Development of a data analysis platform for characterizing functional connectivity networks in rodents

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Utveckling av en dataanalys rutin för att karakterisera funktionella nätverk hos gagnare

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1 Abstracts

1.1 Abstract
This document addresses the development and implementation of a routine for analyzing resting-state functional Magnetic Resonance Imaging (rs-fMRI) data in rodents. Even though resting-state connectivity is studied in humans already for several years with diverse applications in mental disorders or degenerative brain diseases, the interest for this modality is much more recent and less common in rodents. The goal of this project is to set an ensemble of tools in order to be able for the experimental MR team of KERIC to analyze rs-fMRI in rodents in a well defined and easy way. During this project several critical choices have been done, one of them is to use the Independent Component Analysis (ICA) in order to process the data rather than a seed-based approach. Also it was decided to use medetomidine as anesthesia rather than isoflurane for the experiments.

The routine developed during this project was applied for a project studying the effects of running on an animal model of depression. The routine is composed of several steps, the preprocessing of the data mainly realized with SPM8, the processing using GIFT and the postprocessing which is some statistic tests on the results from GIFT in order to reveal differences between groups using the 2nd level analysis from SPM8 and the testing the correlations between components using the FNC toolbox.

1.2 Sammandrag
Detta dokument behandlar utvecklingen och implementeringen av en rutin för att analysera bilder från resting-state funktionell Magnetisk Resonenstomografi i gnagare. Även om resting-state connectivity studerats i människor i några år, med olika applikationer i psykiska störningar och neurogenerativa sjukdomar, är intresset för detta område är betydligt nyare bland experimentell förskare som arbetar med gnagare. Målet av denna projekt är att installera en procedur så att KERICs experimentell MR team kan lätt analysera resting-state funktionell MRT data. Under denna projekt har olika viktiga val gjorts, en av dem är att använda Independent Component Analysis procedur för att analysera data framför en seed-baserad teknik. En andra var att använda för anestesi medetomidin och inte isofluran för experiment.

Rutinen som var utvecklad under denna projekt blev användad på data från en projekt som studerar effekter av löpning på depression hos råttorna. Rutinen är delad i några delar, den första är att förbehandla data främst med SPM8, den andra är att använda GIFT för att behandla data och den sista är att testa statistiskt resultat från ICA med SPM8 och att testa korrelation mellan komponenter med FNC.
1.3 Résumé

Ce document traite du développement et de l’implémentation d’une procédure pour analyser les images en relation avec l’imagerie par résonance magnétique fonctionnelle à l’état de repos chez les rongeurs. Bien que la connectivité fonctionnelle cérébrale à l’état de repos est connue et étudiée depuis plusieurs années en recherche clinique avec diverses applications pour les troubles mentaux et les maladies neurodégénératives, l’intérêt pour cette technique est beaucoup plus récent dans le cadre des recherches expérimentales chez le rongeur. Le but de ce projet est d’implémenter un ensemble d’outils afin d’être capable pour l’équipe d’IRM expérimentale du Centre de Recherche Expérimentale et d’Imagerie de l’Institut Karolinska (KERIC) d’analyser les données d’IRM fonctionnelle à l’état de repos chez le rongeur selon une procédure claire et facile à suivre. Au cours de ce projet, plusieurs choix importants ont été faits, l’un d’eux est d’utiliser l’Analyse en Composantes Indépendantes (ICA) afin de traiter les données plutôt que d’employer une approche basé sur le choix d’une région d’intérêt (une « seed ») et de calculer le niveau de corrélation entre celle-ci et les autres régions du cerveau. Il fut aussi choisi d’employer la medetomidine comme anesthésiant plutôt que l’isoflurane pour les expériences.

La routine développée durant ce projet a été mise en application pour un projet étudiant les effets de la course à pied sur un modèle animal pour la dépression. La routine comporte plusieurs étapes, la préparation des données d’abord, réalisée principalement à l’aide de SPM8, le traitement des données à l’aide de GIFT ensuite, et l’analyse des résultats des tests qui se fait à l’aide de tests statistiques réalisées via les analyses de second niveau de SPM8 ainsi que le test des corrélations entre les composantes à l’aide de la boîte à outil FNC.
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3.2 Table of abbreviations

BOLD Blood Oxygen Level Dependent
DTI Diffusion Tensor Imaging
EPI Echo-Planar Imaging
fMRI Functional MRI
FSEMS Fast Spin-Echo MultiSlice
FSL Flinders-Sensitive Line
FWHM Full Width at Half Maximum
ICA Independent Component Analysis
MRI Magnetic Resonance Imaging
PCA Principal Component Analysis
rs-fMRI Resting-state functional MRI
SPM Statistical Parametric Mapping
4 Introduction

4.1 Subject of the research

The development of the routine to analyze resting-state functional MRI datasets was done within the context of a longitudinal study on an animal model prone to develop depression, The Flinders Sensitive Line. Hence, the data used in the project relates to the basic mechanism of depression and depression treatment.

4.1.1 Depression

Depression, also known as Major Depressive Disorder (MDD) or major depression, is a mental disorder characterized by episodes of low mood accompanied by low-self-esteem and loss of interest and pleasure in normally enjoyable activities.

It is estimated that depression affects more than 300 million people in the world, what is about 4 to 5 percents of the global population (1). There is thus a need for a better understanding of this disease.

It has been observed that people suffering from severe depression have a less voluminous hippocampus as well as a less voluminous anterior cingulate cortex (2). A more recent finding has shown using resting-state functional Magnetic Resonance Imaging (rs-fMRI) that the functional connectivity networks in the brain were altered in severely depressed patients compared to healthy controls to the degree that it was possible to determine from the data acquired whether or not the patient was depressed with a high accuracy (3).

It has also been shown that physical activity is a good way to prevent the development of depression. Therefore people with regular physical activity tend to have a larger hippocampus volume than the people who do not.

4.1.2 Flinders Sensitive Line rats, an animal model for depression

The Flinders Sensitive Line rats or FSL rats is a strain of rats that are selected for their increased responses to the anticholinesterase DFP. This strain has been therefore proposed as an animal model for depression as they expose similar symptoms and behavioral patterns as patients suffering of major depression (4).

Studies conducted prior to this thesis research has shown that FSL rats who had access to a running-wheel had a larger hippocampus volume (waiting for publication) as well as a reduced depressive behavior compared to the ones who did not have access to a running-wheel (5) (6), what matches with the clinical findings linking depression and a decreased hippocampus volume (2).

As a next step the goal is to investigate if antidepressive running provokes also changes in the functional connectivity networks of the rats. As the animals where single-housed for the duration of the study, a stressful and depression-inducing experience for rats, that are social animals, another goal is to investigate if single-housing provokes changes in rats’ resting-state networks.

