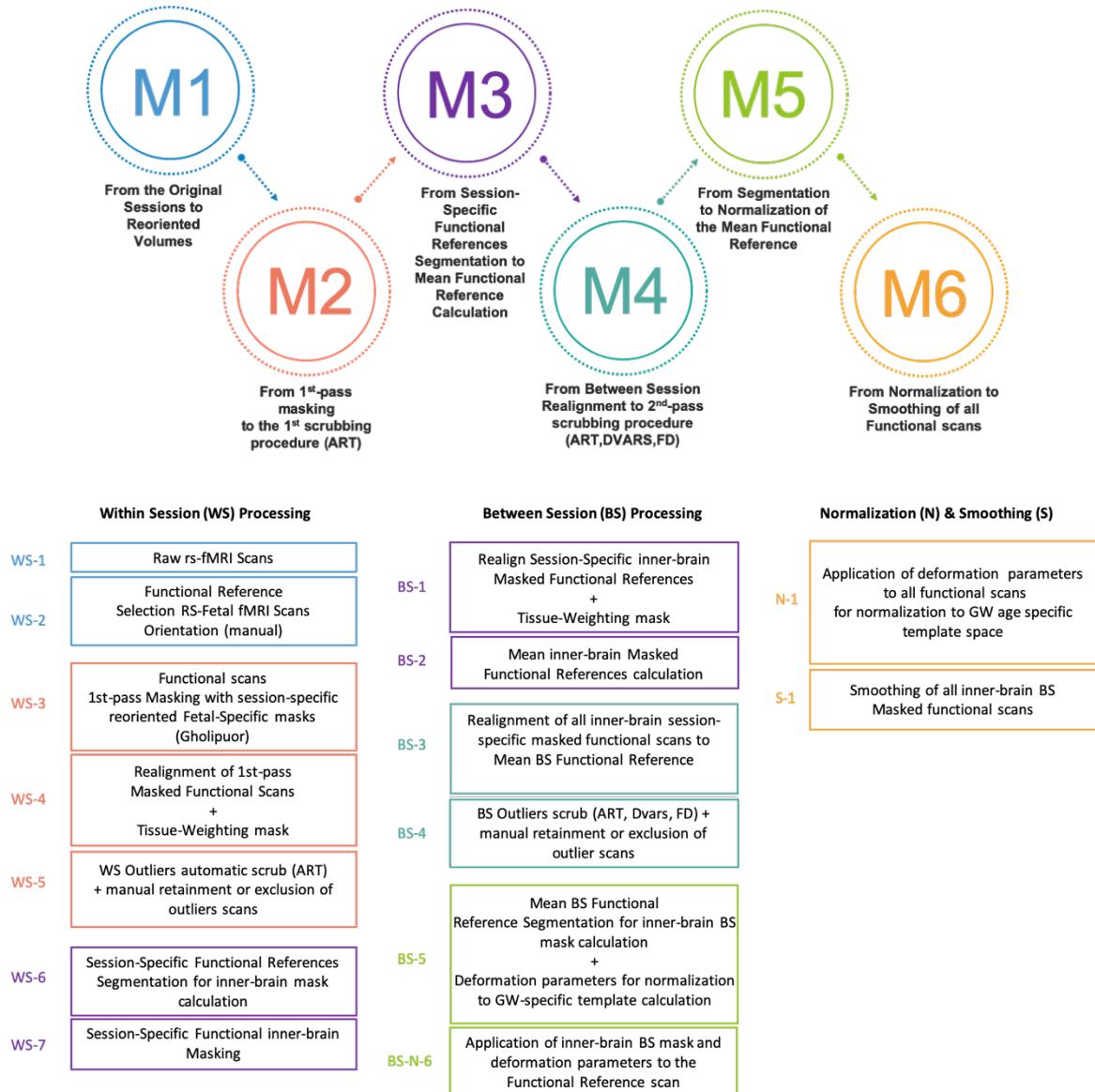


Figure 1: Flowchart of RS-FetMRI of module 1.

RS-FetMRI Usage

The entire RS-FetMRI package is composed of a main script, called '*RSFetfMRI.m*', which is subdivided into six modules (Figure 1), a MATLAB custom-built function '*Create_template.m*' and an automatic modified version of the Artifact Detection Tool (https://www.nitrc.org/projects/artifact_detect) called '*art.m*' accompanied with relative configuration files. The RS-FetMRI script uses a wide range of SPM functions for image processing and image visualization. It also contains a folder called '*Templates*' that includes three subfolders ('*Template_for_session*', '*Template_Priors_Seg*' and '*Template_orig*') containing different Nifti files useful for 1st-pass masking, segmentation and visualization. In particular, as explained in the 'Installation and Requirements' paragraph, the '*Template_orig*' files need to be downloaded from a different website.

Installation and Requirements

The RS-FetMRI package can be downloaded from the open-source repository on GitHub (<https://github.com/NicoloPecco/RS-FetMRI>). It can also be found in the SPM repository toolboxes (...). The RS-FetMRI package can be used on any computer operating system where Matlab2013 (or above) and SPM12 (or above) have been installed. **The user must download the CRL Fetal Brain Atlas images** – GW 21 through 37 – from <http://crl.med.harvard.edu/research/fetal_brain_atlas/> and then rename all of the files as demonstrated in the example for a 21st Gestational Week (GW) below:

Fetal Brain Atlas weeks 21 → STA21.nii

All of the 'STA*.nii' files must be placed inside the subfolder '*Template_orig*' which is found within the '*Templates*' folder.

Initialization

Enter the '*art*' folder within the '*RS-FetMRI*' package. This folder contains **three configuration files** ('*.cfg*'). Within each of the configuration file, **three paths** ('*image_dir*', '*motion_dir*' and '*mask_file*') need to be modified before any analysis can be exploited (**See supplementary Initialization**).

If the ART toolbox was previously installed, it should be removed from the MATLAB paths and then updated with the new script path instead.

Once the configuration files were modified, open Matlab software and **set the current folder** within the '*RS-FetMRI*' folder and press the run button.

Test Set

The RS-FetMRI package also includes a complete Test Set. The subject under examination was at the 35th GW. All of the images present in this manual were selected from this subject.

The '*TestSet*' folder can be downloaded from the open-source repository in GitHub (<https://github.com/NicoloPecco/RS-FetMRI>). Due to repository memory size, only the first two session of this subject were loaded. If the user would like to try the analysis on this subject, they should simply download the material, unzip the '*TestSet*' file and keep the '*RS-FetMRI*' first folder ('*M1_PP_01_OrigVol*') and move the others in a different repository. Please note that initialization is required to try the TestSet.

All of the parameters inserted during the processing are reported in the **supplementary Test Set parameters**.

Implementation of the Six Modules

In this section, a detailed explanation of each module, accompanied by flowcharts and images, is reported. All of the images that are presented in this manual were selected from a 35 GW subject. The layout and description of each module will follow the structure of Figure 1.

M1: From the Original Sessions to Reoriented Volumes

Folders: M1_PP_01_OrigVol, M1_PP_02_4Dto3D, M1_PP_03_Reorient, M1_PP_04_Rename.

WS-1

Once each step of the initialization paragraph has been completed, MATLAB can be opened at the RS-FetMRI directory and the main script ‘*RS-FetMRI.m*’ can be launched. All of the folders that will be useful for the entire processing procedure will be created in the current directory and then followed by an SPM pop-up window (Figure 2) which will ask the user to insert the input files into the ‘*M1_PP_01_OrigVol*’ folder.

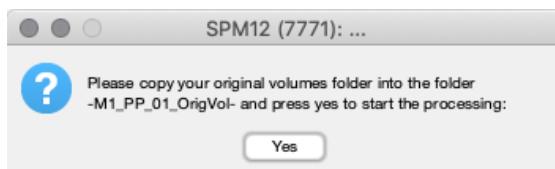


Figure 2: SPM instruction window: insert 4D data file(s) into the ‘*M1_PP_01_OrigVol*’ folder.

The user can insert:

- 1) One folder that contains the 4D Nifti raw file, launching Single Session Analysis;
- 2) Multiple folders, each of which contains a 4D Nifti raw file, launching Multiple Sessions Analysis.

Be aware that the number of 3D fetal rs-fMRI volumes per session in each 4D Nifti file is arbitrary, meaning that the processing pipeline does not have a maximum number of volumes that can be processed per session. Once the volumes are added, the user can press ‘Yes’ and the processing will automatically start. Each 4D-session volume is renamed with 4 digits, starting from ‘1001’, inside the ‘*M1_PP_02_4Dto3D*’ folder, while keeping the original volumes intact. The 4D-volumes are 3D-converted and the single 3D volumes are placed into a session-specific subfolder inside the ‘*M1_PP_02_4Dto3D*’ folder.

WS-2

An SPM pop-up window will give the instructions on how to 1) choose the WS reference volume, 2) reorient the chosen reference volume, and 3) apply the transformation parameters to all of the volumes of the current session (Figure 3A). The SPM check registration function is used to display six images that are equally spaced throughout the session (Figure 4A). The user must choose the WS reference volume for each session by choosing the most representative volume for the current session. When selecting the reference volume, we strongly recommend to avoid selecting a volume where the fetal brain image is cut; at the very least, select a reference image with the least amount of brain tissue missing with respect to the overall session. The user must select one image from the SPM drop-down menu (Figure 3B).

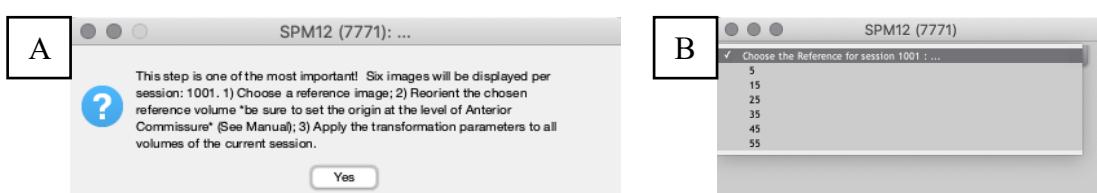


Figure 3: A) SPM instruction window on how to reorient Reference images. B) SPM drop-down menu to select the current reference images. The numbers that can be chosen depend on the number of 3D volumes per session.

Once selected, the SPM window will update, showing only the selected reference volume (Figure 4B). The user must reorient each plane of the chosen reference volume by adjusting the roll (i.e. y-axis), pitch (i.e. z-axis) and yaw (i.e. x-axis) values in the SPM window until the orientation of the reference volume (Figure 4C) matches the standard template image (Figure 5). Once reoriented, and before applying the transformation to all volumes, the user must **set the origin** at the level of the **Anterior Commissure** (AC) (Figure 4C) and then press the ‘Set Origin’ button. The user can apply the transformation matrix to all of the volumes of the relative session by clicking the ‘Reorient...’ button and then selecting the volumes to reorient. When reorienting the first session, it is recommended to apply the reorientation matrix to all sessions, as it is hypothesized that all of the following sessions will need a similar amount of reorientation. This can save a lot of time when reorienting the following sessions.