In order to achieve these goals, this thesis research aimed to build the routine and tools in order to identify and compare the resting-state functional connectivity networks in rodents.
4.2 Concepts related to functional MRI

In order to further understand the content of the current report, it is necessary to introduce the Blood Oxygen Level Dependent (BOLD) contrast and resting state functional MRI (rs-fMRI), two important concepts relative to functional Magnetic Resonance Imaging (fMRI).

4.2.1 Functional MRI and the Blood Oxygen Level Dependent effect

Functional MRI is an MRI procedure that measures the brain activity by detecting changes in blood flow. The most common way to achieve this is to use the Blood Oxygen Level Dependent (BOLD) contrast.

When a brain area is active, its consumption of oxygen increases, leading to an increase of deoxyhemoglobin in the nearby blood vessels. After a few seconds, as a reaction, the blood flow increases, bringing a large quantity of oxygenated blood that overcomes the consumption of oxygen in the area.

The magnetic properties of the hemoglobin differs depending if it is bound to a dioxygen molecule or not, oxyhemoglobin being diamagnetic when deoxyhemoglobin is paramagnetic. Thus, deoxyhemoglobin’s magnetic field will decrease the relaxation time $T_2^*$ of the surrounding protons (7), hence decreasing the MR signal in the associated voxels when using a sequence sensitive to $T_2^*$ such as Echo Planar Imaging sequence (or EPI sequence). However, within a few seconds the vessels dilate and more oxygenated blood is recruited. The flush of oxygenated blood with diamagnetic hemoglobin increases the $T_2^*$ and the signal intensity. The increase of the blood flow will last up to 30 seconds and typically dominate the initial signal drop, making activated regions more intense. The MR-intensity changes due to the hemodynamics are called the Blood Oxygen Level Dependent contrast or BOLD effect and it typically has a signal accounting for 2 to 5% of the total intensity of a voxel.

fMRI scans are usually done to improve the knowledge of the areas of the brain that are related to a certain task, a certain activity or a pathology. To achieve this goal, it is most of the time asked to a subject to realize a particular task (tapping the finger, pushing a button) or a stimulus (watching images, listening sounds) is applied to him or her while the acquisition is going on.

This is a problem when one studies a pathology with disabled or sick patients are addressed as they might have difficulties to achieve the required task. Another problem is the fact that the results of the study are often depending on the manipulator conducting the study as a lot depends on the understanding of the task to achieve by the patient or on the way the manipulator applies the stimulus, bringing extra sources of variance to the results of the study.

When doing fMRI on animals, the manipulator has an even greater influence on the results as studies can be conducted only using stimuli induced by the manipulator on the subjects.

A good way to address these issues is to use resting-state functional MRI.
4.2.2 Resting-state functional MRI

Resting-state functional MRI is the use of fMRI when the subject is asked to relax and to not think about anything and no other stimuli or no task is required from him or her. In the case of animal experiments, the animal is under anesthesia and no stimulus is applied while using fMRI procedure.

The main purpose of rs-fMRI is to identify patterns in the brain activity from the spontaneous fluctuations in the measured Blood Oxygen Level Dependent (BOLD) MR-signal. In theory, if two areas of the brain are connected together, their BOLD fluctuations should covary in the time with or without a short delay, i.e. a lag time.

The applications to this modality have been mainly developed for humans in the past ten years to study mental disorders, degenerative neural diseases as Alzheimer disease, strokes and many others. Identification of resting-state networks within the brain has been also conducted, identifying more than 10 different networks involving many different brain regions within the human brain.

More than ninety percents of the neurological drug candidates fail due to a lack of a proven positive effect, what costs to the research and the pharmalogical industry a large amount of time and money, therefore there is a need for new and better methods to predict efficacy of those candidates before this stage. Hence more focus has been brought to the application of rs-fMRI to preclinical studies on animals and in particular on rodents. Rs-fMRI on rodents will allow a better understanding of pathologies involving the brain as well as a better understanding of the animal models and a more objective way to evaluate them. Rs-fMRI will also help in a close future to evaluate better the efficacy of the drugs compared to many current tests done nowadays such as the forced swim test in depression.

4.2.3 Previous research in resting-state functional MRI

Many studies were conducted using resting-state functional MRI in clinical research for more than ten years, those had mainly their focus on mental disorders such as autism (8), schizophrenia (9) or depression (3) but also on degenerative diseases, like Alzheimer disease (10), or on addiction (11).

However the shift towards resting-state connectivity in research involving rodents is more recent (12) (13) (14).

No clear consensus is reached on which method should be used to conduct the analysis on rodents. Various methods to analyze the data exist, the most widely used are:

- seed-based approaches, for which a Region of Interest (ROI) is defined before to watch at the correlation of its signal with other areas of the brain (8),
- the classification of the regions of the brain based on correlations between all the regions of the brain pairwise (13),
- the Independent Component Analysis (ICA) (12) (9), a multivariate statistical approach.

In this project it was chosen to use ICA, as a data driven approach rather than a hypothesis driven approach may be better suited for exploring the characteristics of this hitherto less well studied modality for studying brain function.
5 Thesis research project
This chapter describes the routine developed during the project as well as the practical implementation of the routine during experiments.

5.1 Design of the experiment
The study was done according to the following design: sixteen FSL rats have been divided in two groups, where half of the rats had access to a running wheel during single housing while the other half did not have access to a running wheel.

All the rats were scanned before and after the single-housing as the goal was to reveal changes related to antidepressive running but also changes related to single-housing.

The two scan sessions were done in the same way. The rats were first anesthetized using isoflurane, an anesthetic gas and positioned in the scanner. Thereafter the animals were given a bolus injection of 0.1 mg/kg of medetomidine through a subcutaneous catheter and the isoflurane was discontinued. Five minutes after the bolus a slow infusion of 0.05 mg/kg/h was initiated. Medetomidine is considered the anesthesia of choice for rs-fMRI but is not efficient for inducing anesthesia, which is the reason for the two step anesthetization. However, isoflurane is vasodilating, and decreases the BOLD contrast. To ensure that the effect of isoflurane was gone, the acquisition of the rs-fMRI datasets was commenced 90 minutes after the discontinuation of isoflurane. During the waiting time all prior scans, the planning of the slice, the optimisation of the magnetic field (also called shimming) and the acquisition of the reference scan were done.

After one hour and a half the first fMRI dataset (also called BOLD dataset later in this report) were acquired.

Then a scan using another modality called Arterial Spin Labeling was acquired. This modality enables the determination the Cerebral Blood Flow for the different areas of the brain by using an extra coil to label the blood in the arteries of the neck before to watch where this blood goes.