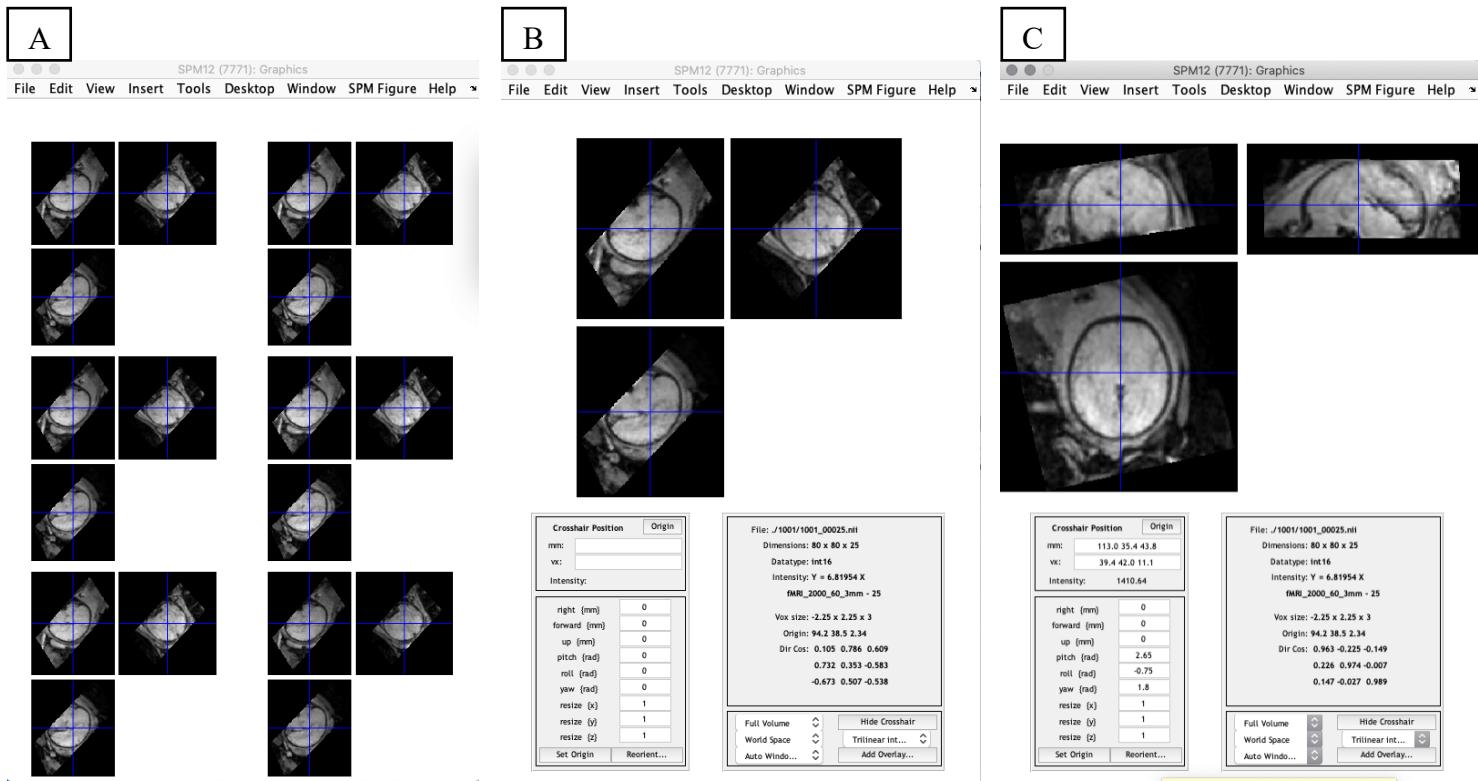


Figure 4: A) Six images are displayed in the SPM display window; the user should select the reference volume for the current session from the number (left click over the image to display the number) in the SPM drop-down menu. B) The chosen image will be displayed and the user should reorient each plane of the volume by modifying the roll, pitch and yaw axes until the orientation matches the template. C) Finally, the user must set the origin at the level of the Anterior Commissure and reorient all session images.

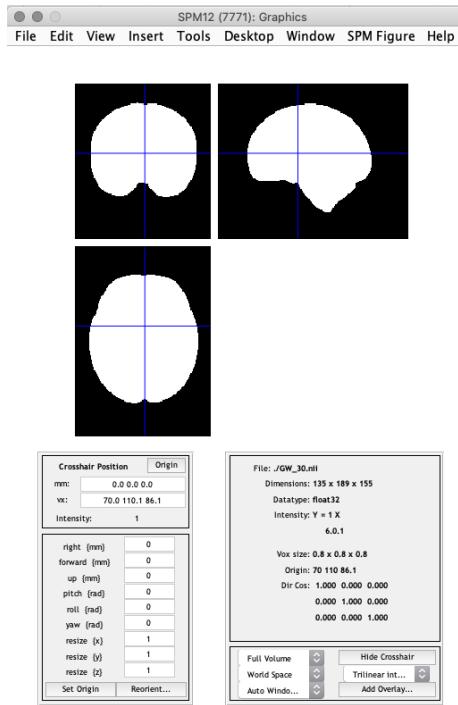


Figure 5: Example of a GW template, displayed here is a 30th GW. These template images have a default origin at the level of the AC. The user should use these templates as a guide when setting the origin for all of the reference images.

SPM will open a pop-up display window with the reoriented images (Figure 6A) and the user can decide to proceed or to reorient/change the reference images by pressing the ‘Continue’ or the ‘Reorient’ button, respectively (Figure 6C). Reoriented images can be found in the ‘M1_PP_03_Reorient’ folder. Each reference volume, inserted previously by the user, is automatically placed as the first volume of the relative session; this is a necessary step for ART (Artifact Detection tool) in M2. The final dataset of M1 can be found inside the ‘M1_PP_04_Rename’ folder. The ‘M1_PP_04_Rename’ contains as many subfolders as the initial number of sessions and then the relative 3D session volumes can be found within each subfolder (i.e. ‘1001_001.nii’, first volume of the first session).

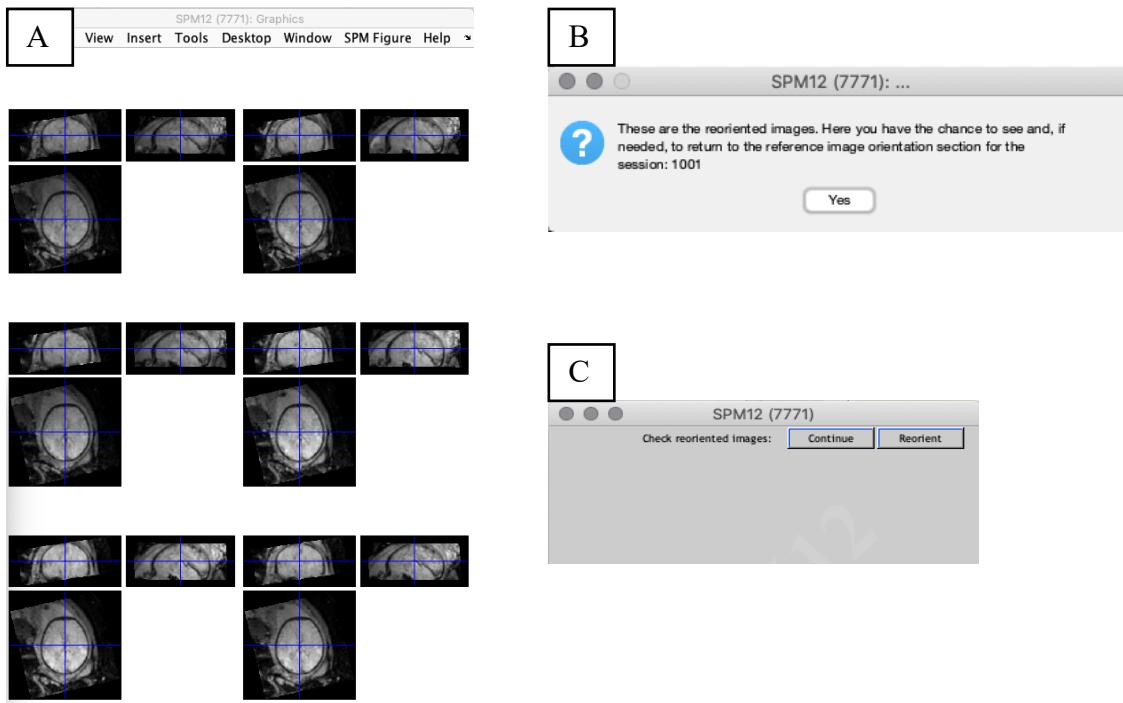


Figure 6: **A)** Reoriented images for the current session. **B)** SPM instruction window to reorient the images again. **C)** The user can decide to continue with the processing or to reorient the current session images.

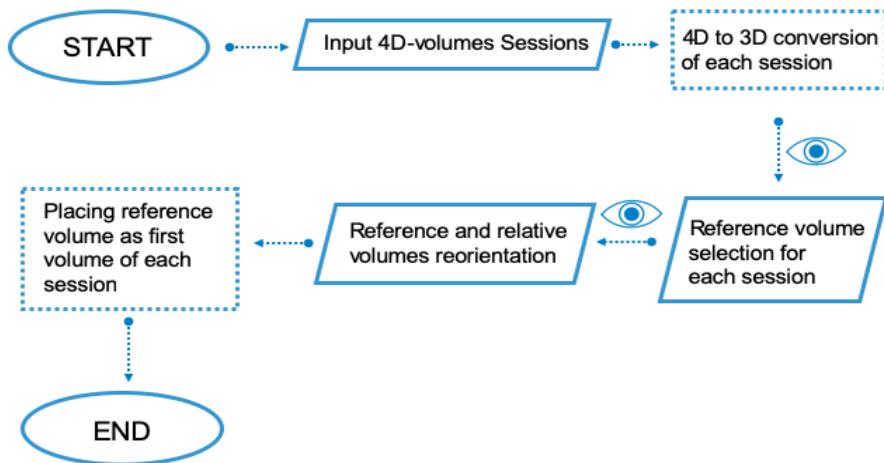


Figure 7: Module 1 RS-FetMRI flowchart.

M2: From 1st-pass masking to the 1st scrubbing procedure (ART)

Folders: M2_WS_01_Mask, 2_WS_02_Realign, M2_WS_03_Scrub, M2_WS_04_Rename.

WS-3

M2 begins with the creation of 68 session-specific template masks using the '*Creation_template.m*', a custom-built MATLAB function. This function uses the WS reference volumes from each session and all of the original templates for each GW (from the 21st to 37th) as input (subfolder '*Template_for_session*' inside the '*Template*').

First, each template origin is set at the AC by default to ensure that it matches with the user's selected origin. Each WS reference volume is used, iteratively and independently, as the source for co-registering all the templates for resampling to the same voxel resolution and matrix dimension as the user's images.

Second, session-specific template masks are smoothed with a [2 2 2] kernel (sm-2) and binarized above a fixed threshold. Three more templates are generated by smoothing each co-registered template with 3 different kernels: [4 4 4] (sm-4), [6 6 6] (sm-6), and [8 8 8] (sm-8). Session-specific template masks can be found within two dedicated subfolders called '*template*' and '*template_anp*' inside the relative session folder within the '*M2_WS_01_Mask*' folder.

Third, differences in x, y and z origin coordinates between each WS reference volume and template are calculated and the templates are translated for each session to achieve maximum overlap between the template masks and the fetal brain image.

Fourth, SPM check-registration visualization is prompted for the first session displaying the WS reference volume in the top left corner and all of the sm-2 template masks (Figure 9A). The user, helped by the cursor and by the SPM zoom function, chooses from the SPM drop-down menu (Figure 10B) the **1st-pass session-specific template mask** which includes the entire fetal brain, leaving out abdominal maternal tissue (Figure 9B-C).

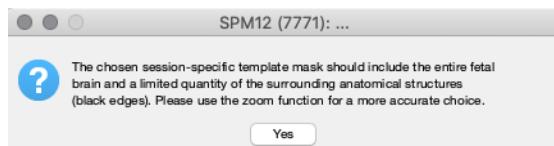


Figure 8: SPM instruction window on how to select the 1st-pass session-specific template mask.

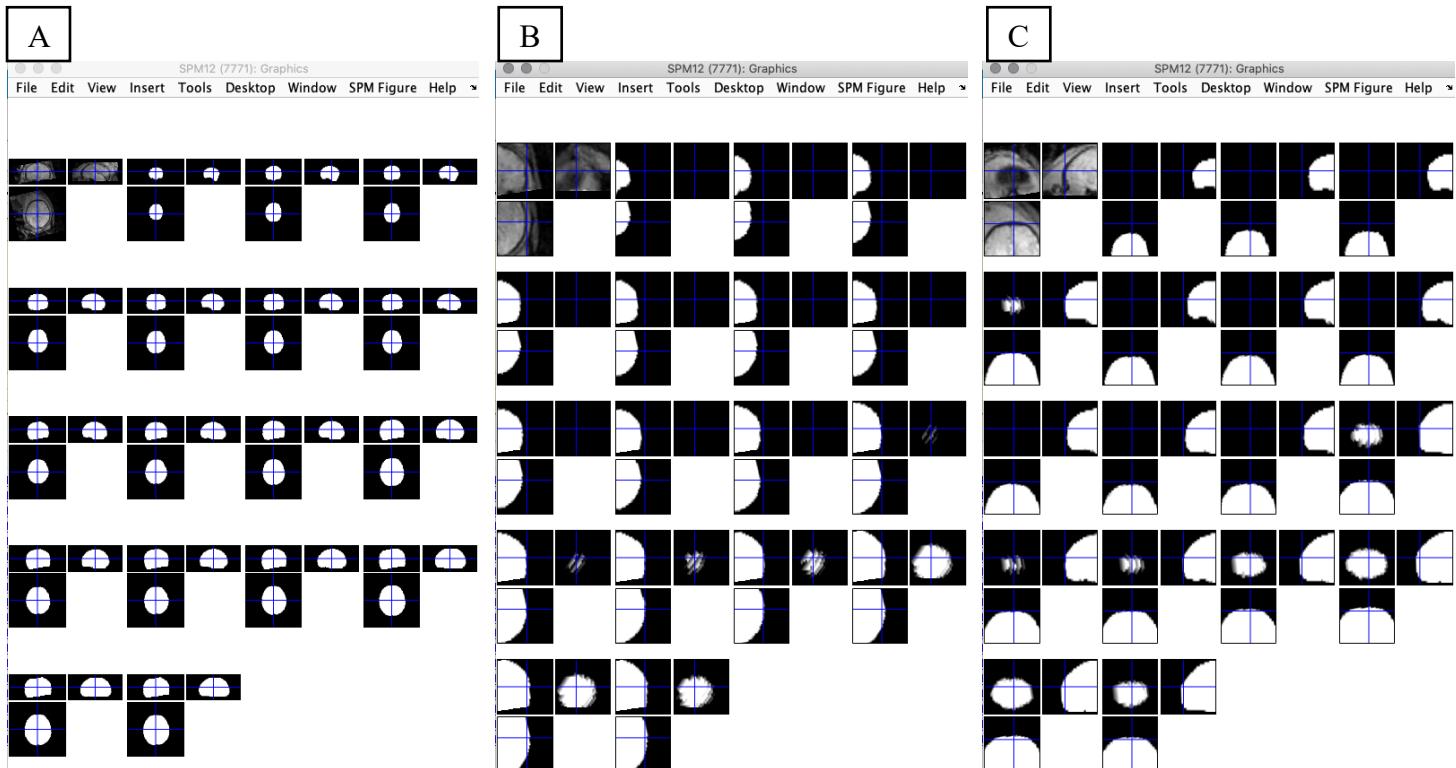


Figure 9: A) SPM check-registration visualization displaying the WS reference volume in the top left corner for the current session and all of the sm-2 template masks. B-C) SPM zoom function (80x80) to better highlight the edge of the fetal brain and to better select the 1st-pass session-specific template mask.