A second BOLD dataset was acquired after the Arterial Spin Labeling dataset (about half an hour after the first BOLD dataset).

5.2 The routine
The main focus of this thesis project was to develop a routine that aimed to extract the resting-state networks from the datasets acquired and be able to compare them for the different subjects.

For making it successful, several sources of noise or variance have to be considered. Firstly the animals might move during the acquisition of the datasets, also they breathe and their heart is beating, all of this might affect the images acquired by the datasets through motion or change in the intensity of the images.

Secondly the noise from the coils and all the chain of the receivers has to be considered. Indeed we expect a BOLD contrast that accounts for less than 5 percents of the total intensity of the image, when thermal noise might also be in that range.
The differences in size and weights of the subjects were also taken into consideration and moreover we tried to reduce the influence of the geometric distortions that are intrinsic of the EPI images.

Each of these problems is addressed by the routine through different techniques that should be used all in the right order.

First to reduce the influence from the geometric distortions, a maximum entropy method has been considered. Also another type of image reconstruction called SENSE has been investigated to unwarp possible aliasing artifact and to increase the signal to noise ratio (SNR).

Then, to account for motion during the acquisition of the datasets, the images are realigned for all the different time points. The realignment procedure may also mitigate potential problems associated with heating of the gradients and drift of the magnetic field.

The BOLD datasets are then fitted with a structural image of the same subject, before to match all the subjects’ data together using a common template in order to reduce the influence of the differences in morphology of the different animals.

Datasets are then smoothed to reduce the influence of the thermal noise and filter through a band-pass filter to reduce the influence from the morphological noise (heart beats mainly).

Datasets are finally processed through using an Independent Component Analysis in order to reveal the resting state networks.

The results from this analysis are then compared using two different methods, second order statistical parametric mapping and a correlation-lag test.

All these steps are summarized in the diagram presented in the figure 1.
The main improvement of this routine compared to before studies is the application of rs-fMRI to watch at the effects of a treatment, voluntary wheel-running here using the results of the routine, the maps of the connectivity networks obtained using ICA, to make statistical comparisons. A comparison between strains of rats could be done using the same routine. This is a step further than what was done so far in the field of animal rs-fMRI.

The tools used in order to achieve this routine are all presented in the Appendix D at the end of this report.
6 Methods used in the routine
In this chapter the methods and tools used in the routine presented in 5.2 are presented. The first part will present the different steps of the preprocessing of the datasets before to present in a second part the methods related to the processing and revealing of the resting-state connectivity networks. The third part will present the steps that are not strictly part of the routine but are necessary to exploit the results and have been called here postprocessing. Finally the last part will describe an important technique for the success of the routine, the skull-stripping.

6.1 Preprocessing steps

6.1.1 Image reconstruction
The image reconstruction is the first step taking the raw (k-space) EPI data from the scanner and removing artifacts from the data taking in account the variation in the sensitivity of the coils and variation of the magnetic field. We use also a reference volume for the same subject acquired with FSEMS (Fast Spin-Echo MultiSlice) sequence.

6.1.1.1 SENSE reconstruction
The SENSE reconstruction is a technique that allows reducing acquisition time in MRI. This technique uses the sensitivity information from the different channels of the coils (the volume coil and the surface coil) to reconstruct an image acquired with a reduced field of view, and having as a consequence a folded image as a result of the acquisition. The goal of SENSE is to make the correction of these data in order to unfold the data acquired by solving a set of linear equations:

\[ E \cdot f = d \]

Where \( d \) is the vector containing all the k-space data acquired from all channels, \( f \) the unknown vector containing the desired full field of view data and \( E \) the encoding matrix using the sensitivity values of the matrix for the full field of view (15).

During the thesis research a program for the SENSE reconstruction was written. This program takes the fid file (k-space encoded data), separates the values obtained by each of the 4 channels of the surface coil and treats them separately. The Fourier transformation is done for each channel data before to use the results to solve a system of equations with the sensitivity maps calculated from 2 reference images acquired by a volume coil and the 4 channel surface coil as coefficients.

6.1.1.2 Maximum entropy algorithm
Maximum entropy method (MEM) is a method to reconstruct the real image from the data that were acquired with some distortions. The principle is to maximize the entropy of the data taking into accounts some constraints such as a reference image in order to get the image that is representing the best the state of knowledge we have.

Entropy is a statistical notion defined by:
\[ E = - \sum_{i=0}^{n} p_i \ln \left( \frac{p_i}{r_i} \right) \]

Eq. 2

Where \( p_i \) and \( r_i \) are respectively the probabilities (or relative intensity in this case) of the distribution we are looking at and the one of the reference and \( n \) the number of different possible states (number of pixels).

In theory we must find the distribution \( P \) maximizing \( E \) in order to get the image without distortion.

In practice, we prefer to maximize the Lagrangian data \( L \) defined as:

\[ L = E - \lambda \ast C \]

Eq. 3

With \( C \) a \( \chi^2 \) distribution defined as:

\[ C = \chi^2 = \sum \left( \frac{F_k - p_k}{\sigma_k^2} \right)^2 \]

Eq. 4

With \( F_k \) a fit function and \( \sigma_k \) the standard error at the pixel \( k \).

### 6.1.2 Motion correction - Realignment

One of the problems faced during data acquisition is motion. The subjects can move during acquisition of a time-series of images, resulting in a shift of the object on the images of a few pixels affecting the value of the intensity at a particular position significantly. There may be also so-called pseudo motions, when the magnetic field changes during scanning, either due to a modified distribution of magnetic susceptibilities for example when the animal is filling the lungs with air containing paramagnetic oxygen or a temperature change of the gradient coils.

This problem could be corrected using a realignment algorithm. This is achieved using the `Realign` function of the SPM toolbox in MATLAB.

This function uses a least-square approach and a 6-parameter spatial transformation to realign all the images of a dataset to its first image which is used as a reference. Hence, the algorithm optimizes translation and rotation by reaching the minimum of the sum of squared differences between a reference image and the image from each time point (16).
6.1.3 Fitting functional images to structural image - Coregistration

Another problem that could be faced during the project is when we acquire data for the same subject with different sequences, there might be some shift in the object between the two acquisitions, but also some geometric deformations and some differences in their contrast. The coregistration step aims to correct the geometric deformations and align the images obtained from the two different sequences to fit each other.

In this case, two data were acquired with an EPI sequence with important geometric distortions and a reference scan was acquired with a FSEM sequence. It is important to fit the images of the brain from the EPI sequence with the images from the reference scan so that the regions that are activated during the EPI can be identified, but also in order to be able to align the data from all the subjects together.

We use the Realign function of SPM in order to achieve this purpose. This program uses a procedure (described in the article written by Collignon et al. (17)) in which an objective function is minimized. The program gives the choice of the objective function to be minimized. The Normalized Cross Correlation function was used as the program’s help recommends to do for a coregistration within a modality (here both scan are done using MRI). From the estimation done through the minimization of the function achieved by manipulating a source scan to fit with the structural reference, obtained parameters are used to apply an affine transformation to all the volumes to be coregistered in order to fit the reference image.

6.1.4 Fitting all datasets to a common template - Normalization

Another challenge of this analysis is that the brains of the different rats in a study may have different size and shape, although the differences are rather small in comparison to humans, due to the fact that the rats are all of similar age, have been exposed to similar environment and are genetically very similar. When investigating group differences it is thus necessary to determine how to match voxels from different subjects, which is the spatial normalization problem.

After the datasets from each subject have been coregistered, they should be normalized. The normalization step aims to fit all the brain images of all the different subjects to a common template, i.e. the « normal » brain so that all the brains that have originally different dimensions have their structures fitting each other. This is needed since the goal of this study is to realize a group analysis where all the data are processed in the same time and in the same way.

To fit all the subjects’ data to a common template we use the Normalize procedure in SPM. Generally the algorithms used in this procedure work by minimizing the sum of square differences between the image to be normalized and a template. To obtain an unbiased result, the contrast of the template should be similar to the contrast in the image to be normalized. The normalization procedure works in 2 steps. The first step is to determine the optimum 12-parameter affine transformation. The second step is the estimation of nonlinear deformations which are defined by a linear combination of three dimensional discrete cosine transform basis functions (18).
6.1.5 Noise reduction strategies

All variations in the data which do not reflect brain activity may be considered as noise. During the acquisition of the data, there are many sources of noise that can disturb the results, from the thermal noise of the sensors, i.e. the coils, to the influence of the breathing and the cardiac rhythm (also called physiological noise). Also there may be little inconsistencies in the results of the normalize procedure. Variations in the data from regions not within the brain do not reflect brain activity and is considered as noise. To reduce the influence of those nuisances on the results we must filter them from the BOLD signal.

Hence three different strategies are used:

- the images are smoothed spatially,
- the signals for each position (voxel) are filtered temporally, removing the contribution from the structures of the body (continuous part of the signal) and the high-frequency signal that are under the response time necessary to the BOLD effect (about 7 to 10 seconds).
- The last strategy is the Principal Component Analysis that will be described in 6.2.

First and second strategies are described below.

6.1.5.1 Band-pass filter

A first strategy to reduce the noise is to filter temporally the data. This has for goal to reduce the influence from physiological noise.

For this purpose a band pass filter (0.01 Hz - 0.1 Hz) was programmed using the Fourier transform function.

This program takes all the images of a dataset, create a 4D matrix (with the dimensions: x, y, z and t), make the Fourier transform in the dimension t for each given position (x, y, z), this corresponds to make the Fourier transform of the timecourse for each voxel of the dataset. Then the program sets all the values in the resulting 1D matrix that are over and under the allowed frequency band to zero, make the inverse Fourier transform of the resulting matrix before to save the dataset obtained into a new file.

Chosen frequencies are similar to the ones seen in other articles (12) (13) treating about rs-fMRI and it seems to be a good choice of frequency to filter out the influence of the cardiac rhythm (frequency range of 200-600 beats/min for a rat (over 5Hz)) and breathing (frequency range between 20 and 90 beats/minute under anesthesia and between 50 and 85 beats/minute during acquisitions of BOLD datasets (over 0.3Hz in the case of 20 beats per minute)).

6.1.5.2 Smoothing:

A second strategy to reduce the noise is to smooth spatially the images using a Gaussian kernel. This procedure aims to reduce the thermal noise and the inconsistencies from the results of normalization by blurring the resulting images.

This is done using the Smooth function in the SPM toolbox. This procedure applies a Gaussian filter to each volume (the stack of slice correspondent to a time point) with the Full Width at Half Maximum (FWHM) set by the user. Here a FWHM of 0.5mm within the slice and 1mm between slices were used as they are similar to the voxel dimensions of the data acquired using the EPI sequence. The success
of this approach relies on that variations in the BOLD signal for adjacent voxels should be related and the thermal noise is random (following a Gaussian distribution).

6.2 Processing steps

6.2.1 Concatenation of the data: Principal Component Analysis
Once all the previous steps described in 6.1 are done, we have a set of images that are fitted to the same template and ready for the processing. However the amount of data is large and the data still contains some noise and some irrelevant signals. Therefore an ultimate preprocessing step is needed in order to reduce the amount of data to be treated by the Independent Component Analysis. This preprocessing step is the Principal Component Analysis (PCA).

The PCA is a mathematical procedure that decomposes some observations, the functional volumes for each timepoint, into a set of linearly uncorrelated variables, the principal components. The number of components is inferior in any case to the number of observations (in our case the number of timepoints, i.e. 300). This way, the part of the signals that cannot be obtained by a combination of the basis created during this procedure are filtered out, those will be mainly contributions due to noise or minor contributions as the components are obtained in the order of their contribution to the total variance of the data.

In case of a group analysis like in the current study, two PCA are applied to the data, the first is applied to the data from each subject separately and then a second is done on the results from this PCA. These two steps (the PCA on individual subjects’ data and the group PCA) are implemented as preprocessing steps within the GIFT toolbox that does the Independent Component Analysis.

6.2.2 Independent Component Analysis
In order to reveal the networks, a statistic method called Independent Component Analysis (ICA) is used. It is a method for finding underlying factors or components from multivariate (multidimensional) statistical data such as the evolution of the intensity of pixels over the time (the timecourse of each voxel). It is a blind source separation method, meaning that it separates the signals into distinct components and locate the source of the signals. A typical example of the problem it solves is the separation of the voices of different people talking in the same time within a crowd.
What distinguishes ICA from other methods for functional MRI processing is that we do not need to introduce any prior paradigm or epoch nor identify any region of interest as a seed for the analysis.

The procedure can be described by the following simple equation:

\[ X = A.S \]

Eq. 5

Where \( X \) is the matrix of the observations (the timecourse of each voxel), \( S \) the resulting Spatial Maps of the different components and \( A \) the timecourses of these components. In the case of the crowd, \( X \) would be what each person in the crowd hears (or what each microphone records over time), \( A \) would be the separated voices of the different speakers and \( S \) would be how much of each of these voices is recorded by each microphone or heard by each person in the crowd. The combination of the separated voice and the maps of where the voices spread are called independent components.

This procedure is applied on the data resulting from the PCAs.