Once chosen, a new SPM window will display the WS reference volume from the first session, along with the sm-2, sm-4, sm-6 and sm-8 template masks (Figure 10A). Here, the user can select the optimal session-specific template mask after inspecting full fetal brain coverage brain morphology asymmetries in the x-, y- or z- planes from the SPM drop-down menu (Figure 10C).

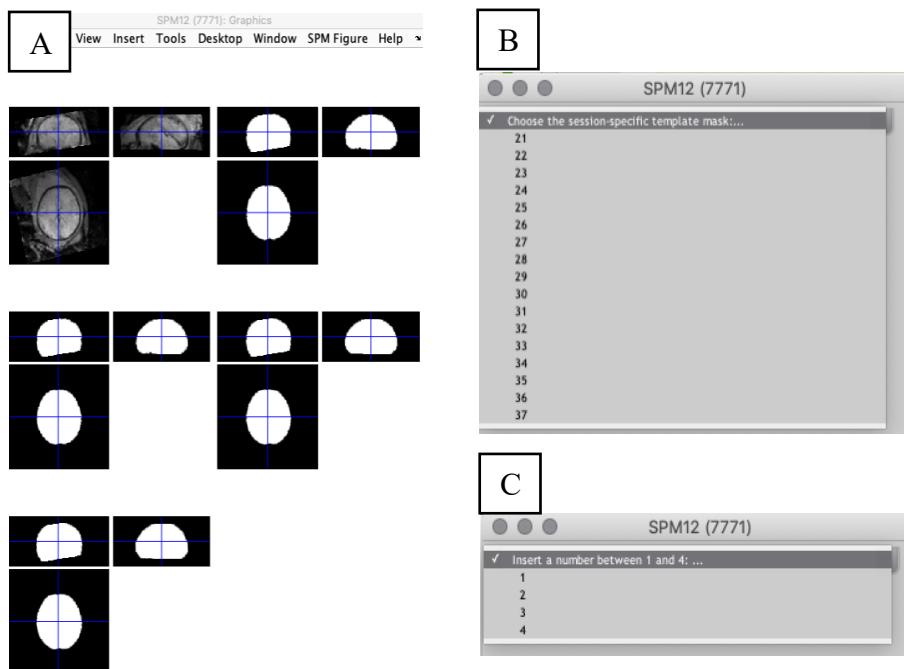


Figure 10: **A)** WS reference volume of the current session (top-left), the sm-2, sm-4, sm-6 and sm-8 template masks for the final 1st-pass session-specific template selection. **B)** SPM drop-down menu to select the 1st-pass session-specific template. **C)** SPM drop-down menu to select the final 1st-pass session-specific template.

All of the functional scans will be masked with the respective co-registered template mask. The 1st-pass masked images will be given ‘m’ as a prefix and they will be placed in the ‘M2_WS_01_Mask’ folder (i.e. ‘m1001_001.nii’, as the first volume of the first session). Sequential visualization of each reference volume, the final template mask, the 1st-pass Masked reference volume and random session images, are shown in an SPM display window (Figure 11A). After visual inspection, the user must press the ‘Continue’ button to proceed with the processing (Figure 11B).

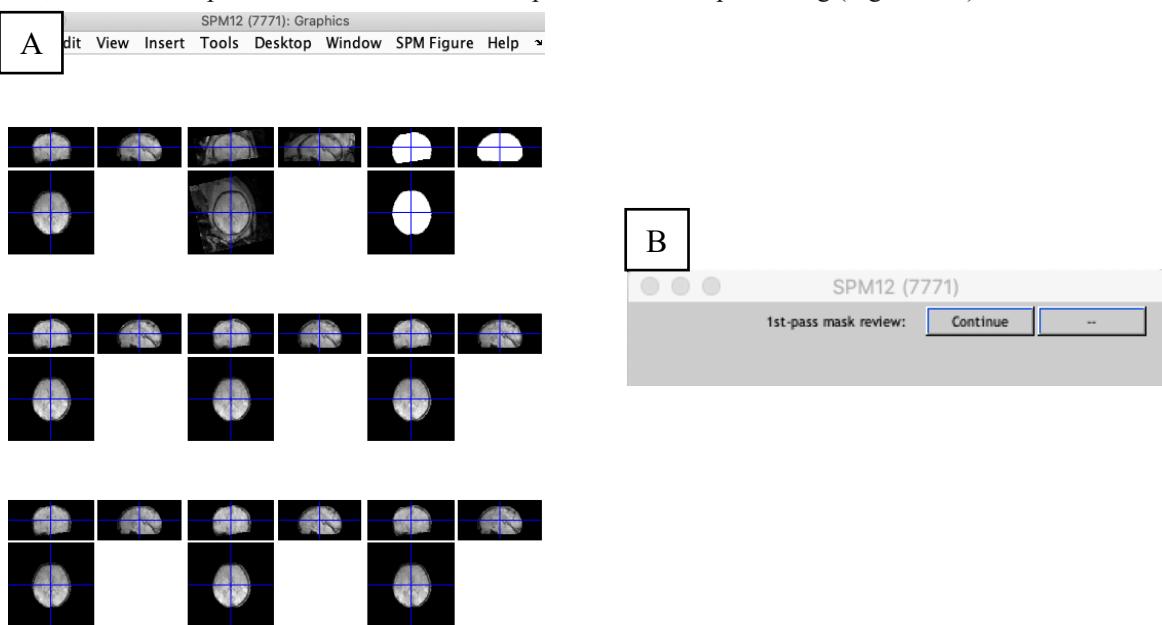


Figure 11: **A)** SPM check-registration visualization displaying the WS 1st-pass session specific masked reference volume in the top left corner, the WS reference not-masked volume, the 1st-pass pass session specific masked and a random 1st-pass session specific masked image from the current session. **B)** SPM input for visual inspection.

WS-4

The SPM display window is updated and the user is asked to select a tissue-weighting mask for realignment among all of the GW atlas masks shown (Figure 12A). This tissue-weighted mask constrains the WS realignment to the fetal inner-brain voxels and increases the accuracy of motion estimates. Therefore, the selection of the tissue-weighted mask (Figure 12C) should completely fit within the fetal brain (Figure 12B). During the realignment algorithm, translation and rotation parameters are calculated for each session with respect to the WS reference volume and reported in a ‘rp*.txt’ file. The same parameters are reported in two different SPM graphs (Figure 13). A mean WS image is created by averaging all of the volumes for each session. Realigned images are renamed with ‘r1’ as a prefix inside the ‘M2_WS_02_Realign’ folder (i.e. ‘r1m1001_001.nii’, first volume of the first session).

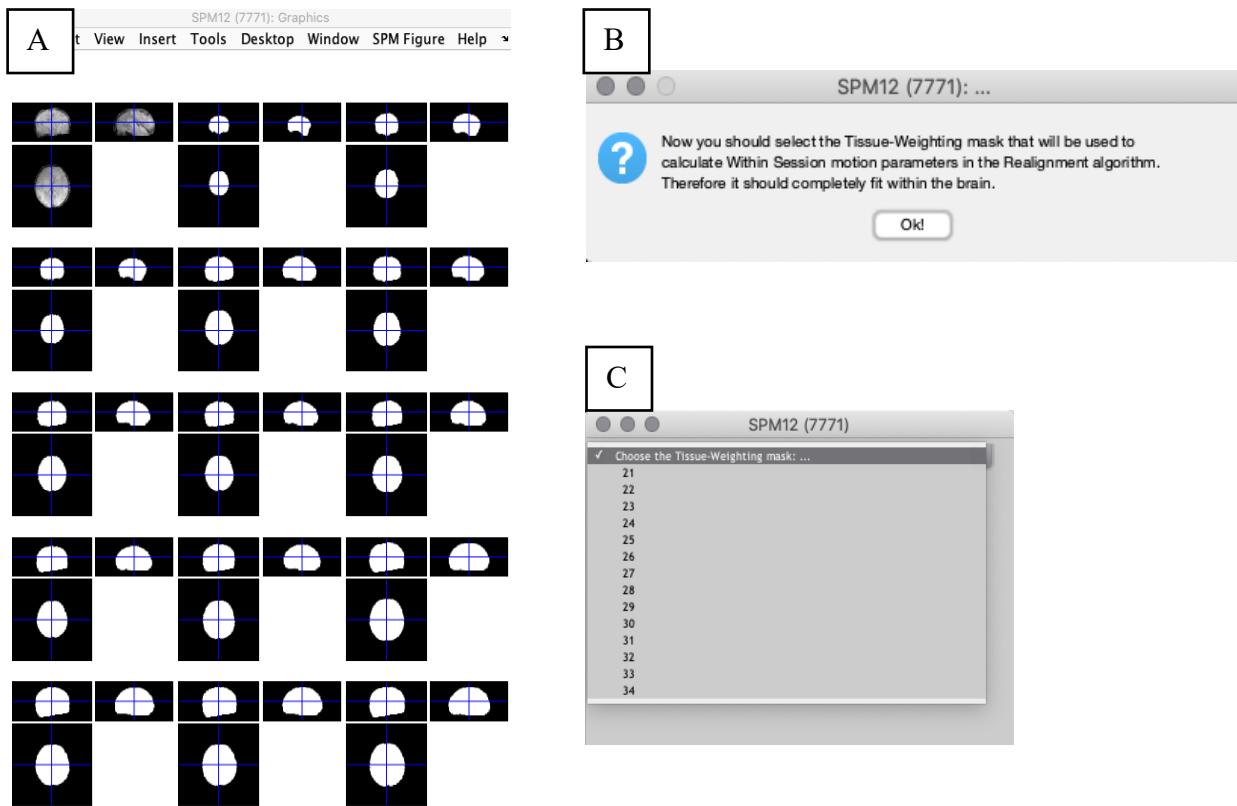


Figure 12: **A)** SPM check-registration visualization displaying the WS reference volume in the top left corner for the current session and the sm-2 template from 21st GW to the previously selected GW for the 1st-pass session-specific template. **B)** SPM instruction on how to select the tissue-weighting mask. **C)** SPM drop-down menu to select the GW of the relative tissue-weighting mask.

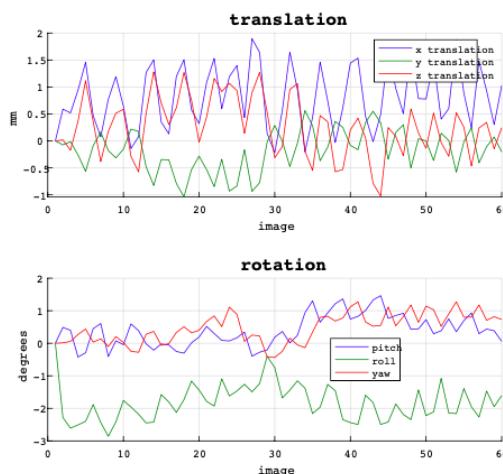


Figure 13: Example of an SPM display window for one session image realignment. For all sessions, two graphs are displayed with translation (top graph) and rotation (bottom graph) parameters. These values are also reported in the rp*.txt file from each session.