![Figure 3](image-url)

The implementation of these two steps PCA and ICA is done by using the Matlab toolbox called GIFT.

6.3 Postprocessing steps

6.3.1 Functional connectivity network identification: Correlation-Lag test

Once the different components are obtained from the ICA analysis, pointing out regions of the brains that are correlated together, it is interesting to see if some components are related to each other and form a network or if they reflect unrelated phenomena. The MATLAB toolbox FNC facilitates the investigation of the relations between the independent components obtained from GIFT. Two variables are taken in account, the correlation and lag between signals (cf. fig. 2). If two components are correlated together for all the subjects of a group and have a low or no lag between them, then they are part of a same network. It is an assessment of the effective connectivity between brain regions.
This procedure has for goal not only to help build the resting state networks within the brain but also to make comparison of the effective connectivity between the groups within a study. Hence, it is possible to make a two sample T-tests between two groups defined using the correlation and lag values to make the test, the same that are used to watch at relations between components within a group. If a systematic difference is observed between the groups, it will be shown in case it fulfills a certain requirement (to be with a p-value under 0.05 for example).

The higher the correlation between the timecourses of two components, the more related they are considered to be. If two timecourses (for two components) are highly correlated, the corresponding brain regions (shown on the corresponding spatial maps by a high value) are functionally connected, the value of the correlation shows then the strength of the connection.

6.3.2 2nd level Statistical Parametric Mapping: T-Tests and Multiple Regression

A T-test is a statistical hypothesis test which is frequently used to test if normally distributed means of samples from different groups may be trivially explained as caused by chance. If two distributions are compared, to test if their means are equal this is called a two sample t-test. In case these observations are done on the same subjects at different times, this is a paired t-test. The t-distribution may also be used as the reference distribution in multiple linear regression and other tests.

For this study, the spatial maps of each component for the different subject are the observations to be used. Those should be used for different test as the observations can be related to different groups.

For example, the influence of wheel-running can be tested by setting a two sample T-test with one group being the results for the rats having access to a running-wheel (“runners”) and as another group the results for the rats not having access to a running-wheel (“non-runners”).

This test can be done through a utility from the GIFT toolbox, however the results have to be checked using the SPM toolbox.
Paired T-test was used to test the influence of single-housing by setting for each subject on one hand results obtained before single-housing and on the other hand the results obtained after single-housing.

A multiple regression was done by using the results for all the subjects at once and defining several covariates in order to test diverse hypothesis. The covariates typically introduced define what results relate to before or after single-housing, to runners or non-runners and to dataset 1 or dataset 2.

These tests can be done using the 2nd-level analysis function from SPM and the results have to be checked using SPM too.

SPM being designed for humans, the checking of the results isn’t convenient with it, so ImageJ is used to inspect the results of the tests in complement to SPM.

![Statistical analysis: Design](image)

**Figure 5**: Design matrix for a multiple regression with for regressors in the order: Data from Runners, Before treatment, Scan 1 or 2 and After treatment. The rows of the matrix represent the images to compare and the columns the covariates. The change in colors represent different values of the regressors for the corresponding images.
6.4 Skull-stripping

A big difference between rodents and human is in the position of the brain in the body. If for humans, the skull is surrounded by little amount of tissues (skin, ears and hair mainly), for rats the skull is surrounded by some muscles among other tissues. This surrounding tissue is not of interest for the study as it does not reflect the cerebral activity of the subjects. Moreover it complicates the task to fit the scans of the brain of the subjects, first when coregistrering and then when normalizing.

This is why it is important to reduce the influence of these tissues on the results of the study. A simple way to achieve is by deleting unnecessary tissues from the scans. This is what is called skull-stripping. The use of skull-stripping helps for coregistering and normalizing the datasets, but it also allows having a mask to use for the Independent Component Analysis that limits the processing to the brain, improving significantly the performance of the analysis.

Many programs exist to achieve skull-stripping, however those are mainly designed for human brains and no automatic approach has found general acceptance for rodents. This leads to the need to delete the tissues manually using an image treatment software such as ImageJ.

Skull-stripping is achieved in the following steps:

a. Thresholding the image choosing an appropriate low cutoff value for which regions in the images with lower intensity are discarded. The result from this step is a binary image with discarded regions having an intensity of 0 and 1 for the regions kept.

b. Clearing the binary image: The brain is not the only organ with a high intensity on the image, so the regions that are not part of the brain are cleared manually.

c. Masking the original image with the newly created mask. This step gives the same image as the original but without all the surrounding tissues.

d. Cleaning the result from eventual extra tissues.

A macro was given by AZ MR team in order to facilitate the skull-stripping.

Figure 6: Slices of brain before and after skull striping. From left to right there is respectively a reference brain image before and after skull-stripping and the corresponding functional brain image before and after skull-stripping.
7 Results

In this part, the results from the different steps of the routine are presented, and some challenges faced are exposed together with the solutions found for each of them, if any. Those parts will be treated in the order in which they come within the routine when taking the raw data until the final outcomes. First are addressed the image reconstruction attempts, then the realignment step, followed by the coregistration and the normalization, before to continue on the smoothing and the time-filtering. The last part presents the results from the processing steps themselves and finally the postprocessing steps.

7.1 Image reconstruction

The image reconstruction steps, both the SENSE reconstruction and the use of the Maximum Entropy reconstruction have for goal to improve the quality of the images obtained from the EPI sequence by rebuilding the volumes and removing folding artifacts for the first and by correcting the problems of geometric deformations for the second.

The program written for SENSE reconstruction did not work out as it was expected, despite attempts to use smoothed image to make the sensitivity maps in order to reduce possible problems due to their noise. The attempts to solve the problems were not pursued as the program was not necessary for making the resting-state fMRI data treatment successful and were rather a side task.

About the Maximum Entropy algorithm, even though many documents about the method were read as well as scientific articles, no serious draft got written. An article suggesting the inefficiency of the method for MRI applications (20) and the timing made that the focus of the project got towards the rest of the project and the analysis of the resting-state fMRI data.

7.2 Realignment

The realignment is the step during which the data of a session are realigned together. In this case the first image chronologically was chosen as a reference to align all the images. There was no reason to use any other images in particular as they looked all very similar and the motions seemed minor.

This was confirmed by the graph (cf. Figure 7: Translation and Rotation of a subject over the two acquisition sessions (300 volumes each), we see a glitch after the 300th image, corresponding to the transition between the two acquisitions. for an example for one of the subjects) of the motions given by the estimation done during the execution of the Realign function.