WS-5

The output files of the SPM realigned algorithm will be used in the **1st-pass scrubbing procedure** by the Artifact Detection Toolbox to estimate the frame-to-mean displacement with volumes considered as outliers if they show a global signal intensity variation exceeding 1.5 STD (with respect to the mean global intensity) and/or if showing motion greater than 4 mm in any direction. Scrubbed volumes by ART will be moved into a session-specific separate subfolder called ‘scrubbed_volumes’ inside the relative session folder in the ‘M2_WS_03_Scrub’ folder. ART also produces two ‘.mat’ files: ‘art_regression_outliers’ which includes outlier volumes and ‘art_regression_outliers_and_movement’ which includes outliers as well as movement parameters. An SPM pop-up window will show the instructions on how to deal with the scrubbed volumes (Figure 14C). After pressing the ‘Ok!’ button, a new SPM display window will open showing the reference volume in the top left corner followed by the session-specific scrubbed volumes (Figure 14A). The user can manually retain/exclude all scrubbed volumes by selecting ‘Yes’ or ‘No’, accordingly, or the ‘OnebyOne’ button to analyze each volume individually (Figure 14D). In this last case, the window is updated with the reference volume at the top and then each scrubbed volume at the bottom giving the user the opportunity to scrub or keep the single volume by pressing ‘Yes’ or ‘No’, accordingly, in the SPM window (Figure 14E).

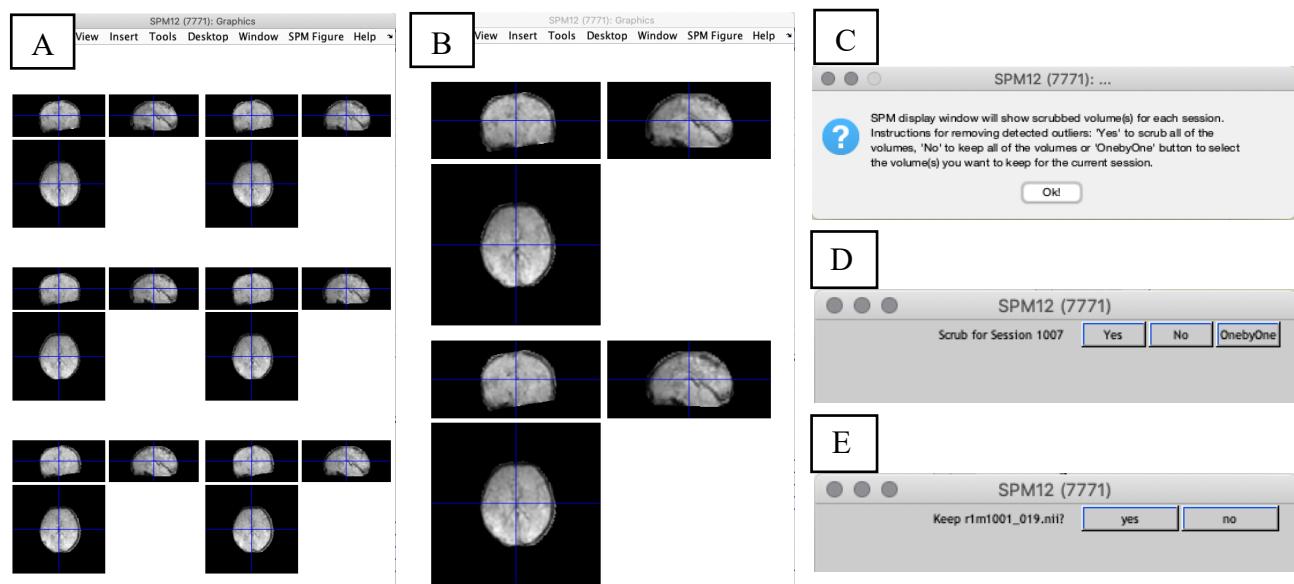


Figure 14: A) SPM display window is opened showing the reference volume in the top left corner followed by the scrubbed volumes for the current session. C) SPM instructions on how to manually exclude/retain scrubbed volumes D) The user can scrub or keep all scrubbed volumes by pressing ‘Yes’ or ‘No’ accordingly. Alternatively, the user can press the ‘OnebyOne’ button to analyze each volume individually and the SPM display window is updated (B) showing the reference volume of the current session on the top and the scrubbed volume on the bottom.

At the end of M2, surviving volumes are renamed and placed inside the ‘M2_WS_04_Rename’ folder. Sessions with less than 1/3 of the original volumes will not be included in the subsequent modules. A pop-up message informs the user on the number of remaining sessions (Figure 15).

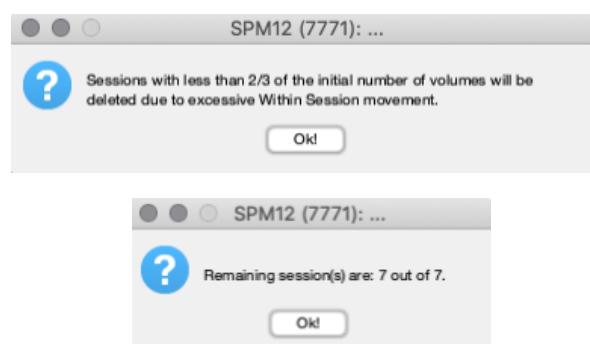


Figure 15: SPM informational message of session-exclusion criteria (top) and SPM information message of number of remaining sessions (bottom).

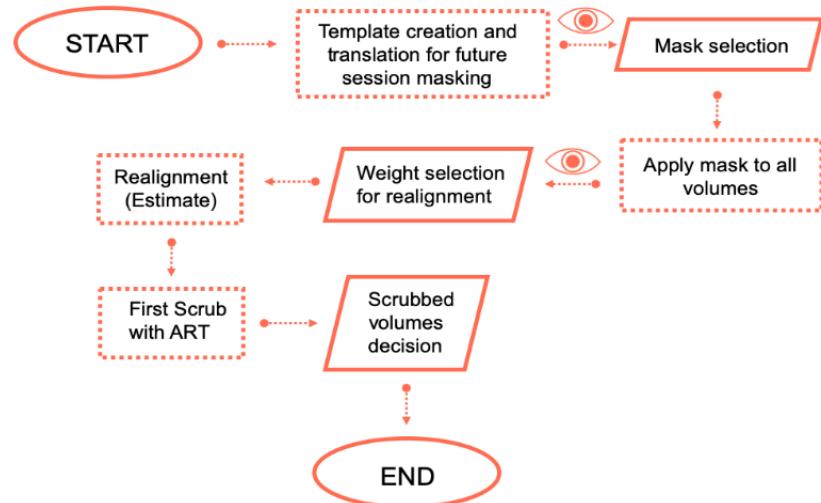


Figure 16: Module 2 RS-FetMRI flowchart.

M3: From Session-Specific Functional Reference Segmentation to Mean Functional Reference calculation

Folders: M3_WS_01_SegRefVols, M3_WS_02_MaskRefVols, M3_WS_03_RealignMaskRefVols, M3_WS_04_Rename.

WS-6 and WS-7

The goal in M3 is to refine 1st-pass session-specific inner-brain masks and to remove the majority of the maternal tissue from around the fetal brain, thereby facilitating the segmentation algorithm. First, the user will be prompted to accurately check the atlas mask brain coverage (Figure 17A) and either confirm the chosen mask from M2 by pressing the ‘No’ button (Figure 17B) or to further select another atlas mask with the “best-fit” fetal brain coverage, and then rerun the masking procedure only on that session-specific reference volume by pressing the ‘Yes’ button (Figure 17B).

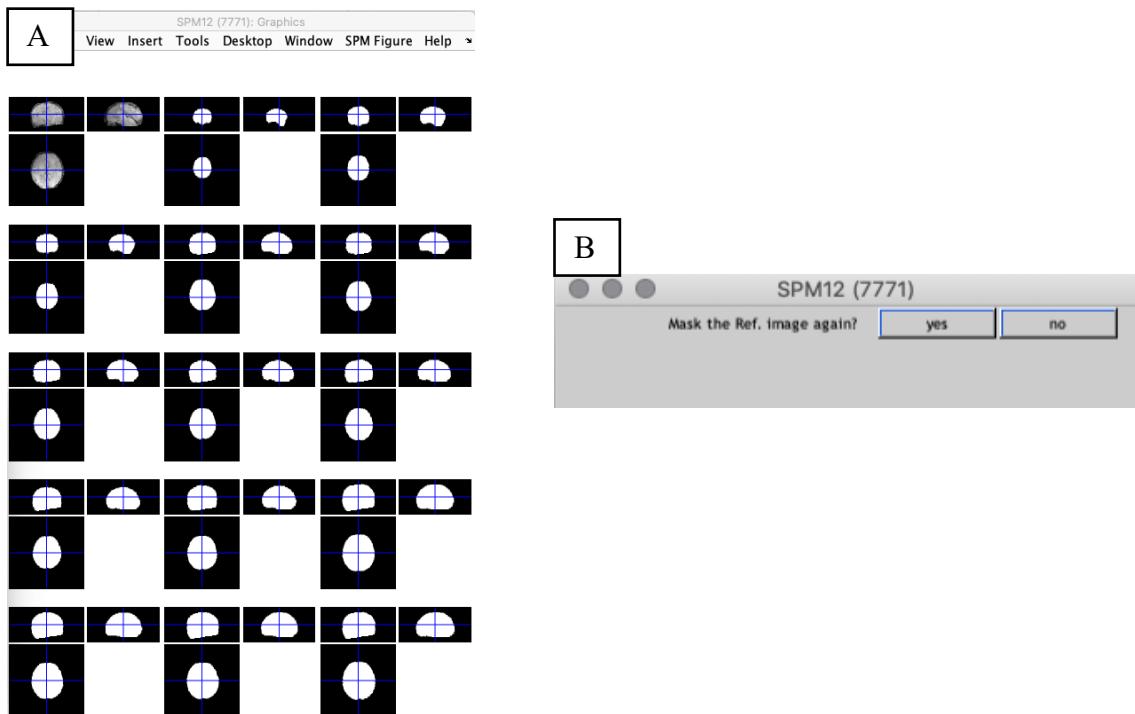


Figure 17: A) SPM check-registration visualization displaying the WS reference volume in the top left corner for the current session and all of the sm-2 template masks. B) SPM input window asking if the user wants to mask the reference again ('Yes') or if the to proceed with segmentation ('no').

When all of the reference volumes are masked, a pop-up window will remind the user of the previously chosen GW in M2 (Figure 18A). Next, the user can accept or change the GW that will be used in the segmentation algorithm by selecting the ‘Yes’ or ‘No’ button accordingly (Figure 18B). In the latter case, two consecutive SPM drop-down menus allow the user to select a specific GW value (i.e. numerical value between 21 and 37) for the GW-specific brain fetal tissue and structure maps created using C1:C7 preprocessed tissue classes (Figure 18C) (i.e. 1 - cortical plate and cerebellum, 2 - white matter (WM), 3 - cerebro-spinal fluid (CSF), 4 - deep grey matter (DGM), 5 - hippocampus, 6 - amygdala, 7 - brainstem) generated from Fetal Brain Atlas tissue and regional segmentations (Gholipour et al., 2017) and inner and outer brain space, respectively C8 and C9 (Figure 18D).

Here, it is strongly recommended to use the GW that was used to mask the images in the previous module (Figure 18). Once the two GWs values on the SPM drop-down menu have been selected (Figure 18C-D), the segmentation algorithm will start running and the outputs (i.e., ‘c9_mask.nii’ and ‘sc9_mask.nii’) will be found inside the folder ‘M3_WS_01_SegRefVols’ of each session.

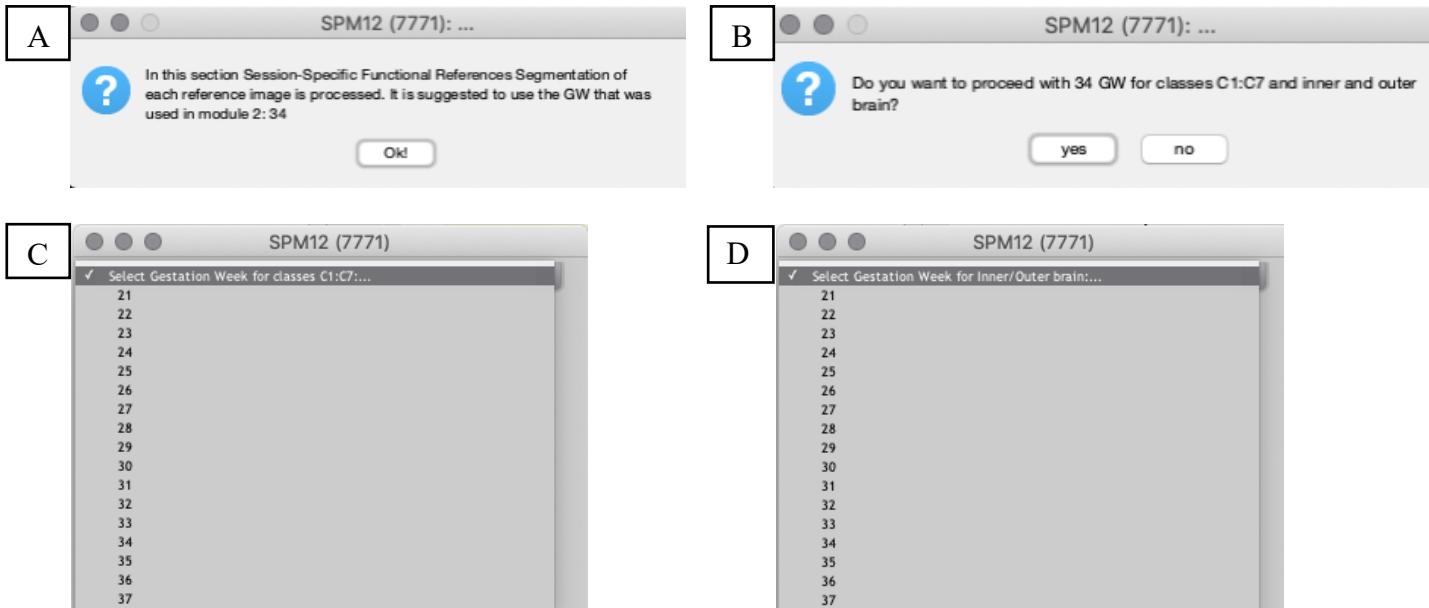


Figure 18: **A)** SPM information message on which GW should be used for the segmentation of the reference volumes. **B)** If the user wants to proceed with the previous GW (i.e., ‘Yes’) segmentation algorithm will start running. In the event that the user selects the ‘No’ button, GW for C1:C7 classes and GW for inner/outer brain will be requested. **C-D)** SPM drop down menu to select the GW for Classes C1:C7 and inner/outer brain respectively.

The segmentation algorithm output, called ‘*c9_mask*’, represents the inverted session-specific inner-brain mask of the fetal brain. This output will be inverted and smoothed with a [2 2 2] kernel, producing the final session-specific inner-brain mask called ‘*sc9_mask*’. Sequential visualization of each WS reference volume and the relative session-specific inner-brain mask are shown in an SPM display window for visual inspection (Figure 19A). The session-specific inner-brain mask is then applied to all of the session volumes inside the folder ‘*M3_WS_02_SegRefVols*’. Masked images will be placed in the ‘*M3_WS_02_MaskRefVols*’ folder with an ‘*m*’ as a prefix (e.g., ‘*mr1m1001_001.nii*’, the first volume of the first session).

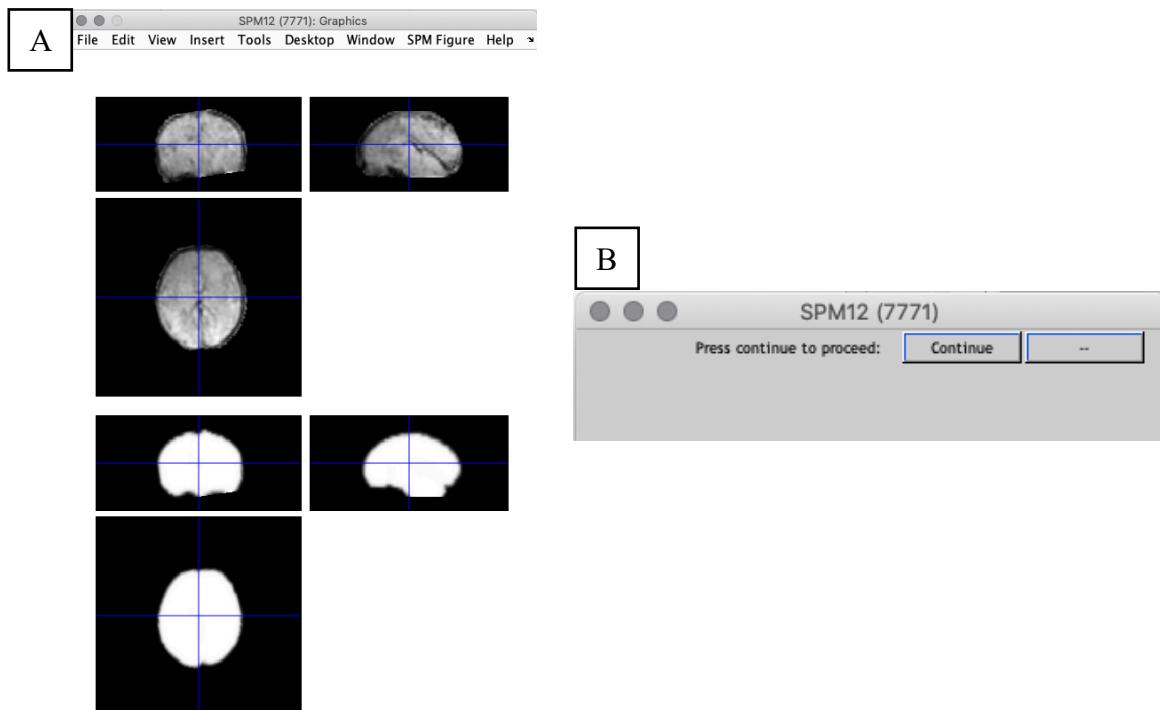


Figure 19: **A)** SPM sequential visualization of each WS reference volume and their relative session-specific inner-brain mask is shown in an SPM display window for visual inspection. **B)** SPM input window to proceed with the visual inspection of the next session.

BS-1 and BS-2

Session-specific inner-brain masked functional reference volumes are automatically realigned through the SPM realign algorithm (Figure 20). The algorithm, through the use of the previously selected tissue-weighting mask, created the ‘rp*.txt’ file as well as the between-session representative “template” functional volume (‘Mean_fMRI_Ref_Vol’) within the folder ‘M3_WS_03_RealignMaskRefVol’. The between-session representative “template” functional volume is then placed at the first position of the first session because it will be used as the reference volume for the between-session realignment in M4.

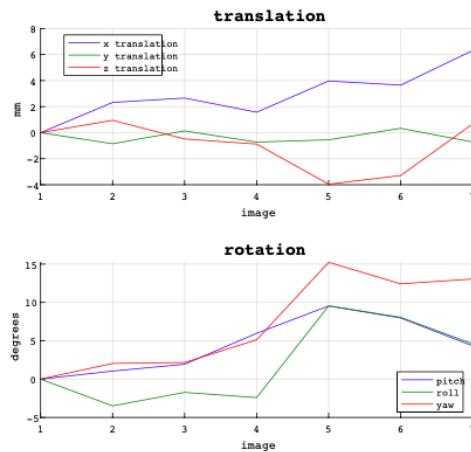


Figure 20: Example of an SPM realign display window for WS reference volume realignment (left). Two graphs are displayed with translation (top graph) and rotation (bottom graph) parameters.

An SPM display window shows the between-session representative “template” functional volume and all of the reference volumes (Figure 21). At the end of M3, all of the volumes will be renamed sequentially within the folder ‘M3_WS_05_Rename’.

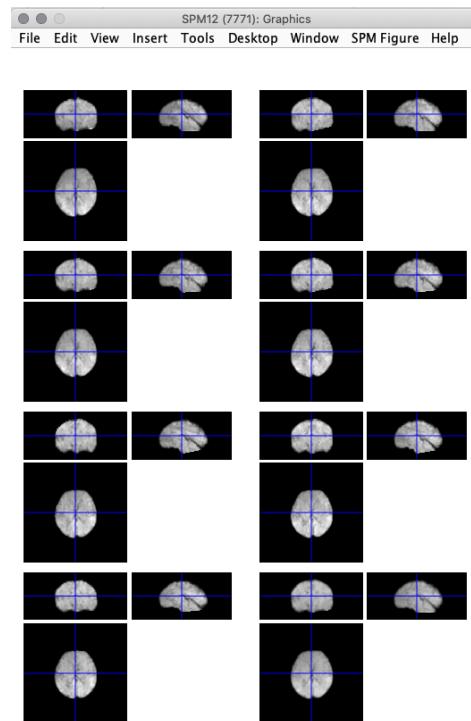


Figure 21: SPM check-registration visualization displaying the realigned WS reference volumes and the BS representative “template” functional volume in the bottom-right corner.

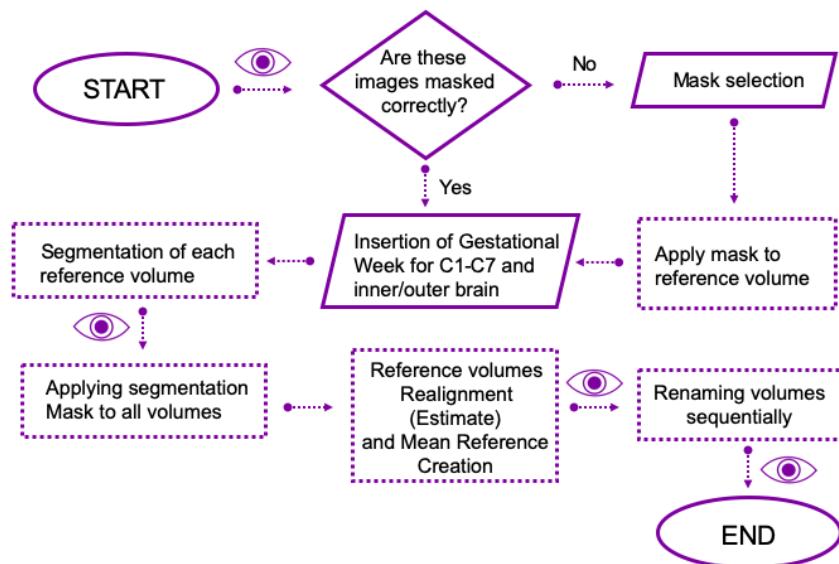


Figure 11: Module 3 RS-FetMRI flowchart.

M4: From Between Session Realignment to 2nd-pass scrubbing procedure (ART, DVARS, FD):

Folders: M4_BS_01_Realign_Reslice, M4_BS_02_Scrub, M4_BS_03_Rename.

BS-3

M4 starts by prompting the choice for the BS realignment by either using the “reslice” option, thereby progressing on to single-subject statistical analysis in the subject’s anatomy, or for “without reslicing”, which is suggested for “group-based” analysis. These two options can be selected by pressing the ‘Yes’ or ‘No’ button in the SPM input window, accordingly (Figure 22). In both cases, images will be renamed with ‘r2’ as a prefix after realignment within the ‘M4_BS_02_Scrub’ folder (i.e., ‘r2mr1m1001_001.nii’, first volume of the first session).

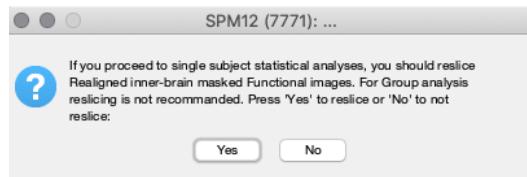


Figure 22: SPM information message on how to choose the reslicing option for the realignment algorithm.

First, the SPM Realign algorithm calculates the between-session translation and rotation parameters (i.e., *rp* files) following the realignment of all of the functional scans to the between-session representative “template” functional volume (i.e. Realign to First) (Figure 23).

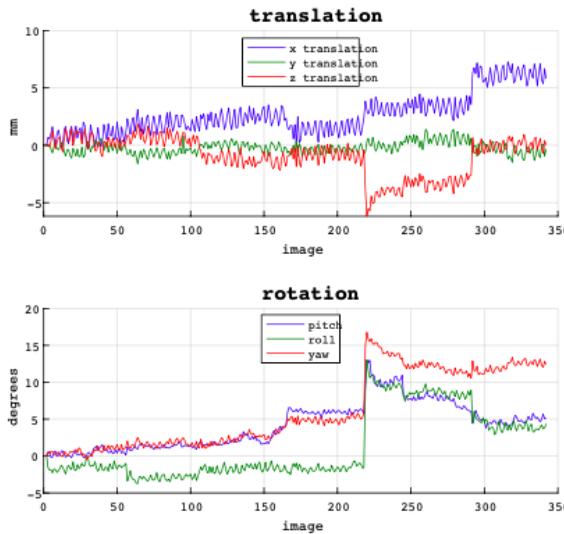


Figure 23: Example of an SPM realign display window for the realignment of all of the volumes with respect to the between-session representative “template” functional volume. Two graphs display with translation (top graph) and rotation (bottom graph) parameters.

Next an SPM window will display the between-session realigned masked reference WS volume and between-session representative “template” functional volume for visual inspection and check-registration purposes (Figure 24).