It was estimated by the function that the subjects were moving by less than 200 microns during the whole scans in any of the directions and about 0.1 degree for its rotation (cf. Figure 7: Translation and Rotation of a subject over the two acquisition sessions (300 volumes each), we see a glitch after the 300th image, corresponding to the transition between the two acquisitions., equivalent to less than the dimension of a voxel (i.e. 0.5*0.5*1 mm³). This could be expected as the rats were anesthetized and have been fixed to the rig using a tooth bar and ear bars (those bars are little plastic objects that are meant to mechanically fix the animals in order to avoid any involuntary
motions, those objects are meant to break in case the animal make a big movement so that the animal does not get hurt).

![Translation and Rotation](image)

Figure 7: Translation and Rotation of a subject over the two acquisition sessions (300 volumes each), we see a glitch after the 300th image, corresponding to the transition between the two acquisitions.

### 7.3 Coregistration

The coregistration step is used to fit the functional MRI data acquired with an EPI sequence with a structural image acquired for the same subject with a fsems sequence after having realigned the fMRI data together.

This step was not included at first in the routine as it was not simple to make it work and seemed as not necessary as all the rats should be very similar in size, age and other characteristics.

But after a first trial to process all the data together, two major problems were faced:

a. how to know where the components displayed really are within the brain (the EPI images have some geometric distortions)

b. how to know if the components points are corresponding to the same regions of the brain for all the different rats (We realized that even if the rat colony should be very similar, rats had very different sizes and weights, but also different sizes for the brains).

To address the first problem (a), the coregistration step seems to be a good solution, for the second problem (b) it is a necessary first step to address it before to use the normalization step.
Several trials were done to realize the coregistration using the structural image as a reference and the mean image of the realignment step as source image without any other particular precautions, they all failed. Since this function was working with the example data given with the toolbox using a human brain, this having for characteristics to have no tissue or almost none surrounding the brain, I came to the conclusion that it is needed to skull-strip my reference and my source images. A major improvement can be seen in the condition that the skull-striping is done carefully (cf. fig.5).

![Figure 8: Importance of good-skull-striping of the source images for coregistration. On the top left: a badly skull-striped slice. On the top in the middle: the same slice well skull-striped. On the top right: a slice of the volume before coregistration. On the bottom left: the same slice coregistered with the source image having the bad skull-striping. On the bottom in the middle: the same but coregistered with the source being well skull-striped. On the bottom right: the corresponding slice of the reference volume.](image)

A last issue related to the size of the outcome data was however faced. The Coregister function gives, as an outcome, images of similar size and resolution as the reference image. A reference image that has for size 512*512*11 voxels was used at first to coregister 600 images of 64*64*11 voxels for 27 different sessions, a problem of space on the hard drive was faced in the same time as a very long processing time. It was calculated that about 170 GB of hard disk space was needed to store the output of this processing step. This was clearly impossible.

An attempt to reduce the size of the reference image to the same size as the source image (i.e. 64*64*11) was made. This gave a satisfactory result with the need of only 3GB to store the results.

### 7.4 Normalization

Normalization is a technique that has for goal to fit data from diverse subjects with the same template image in order to fit the anatomical features of the brain of the different subjects together.

This step comes directly after the coregistration and use the structural image of the different subjects (used in the previous step as reference images) as the source images for this step. Those structural images are used to estimate the transformation to apply to the coregistered functional images.

The template image used is a structural image from the pilot study of the project, the image was skull-striped as the ones for the coregistration step.
Normalization of the data permits to align all the data to be treated and thus brings a visible improvement to the results given by ICA. The components spatial edges seem clearer and the regions shown fit the structural image, making it easier to interpret the results (cf. fig.6).

Figure 9: Difference in the results of ICA depending if normalization is done or not during preprocessing steps. a. and b. A component in the striatum without and with normalization respectively. c. and d. A component in the somatosensory cortex without and with normalization respectively. We can see that with normalization the resulting components are sharper and better aligned with the structural used to superpose the results.
7.5 Smoothing and time-filtering

7.5.1 Smoothing
The smoothing step is realized in order to clean the data from the thermal noise, but also to correct the potential mismatch left after normalization. It has been done systematically after the normalization. A 0.6mm in-plane and 1mm between slices for the FWHM for the Gaussian filter were chosen to apply as the resolution of the images to be filtered is 0.5mm in-plane and 1mm for the thickness of the slices. The resulting images are a bit blurrier than the originals, but the noise is in the same time significantly reduced with a Signal Noise Ratio (SNR) of less than 20 before smoothing and of about 50 after (cf. fig.7 and fig.8). The SNR is calculated using the following formula

$$SNR = \frac{\mu}{\sigma}$$

Eq. 6

Where $\mu$ is the mean pixel intensity value over a neighborhood (here in the brain region) and $\sigma$ the standard deviation for the same area

![Figure 10: slice after normalization but before smoothing](image)

![Figure 11: the same slice after smoothing](image)

7.6 Temporal filtering of the data or Time-filtering
As told in the methods, time-filtering is necessary in order to clean the data from the influence of the cardiac rhythm and breathing, as well as slow scanner drift, on the data. A first script was written using a Butterworth band-pass filter with 0.01Hz and 0.1Hz for the filter bandwidth. The filter was working as a moving average. However it had two major drawbacks: It needed the Signal Processing toolbox from Matlab to work limiting the number of computers that could use it, but more importantly, it was screwing the results from the ICA, showing all similar variations at the beginning of the timecourses (cf fig.9).

![Figure 12: Timecourses of diverse components given by ICA when preprocessed with the Butterworth filter](image)
A new script was written using the Fourier transform function fft and its inverse ifft. The two drawbacks from the previous function was solved and the results seemed better (cf fig.10).

![Image](image.png)

**Figure 13:** Timecourses of the same components as for figure 12 given by ICA when preprocessed with the Fourier transform filter

As the FT-implementation of the band-pass filter retains 54 out of 300 frequencies it will improve signal to noise by \( \sqrt{\frac{300}{54}} = 2.35 \) times.

### 7.7 Processing: PCA and ICA

I will describe PCA and ICA together as those are both done at the same time one after the other.

For this step, the toolbox, GIFT, allows the user to change many parameters, from the most basic such as the number of components to use for the ICA analysis or the number of steps and components for the PCA to the algorithm for the spatial reconstruction, the choice of the algorithm to use to make the ICA or the scaling to use for the results.

In this step, the default values were used for most of them except for the preprocessing step for which it was chosen to remove the mean intensity over time for each voxel (the other choices are to remove mean per timepoint, intensity normalization and variance normalization). I chose this option as it is equivalent to not do any change to the data as the mean was already removed via the band-pass filter, when the default option (remove mean per timepoint) would be equivalent to global signal regression that appears to bias the results of the analysis by introducing false negative correlations as suggested by Weissenbacher et al (21).