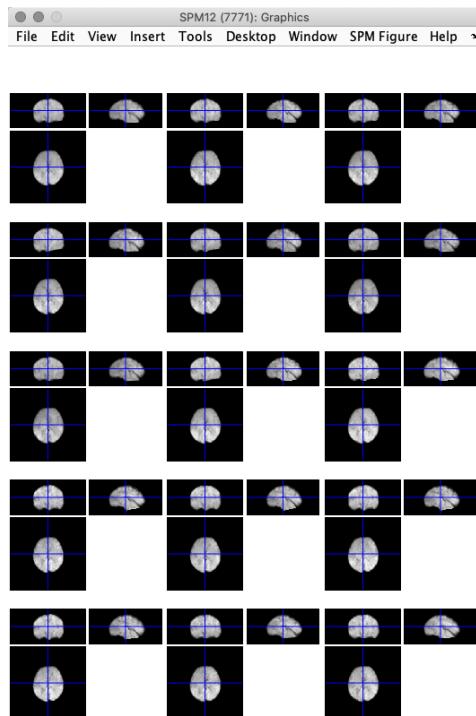


Figure 24: SPM check-registration visualization displaying random BS realigned inner-brain masked volumes and the Mean inner-brain masked functional reference volume in the top-left corner.

BS-4

The unique BS ‘rp*.txt’, as well as the between-session representative “template” functional volume, will be used by ART along with the same global signal intensity variation threshold as in M2 however with an increased motion threshold (5 mm) to calculate the outliers. Next, frame-to-frame estimation of motion (FD) and signal intensity (DVARS) changes are calculated and concatenated (‘concat.txt’ file) with the ART output (Figure 25) for outlier estimation during the **2nd-pass scrubbing procedure**. Scrubbed volumes are moved, as in M2, into a session-specific separate subfolder called ‘scrubbed_volumes’ inside the ‘M4_BS_02_Scrub’ folder. The same process to manually retain/exclude volumes as seen in M2 is offered again here (paragraph WS-5). Recall that a session with less than 1/3 of the initial number of volumes will automatically be removed from the time-series and will not undergo further preprocessing in the following modules (Figure 15).

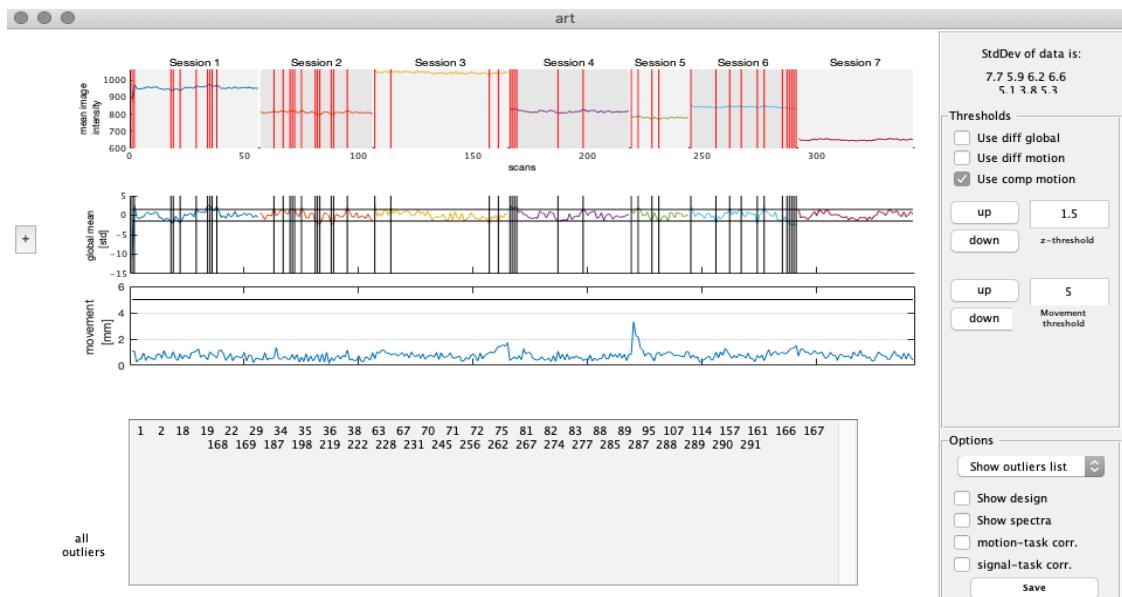


Figure 25: Graphical output from the ART BS. The second and third charts represent the global mean and the movement for all images. Vertical lines along the graphical output represent outliers. Displayed on the right: the z-threshold for the intensity (1.5) and the movement threshold (5mm). Images determined outliers are reported in the ‘all outliers’ table on the bottom.

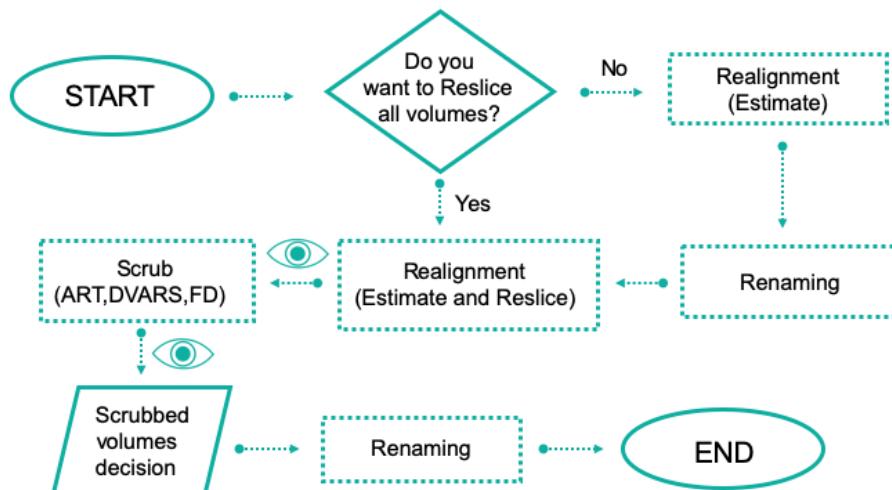


Figure 26: Module 4 RS-FetMRI flowchart.

M5: From Segmentation to Normalization of the Mean Functional Reference

Folders: M5_BS_01_SegMeanRefVol, M5_BS_02_MaskMeanRefVol, M5_BS_03_NormMaskMeanRefVol.

BS-5

M5 starts by prompting the choice for “GW subject-specific” or for “GW median-sample group-based” spatial normalization. The user can press the ‘Group Analysis’ or ‘Single subject’ button and proceed with the analysis (Figure 27). In the first case, the folder names of M5 and M6 are updated by adding a ‘GR’ string (i.e. M5_GR_BS_01_SegMeanRefVol, M5_GR_BS_02_MaskMeanRefVol, M5_GR_BS_03_NormMaskMeanRefVol).

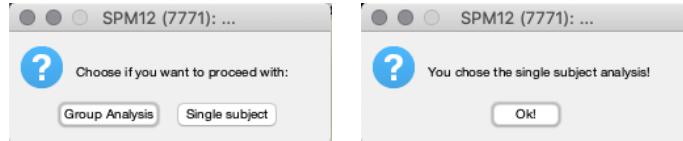


Figure 27: The user can proceed with the Group Analysis or Single Subject Analysis by pressing the respective button.

Next, the user is asked to insert the specific GW value (i.e. numerical value between 21 and 37) for C1:C7 classes and for inner and outer brain in the SPM drop-down menu (Figure 18C-D). In the case of a single subject, the user should select the gestational week of the fetus at rs-fMRI time-series acquisition, whereas in the case of group analysis, it is instead the median gestational week of the entire sample. This GW choice should be rationalized in the same way as in M3. SPMs unified segmentation–normalization algorithm will be used to derive a between-session representative “template” functional mask with the same procedure outlined for M3 and to calculate the deformation parameters based on spatial registration with the specific brain fetal tissue and structure maps created using C1:C7 pre-processed tissue classes. Then an SPM pop-up window allows for visual inspection of the between-session representative “template” functional image and its inner-brain segmentation mask (Figure 28A). The user must press the ‘Continue’ button to proceed with the processing (Figure 18C).

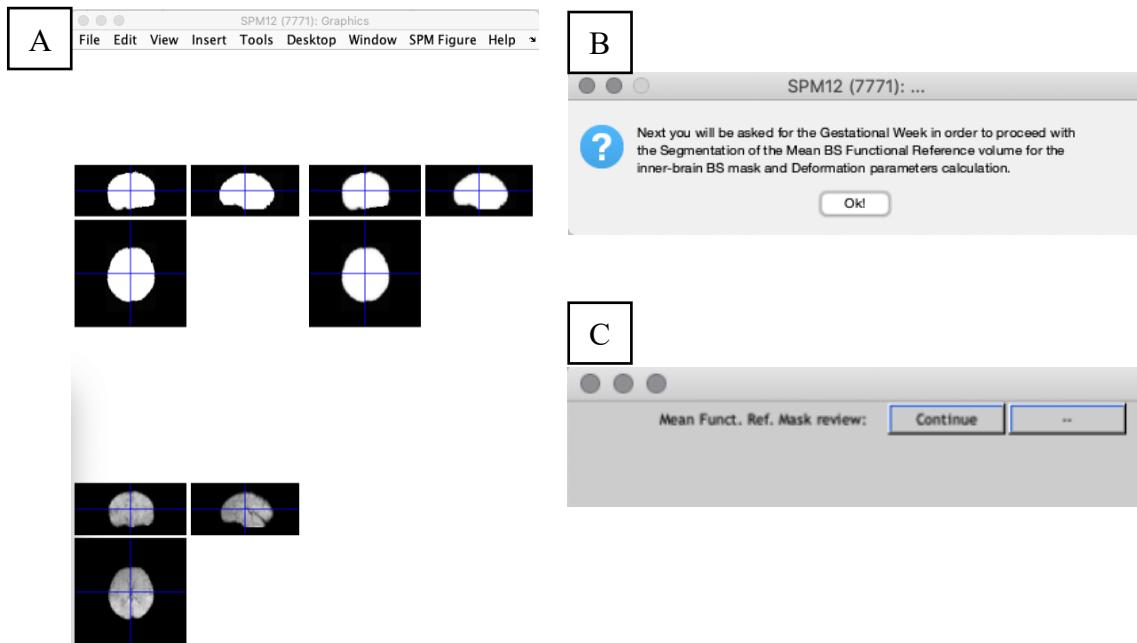


Figure 28: A) SPM instructions. **B)** SPM check-registration visualization displaying the ‘c9’ mask and ‘sc9’ mask and the BS inner-brain reference volume. **C)** SPM window to continue with the processing (bottom-right).

BS-N-6

The between-session representative “template” functional mask and the deformation parameters are obtained and applied to the between-session representative “template” functional volume. The SPM display window shows the between-

session representative “template” functional volume at the top and the CRL Fetal Brain Atlas at the bottom (Figure 29A). This process can be repeated until the user is satisfied with the result, by pressing the ‘Yes’ or ‘No’ button in the SPM input window (Figure 29B). In the event that both group and single subject analysis are desired, it is suggested to terminate the processing with single subject analysis and then re-launch M5 and M6 by choosing group analysis (**See supplementary Group Analysis**).

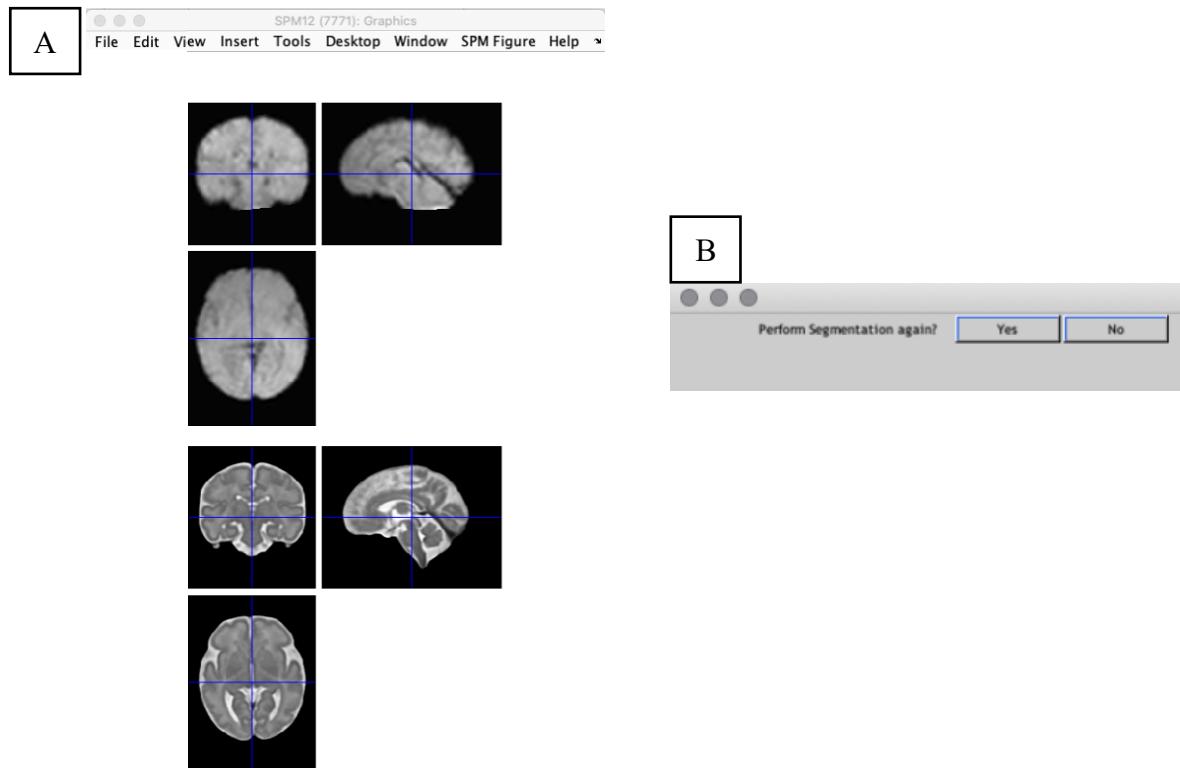


Figure 29: A) BS representative “template” functional volume spatially normalized to GW subject-specific and the corresponding CRL Fetal Brain Atlas images (CRL-FBA) – GW 21 through 37. B) SPM input window to perform the segmentation again or to proceed with the processing.

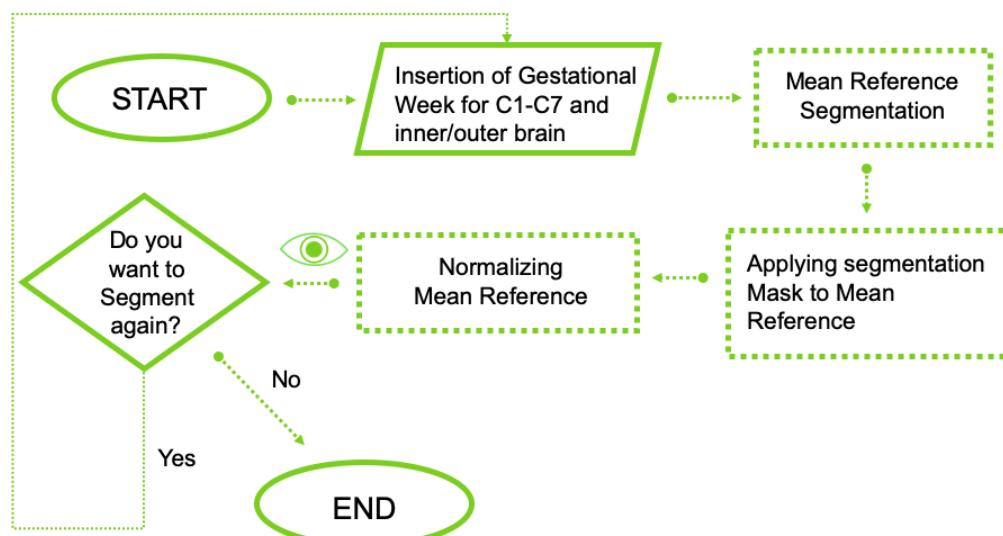


Figure 30: Module 5 RS-FetMRI flowchart.

M6: From Normalization to Smoothing of all Functional Scans

Folders: M6_BS_01_MaskAllVols, M6_BS_02_NormMaskAllVol.

N-1 and S-1

In M6, the between-session representative “template” functional mask is automatically applied to all M4 output volumes through each session (Figure 31B) and the deformation parameters are applied to between-session masked functional volumes (Figure 31D) in order to normalize all volumes in the rs-fMRI time-series to GW subject-specific or median-sample group-based atlas space. Normalized between-session masked volumes (i.e. w*. images) are smoothed (FWHM = 4) in order to compensate for imperfect registration residuals, inter-subject variability in fetal brain anatomy and to increase signal-to-noise ratio in very limited structural space using a small filter size (i.e. 4 mm). SPM visualization of functional masked images as well as visualization of functional normalized images will be prompted.

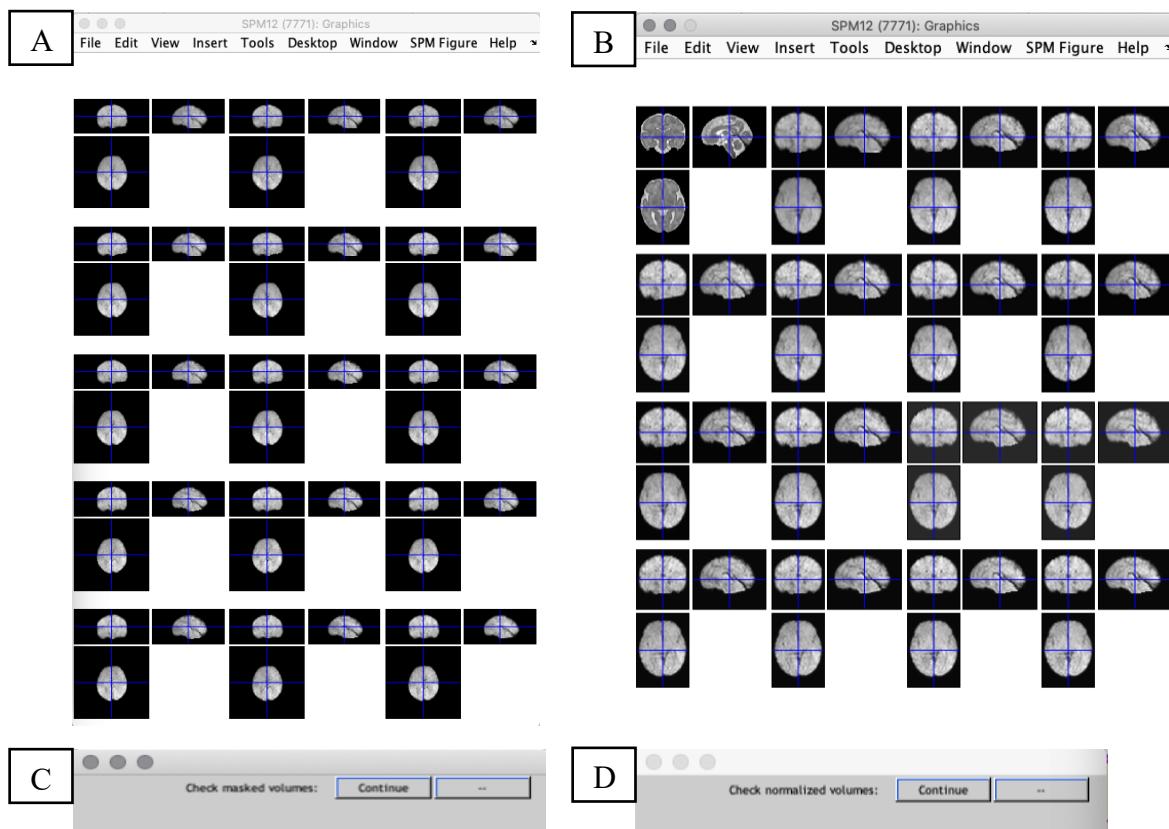


Figure 31: **A)** SPM check-registration visualization displaying the BS inner-brain masked volumes and random masked session volumes; **B)** SPM check-registration visualization displaying the BS inner-brain normalized volumes and random normalized session volumes (GW=35); **C)** The user should visually inspect the masked and normalized images and then press the ‘Continue’ button to proceed with the processing. **D)** The user should visually inspect the masked images and then press the ‘Continue’ button to proceed with the processing.

At the end of M6, a 4D-Nifti file is created by merging all normalized and smoothed volumes (i.e., sw*.nii images) from all sessions and then the entire preprocessed rs-fMRI time-series is displayed in SPM movie mode.

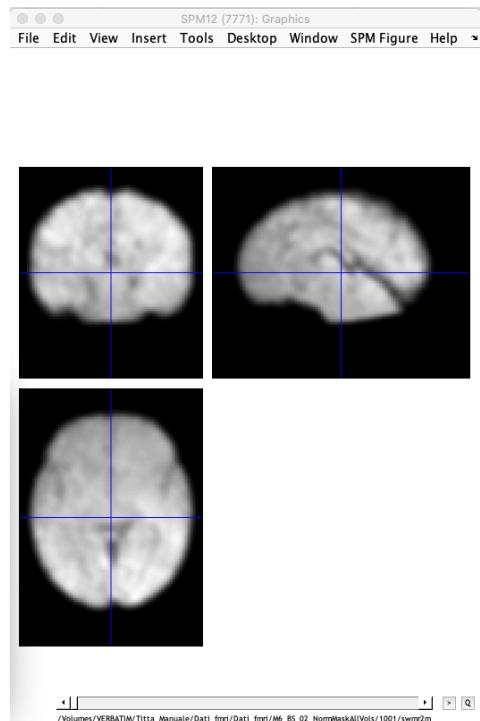


Figure 32: SPM 4D display of all of the normalized images.

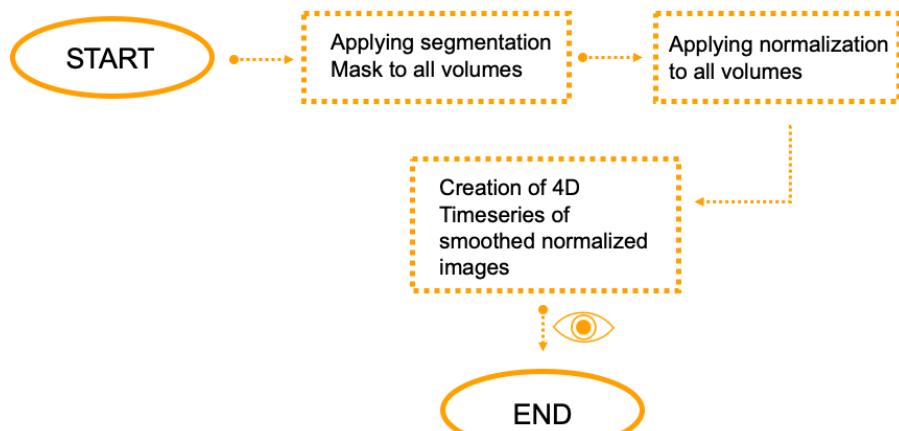


Figure 33: Module 6 RS-FetMRI flowchart.

SUPPLEMENTARY

Supplementary Initialization:

Open the Matlab software and set the current directory within the ‘RS-FetMRI’ folder and enter the ‘art’ folder. Within the ‘art’ folder there are three configuration files (.cfg). In each of these files, three paths (‘image_dir’, ‘motion_dir’ and ‘mask_file’) and they need to be modified.