A major issue in order to get good results from this step is the mask to be used. If the default mask is used, it doesn’t give any conclusive results but empty components as the surrounding tissues are influencing the analysis. It is necessary here to mask out all the non brain tissues. This was done by making a binary mask from the skull-striped reference image. The binary volume was dilated so that it would allow a margin for the size of the brains from the data being used. A major improvement could be seen thanks to this change.

Diverse numbers of components were tried for the ICA but finally 30 components was kept as suggested by Jonckers et al. (12). However it is hard to evaluate the best number of components to be used. If a low number would certainly cut out some interesting components, a too high number
has the inconvenience to make some networks to be divided in two or more different components, and may be catastrophic for many classification algorithms.

It seems also that the choice of the number of components for the first step of the PCA has an influence on the results of the ICA analysis when everything else is kept the same (cf. fig.11).

![Figure 14](image)

*Figure 14: Influence of the first data reduction step on the final result. a. and c. Spatial components obtained with 45 components for the first step of the PCA and 30 components for the ICA analysis. b. and d. Corresponding spatial components but with having 75 components for the first step of the PCA, the rest being the same. It seems that data obtained with 75 components for the PCA show reduced negative areas (blues areas) compared to the ones with 45 components. Positive contributions are though really similar.*

### 7.8 Postprocessing: Identification of the networks.

As explained in the method part, this step aims to evaluate the correlation between the components revealed by the Independent Component Analysis in order to verify if the correlation between two components’ timecourses is significant and consistent within a group of individuals and if there are consistent and significant differences between two groups. This is done by making T-tests on the correlation between the components’ timecourses.

By using the FNC toolbox, it was tried to reveal some significant differences between groups. The toolbox worked and gave some results, so the technique is working.

However the results obtained were hard to interpret and seemed to be not consistent with the hypothesis emitted by the biologists of the team.
Also, as a better understanding of the back-reconstruction of the components for the individuals has arisen, this method revealed to be doubtful when it comes to the results it gives, indeed the individual components being built from the group components, the components’ timecourses for the different individuals are not ensured to be statistically independent.

7.9 Postprocessing: T-test and Multiple regression

Very often in medical sciences, the researcher formulates a hypothesis in the form that the means from two groups are different for some particular property. The standard approach is to measure the particular property for a number of subjects from each group and use a statistical test, most often a T-test, to test if the observed difference in the means may have been caused just by chance, and if it is found unlikely it may be considered to support the hypothesis. The interest of the statistical analysis such as the two samples T-test or the Multiple regression is to compare the results obtained from the processing step, here the Independent Component Analysis and test them against a certain hypothesis.

Here the hypothesis tested was the fact that running has an influence on the functional connectivity of the animals. In order to reveal these differences, components’ spatial maps of the “runners” and of the “non-runners” were used in a two sample T-test.

Several difficulties were faced, first of all what is the confidence window that should be used as a limit to consider that a result is significant and not random, is 95% confidence (p-value < 0.05) enough? Or should it be 99.9% confidence (p-value < 0.001)?

A second difficulty was how to interpret the results. If I have a positive value, does it mean that the area is better connected in one group compared to the others?

The maps obtained from the analysis were not showing huge differences between the groups as such. This can be probably explained by the fact that the size of the groups (8 animals in each) does not allow for a very strong statistical power unless the differences are important.

A last issue was discovered, some data have some shown sudden large jumps in intensity in their timecourses due to a technical issue, probably related to the break of the transmitter coil during the course of the experiments. This issue is dominating over the BOLD signal and might corrupt the
results obtained by our analysis (cf. fig. 13). An effort was done to save the datasets affected by the technical failure trying different strategies among which the most successful so far was to use the intensity of the navigator echoes acquired simultaneously with the datasets.

Figure 16: Timecourse of a voxel of one of the corrupted datasets. It is clear that the intensity is jumping over time in a random fashion and is dominating the normal fluctuations of the signal.
8 Discussion

This study shows the possibility to create a pipeline for analyzing data from resting state functional MRI on rodents. This routine has been set at KERIC and has shown its efficacy to treat the images in a systematic way. This consists in a few steps: Realignment of the fMRI data, Coregistration of the fMRI data to a reference volume of the same subject, Normalization of all subjects to the same template, Spatial smoothing, time filtering and finally group ICA analysis of the fMRI datasets.

If the process is well defined, there are still few substeps that are not yet automatic but require an arbitrary intervention of a human operator, those are the skull-stripping of the data to be used as source images or references or even as a mask. These operations are time consuming and from the quality in the execution of these steps depends a lot the quality of the results of the analysis. That why these substeps are reduced to a minimum of operations through a macro so that sources of mistakes are reduced as well. However this still needs to be improved in the future. The need to use different softwares is also a problem of this substep as the data are read differently by the both softwares.

One of the major problem to establish a systematic path to analyze the data is the variance between subjects (different sizes and weights as well as different size of the brain), and the differences in the conditions in which the experiments are done. To solve this, several experiments were conducted during this study to try a new approach that aims to reduce as much as possible the variance in the acquisition of the images by using few recognizable structures of the rat brain to align the images, center them and make the slice planning so that the images would be as similar as possible. However, we observed some differences in the results of the images due to differences in the size of the brains of the rats despite the fact they were all of the same strain, the same gender and the same age as well as they were handled all in a similar way.

There are several sources of variance in the results from our datasets with which we had to deal with: size of the brains and their orientation, physiological nuisances such as the respiration and the heart rhythm, motions during the acquisition of the datasets, slow scanner drift and thermal noise, also some differences in the weight and physical condition of the animals might have affected the way they react to anesthesia. A last source of variance might be the sensitivity of the animals to the noise within the machine (the EPI sequence is really loud, more than 100dB and the animal even anesthetized might react to it).

Therefore several steps of the routine aims to reduce the effects of these sources of variance, firstly the realignment of the volume has for goal to solve the problem of the motion as does the physical fixation of the animal to the rig.

Secondly, the coregistration and the normalization steps reduce the effect of the variation in size and orientation of the brains of the animals by fitting them to a same reference image. We can however question the efficacy of these steps as the geometric distortion of the brains around the ear channels are such that the general shape of the brains are very different between the reference image acquired with FSEMS and the EPI images. A proper study to evaluate the efficacy of these steps should be done. Also the best parameters to use should be investigated further for these methods.

The spatial smoothing is done to reduce the thermal noise with a great efficacy, increasing the Signal to Noise Ratio from 20 to 50. And the time-filtering reduce the influence of the physiological noise by
filtering out their nominal frequencies as well as the first harmonics. However it would be good in the future studies to record the breathing and the cardiac pulses in order to use it later to evaluate their influence on the results of ICA and to use these signals as references to sort these results temporally. It should also be done for the sounds within the camera’s room.