The following image is relative to the ‘File_1Srb.cfg’ file. Here, squared in red, the three paths that need to be modified.

```

sessions: 1                      # number of sessions
global_mean: 2                   # global mean type (1: Standard 2: User-defined mask)
global_threshold: 1.5             # thresholds for outlier detection
motion_threshold: 4.0
motion_file_type: 0               # motion file type (0: SPM .txt file 1: FSL .par file 2:Siemens .txt file)
motion_fname_from_image_fname: 0   # 1/0: derive motion filename from data filename
#spm_file: ./2back/2bmodel/SPM.mat # location of SPM.mat file (comment this line if you do not wish to estimate number of outliers
image_dir: /Volumes/VERBATIM/Dati_fmri/RS-FetMRI/M2_WS_03_Scrub/9999/           # functional and movement data folders (comment
motion_dir: /Volumes/VERBATIM/Dati_fmri/RS-FetMRI/M2_WS_03_Scrub/9999/
mask_file: /Volumes/VERBATIM/Dati_fmri/RS-FetMRI/M2_WS_03_Scrub/9999/meanm9999_001.nii
end

# Functional data file(s) for each session
# example use:
# session 1 image file01.img file02.img file03.img file04.img file05.img file06.img      # selects specific .img files
# session 1 image file???.img    # selects multiple .img files (?? refers to two-digit numbers with zero-padding to the left)
# session 1 image file.nii     # selects single 4-d .nii file

session 1 image r1m9999_???.nii
#session 2 image 2brun2/2brun2_???.img

# Movement file for each session (comment if motion_fname_from_image_fname is set to 1)

session 1 motion rp_m9999_001.txt
#session 2 motion 2brun2/rp_2brun2_001.txt

end

```

Suppose that the user placed the ‘RS-FetMRI’ folder in the following path ‘/User/My_fMRI_DATA/’. Then the user should replace the string before ‘RS-FetMRI’ (/Volumes/VERBATIM/Dati_fmri/) with ‘/User/My_fMRI_DATA/’:

- File_1Srb.cfg: Update each path maintaining this folder at the end:

1. image_dir: /User/My_fMRI_DATA/M2_WS_03_Scrub/9999/
2. motion_dir: /User/My_fMRI_DATA/M2_WS_03_Scrub/9999/
3. mask_file: /User/My_fMRI_DATA/M2_WS_03_Scrub/9999/meanm9999_001.nii

- File_2Scrb_orig.cfg AND File_2Scrb.cfg:

1. image_dir: /User/My_fMRI_DATA//M4_BS_02_Scrub/
2. motion_dir: /User/My_fMRI_DATA//M4_BS_02_Scrub/Motion_file/
3. mask_file: /User/My_fMRI_DATA//M4_BS_02_Scrub/9999/r2mr1m9999_0001.nii

Supplementary TestSet parameters:

Input parameters for the analysis of the TestSet are reported below:

Module	Input parameters	Single Subject Analysis	Group Analysis
M1	WS Reference Volume for each session:	25, 35	25, 35
M2	1 st -pass Masking (GW value) and second choice	GW (sm-2) = 34; Second choice (sm-8) n=4	GW (sm-2) = 34; Second choice (sm-8) n=4
M2	Tissue-weighting mask (GW value)	33	33
M3	Segmentation WS (GW value)	C1:C7 = 35 Inner/outer = 35	C1:C7 = 35 Inner/outer = 35
M4	Reslice	No	Yes
M5	Analysis	Single Subject	Group Analysis
M5	Segmentation BS (GW value)	C1:C7 = 35 Inner/outer = 35	C1:C7 = 33 Inner/outer = 33

Table S1: input parameters inserted for the processing of the TestSet subject for Group and Single Subject Analyses.

Single session analysis:

As already stated in the WS-1 subparagraph, the user can insert only one folder inside the '*M1_PP_01_OrigVol*,' that contains the 4D Nifti raw file to proceed with Single session Analysis. In this case, the M3 and M4 modules are skipped and their folders removed. A pop-up message will be prompted by SPM, warning the user that the processing will jump from M2 to M5 (Figure S1).



Figure S1: SPM message informing the user that the M3 and M4 modules will be skipped because only one session was detected.

Art Folder:

The 'Art' folder includes the art script and three configuration files used in fetal image processing, one in module 2 and two in module 4. In these files, the paths for the image are hard coded therefore they need to be changed. This folder contains:

File	Description
<i>art.m</i>	An automatic version of the Artifact detection tool (ART) on SPM.
<i>File_1Scrub.cfg</i>	Configuration file that will be used in M2 for the art function. This file is overwritten with the correct number of sessions and the correct session names. At the end of the art processing, this file is reinitialized to its initial configuration for further analysis. As already explained in the initialization paragraph, this file contains hard coded paths that only need to be modified once.
<i>File_2Scrb_orig.cfg</i>	Initial configuration that will be modified and used in module 4 for the art function. This file is loaded in reading mode into the MATLAB environment and then it is modified with the correct number of sessions and the correct session names. The resulting modified file is then saved in the File_2Scrub.cfg that will be used for art configuration.
<i>File_2Scrub.cfg</i>	This file contains the final configuration for Art of module 4.

Supplementary Table S1: Files inside the art folder.

Realignment Algorithm (Estimate):

Estimation refers to how to get the optimal transformation (normally rigid-body transformation with six parameters) from individual images to the reference. As already stated in module 2 after the execution of this step, the header of each input file will be changed to reflect their relative orientation. This step produces two outputs: 1) an ‘rp*.txt’ for each session containing 6 columns that summarize all of the translation and rotation of each image related to the reference image and 2) a mean image created by averaging all of the session images. During this step, the SPM window displays the six motion parameters in two charts (Figure 13); these will be saved for each session as a ‘*.ps’ file. Default parameters for the Realign algorithm are used; they are listed and explained below in Table S2. However, depending on the Module and on the level analysis (WS or BS), some of these parameters can be different.

Parameter	Description	Value
Quality	A value between 0 and 1. The larger the value, the higher the quality of the estimation but the lower speed of the calculation. This value reflects the percentage of voxels taken into account during the estimation. Some of the voxels may contribute less so they can be ignored in the calculation.	0.9
Separation	This is the distance (non-negative) between the points sampled in the reference space. The smaller the value, the more refined in a sampling, and the more accurate in the estimation but slower in the speed.	4
Smoothing	Smoothing the images before transformation parameter estimation. Filter the noise in the images. SPM suggested a 5mm kernel for MRI data.	5
Num. passes	Selection of the reference image is determined here. The reference could be either the first volume or the average volume of all images.	register to mean
Reslice	Only the mean is resliced.	[0,1]
Interpolation	Depending on the complexity of sampling on individual images and the number of voxels considered in the algorithm, the parameter estimation may be affected. The higher degree of interpolation, the more accurate the estimation, albeit at the cost of more time and slower speed. Default 2 nd Degree B-spline.	Depends on Module
Weighting	This image weighting image is chosen by the user and it will be used to give more importance to voxels within the fetal brain.	User selection

Supplementary Table S2: Realignment algorithm (Estimate) default parameters.

Realignment Algorithm (Reslice):

Once the transformation parameters are estimated, there is no new image generated. During the reslice procedure, a series of registered images will match the first image selected voxel-to-voxel and lead to a new series of images. The new images will be named based on their original name however with a specific prefix. Basically, reslice still belongs to the issue of interpolation of points in the original space to infer the value in new space, and in SPM there are several parameters provided to control the algorithm.

Parameter	Description	Value
Resliced images	Refine which images are needed to do reslice operation, and the "mean image" refers to an additional image by averaging all of the resliced ones	[2,1]
Interpolation	SPM suggested that easier approaches (e.g., nearest neighbor) normally perform worse than higher degree methods, which consider more voxels in interpolations.	4
Wrap	This is about the directions in the volumes the values should wrap around in. In MRI scans, the images wrap around in the phase encoded direction. SPM suggests that if the images have been spatially transformed, or you are not sure how to set it up, then it's better to set it with void.	[0,0,0]
Masking	For voxels which need to be sampled from outside the original images, they are set to zero directly over all images.	1
Filename prefix	Prefix to add to the original image name after reslice.	Depends on Module

Supplementary Table S3: Realignment algorithm (Reslice) default parameters.

Segmentation Algorithm:

Segmentation is a crucial step in Fetal rs-fMRI processing. All of the segmentation parameters are already optimized and set in the RS-FetMRI script. However, the user can modify these parameters depending on the image appearance. Indeed, listed below in the table are some retained segmentation parameters that have an impact on segmentation and image processing. MR images are usually corrupted by a smooth, spatially varying artifact that modulates the intensity of the image (bias). These artifacts, although not usually a problem for visual inspection, can impede automated processing of the images.

Parameter	Description	Default
biasreg	Amount of bias regularization. If your data has very little intensity non-uniformity artifact, then the bias regularization should be increased. Other biasreg parameters ranging from 10^{-5} to 10 (included 0 for no regularization) can be used depending on user images.	0.01
biasfwhm	FWHM of Gaussian smoothness of bias. If the intensity non-uniformity is very low in signal variation, then choose a large FWHM. Differently, small FWHM are preferred for signal bias with high variation. Possible values range between 30 and 150mm, including 0 for no correction.	60
mrf	When tissue class images are written out, a few iterations of a simple Markov random field cleanup procedure are run. This parameter describes the strength of the Markov random field. Setting the value to zero will disable the cleanup. Higher value increases the Markov random field cleanup.	1
reg	The amount of regularization determines the trade-off between the three different terms. More regularization gives smoother deformation, where the smoothness measure is determined by the bending energy of the deformation.	[0, 0.001, 0.5, 0.05 0.2]
samp	This encodes the approximate distance between sampled points when estimating the model parameters. Smaller values use more of the data, but the procedure is slower and needs more memory. Determining the ‘best’ settings involve compromising between speed and accuracy.	2

Supplementary Table S4: Segmentation algorithm default parameters.

Group Analysis (GR):

At the beginning of M5 the user is asked to decide between ‘Group Analysis’ or ‘Single Subject Analysis’. The processing for both choices stay the same, the only difference is that the folders (from M5 to M6) will be renamed depending on that choice.

In the event that both ‘Group Analysis’ and ‘Single Subject Analysis’ are desired, there are two possible routes: The first is to run the RS-FetMRI pipeline from start to finish. This choice, however, is time-consuming because the outputs from M1-M4 are the same for both the analyses.

The shortest way is to run the one of the analyses (i.e., ‘Single Subject Analysis’) and then:

- 1) Move the M5 and M6 Folder relative to the ‘Single Subject Analysis’ into another repository;
- 2) Clear all of the variables;
- 3) Load M4 variables (*‘Variables_M4.mat’*) and all the paths (*‘All_paths.mat’*) by double clicking on them.
- 4) Re-launch M5 skipping the ‘else’ cycle.

M2 and M5 are linked by an ‘if - else’ cycle that, depending on the number of sessions inserted in the ‘*M1_PP_01_OrigVol*’, allows the processing to jump from M2 to M5 (i.e., Single Session analysis). For this reason, M5 starts with an ‘else’ cycle that must be skipped if the user launches the script from M5 to repeat the analysis for the ‘Group Analysis’.

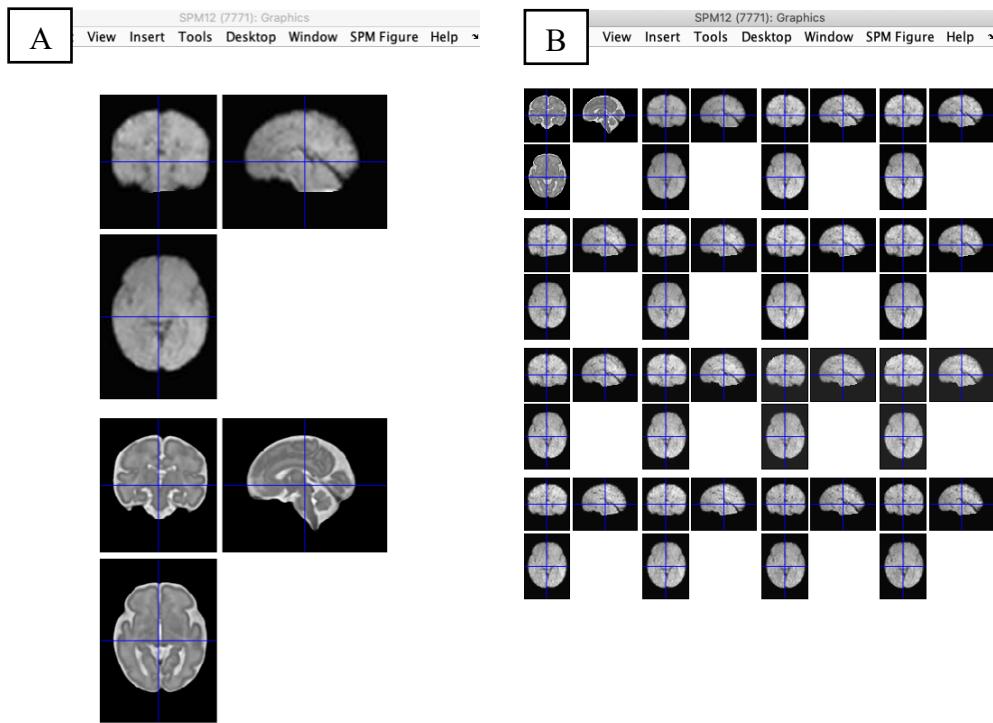


Figure S2: **A)** SPM check-registration visualization displaying the BS inner-brain masked volumes and random masked session volumes; **B)** SPM check-registration visualization displaying the BS inner-brain normalized volumes and random normalized session volumes (GW=33).

Troubleshooting:

In case of any errors during the processing it is suggested to:

- 1) Clear all of the variables in the Matlab Workspace (command: ‘clear all’);
- 2) delete files from the related folder of module that caused the error;
- 3) load the variables from the previous module;
- 4) re-run the processing from the initial module that caused the error until the end of the code.

In the event of failure during the segmentation algorithm inner-brain mask creation, we strongly suggest referring back to and keeping the parameters outlined in table S3 as the default setting and to use a different GW (i.e., the GW that was used in M2). This is because it is rational that the segmentation mask used in module 2 is more suitable to the fetal brain images. However, functional scans are often corrupted by artifact(s) (such as hyperintensity) and high voxel intensity outside of the fetal brain image, which could likely be a source of errors when generating the segmentation image. In the worst case, manual deletion of high voxel intensity outside the fetal brain image or a manual mask may be required.