Another major improvement to the routine would be to fit a Brain Atlas (a set of maps of the brain of an animal) to the reference image so that we would be able to discriminate brain regions during the preprocessing steps but also to identify the functional networks more easily. Similarly, it would be good to adapt the SPM toolbox to be able to use glass brains from rodents (a glass brain is a 3D representation of the brain where only the general outer shape is shown, SPM use a built-in human glass brain fitting the templates provided with the toolbox) to display the results from the statistical analysis. This is the purpose of the toolbox for SPM called SPMMouse, however nothing such exists for SPM8 and it seems that if this toolbox works for making Voxel Based Morphometry (a method to segment and measure the size of a certain brain area and compare it between groups of a study) on rodents, it does not work for making fMRI studies requiring also to use some other tools such as GIFT for example.

Therefore an effort should be put in the future to make an Atlas of rats fitted with MRI images but also to get probability maps for white and grey matter as well as for Cerebral Spinal Fluid (CSF) (a probability map is a set of images showing the composition of the brain for a certain type of tissue, as brain regions are often a mixture of different tissues). This might be achieved within KERIC using the opportunity given by the Diffusion Tensor Imaging (an MRI technique that allows investigating the direction followed by the axons in the nervous system by determining the direction for which the water molecules diffuse) studies done recently for rats and for mice.

A last effort must be done to learn how to use the results from the ICA analysis in order to quantify differences in the functional networks between groups within the studies. A first step has been achieved in this direction by setting some two-sample t-test as well as a multiple regression on the component maps as well as by trying to quantify the correlations between components’ timecourse for the different groups of the study, but this must be continued to harvest the fruits of the efforts put in rs-fMRI as the spatial maps does not have much significance by themselves and are hard to interpret alone.

Another future step is to learn how to use the results from the ICA analysis in correlation with results of other experiments such as behavioral studies, Arterial Spin Labeling experiments, Diffusion Tensor Imaging (DTI), etc. This demands to quantify the obtained results from the data and pushes for the use of an Atlas reference as told earlier in this discussion, but also for the use of the statistical tools.

A last interesting path would be to learn how to use functional connectivity data from resting state analysis to use them for investigating effective connectivity via a seed-based approach or Dynamic Causal Modeling. This would allow to map the different networks of the brain and to get the big picture of the networks of the brain, to build a functional connectome to put in relation with the structural connectome obtained with DTI modalities and histology.
9 Contribution of the project to my personal development

This degree project has been a great experience for me. It has been the occasion to discover and learn a lot about neurology, a field in which I had no knowledge before, but also the occasion to deepen my knowledge in MRI and in image analysis. I definitely learned a lot too in using Matlab and ImageJ, two tools that I was using every day during this project. I learned a lot also about the different multivariate analysis techniques but also about the different statistic tests.

But the project was a good experience not much because of this but rather because it gave me the occasion to discover a new work environment with a different way to deal with interprofessional relations than the ones I had in France, showing me the possibility to have a flatter hierarchical organization and be efficient in working and taking decisions.

It was also a good training for learning on how to plan my time and organize my work and it prepared me to be a better engineer.

Also I realized that my understanding of the whole project changed a lot between the beginning of the project and now and I believe that I’m now ready to lead projects in the development of new tools for functional MRI to a higher level and why not continue the development of this project as a PhD student.
10 Acknowledgements

This period at KERIC has been full of experience related to the lab and this project but also in my personal life and this was really exciting.

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11 Bibliography


12 Appendix A: Instructions for analyzing resting-state functional MRI data

12.1 Preprocessing

12.1.1 Extracting images

12.1.1.1 From .fdf to .img
In order to exploit the data with GIFT, it’s crucial to have Analyze format files, so the conversion of the FDF files is necessary.

Use ImageJ and the plugin Multi FDF Opener. Open the fdf with the plugin and then click save As and choose Analyze7.5 in the menu.

12.1.2 Skull-striping, Making a mask and using it

12.1.2.1 Skull-striping and Making a mask of an .img file
Open the .img file with ImageJ, Duplicate it with the function Image>Duplicate... - Open the window Image>Adjust>Threshold... to threshold your data. Slide the buttons in the bars min and max until you get only what you want to keep. Then press Apply and it will convert your image into a binary image of what you wanted to keep.
- Separate what you want from all the rest using the pencil tool. And then use the Wand tool to create a selection of what you will keep and then use the function Edit>Clear Outside and in the window that appears asking if you want to apply it to all the slices, choose “No”. Repeat this step for each slice.
- Once this is done, use the Image Calculator tool (Process> Image Calculator...). Choose the copy of the image as first image, the binary mask as the second and choose to Multiply in “operation”. Choose also to create a New window, so that if anything is wrong you still have the results from the previous steps.
- The newly created window should display the part you wanted to keep (a brain in our case) without the surrounding tissues. However there should be still a bit of extra tissues locally that you couldn’t clean in the binary. Use the brush or the pencil tool to clean those.
- You have your skull-striped brain image now. You can save it as analyze. You can also make a mask from it by simply applying the function Process>Binary>Make Binary or Image>Adjust>Threshold.

Note: Don’t forget to save the result as analyze file before to close it as ImageJ doesn’t save files by itself at each operation. Also be careful to save backup files before to do any thresholding or binary files since you cannot cancel the operation afterwards.

12.1.2.2 Using the mask
This is a really simple step, take your data to mask out and multiplies it with the mask file via ImageJ. For this go to Process>Image Calculator... and choose the corresponding open images. Don’t forget to save as analyze file the result before to close it.

12.1.3 Using SPM8 : Realigning, Coregistering, Normalising, Smoothing
To open SPM, type addpath 'path of folder of SPM8 on the computer'.
Then type `spm fmri`. Three windows should appear, one large on the right called Graphics, one called Menu on the top left and a third on the bottom left simply called SPM8.

### 12.1.3.1 Realignment

Choose in the Menu window drop down menu Realign: Estimate and Reslice. A batch window appears (cf. fig 1). Click on Data and then click on “New session” to add a “Session”. Click on “Session”, then Right click and choose in the contextual menu “Select files”. A new window appears, choose the files you want to realign together, in our case those are the EPI images of one session.

If you have several sessions for the same subject, Repeat the previous steps and add as many sessions as the number of sessions acquired for the same subject.

Note that the first file selected in the first session is the one that is used as reference to realign all the data. So in case you have two sessions acquired one just after the other, it might be wise to choose the last file of the first session as first as the difference due to the motion between this last timepoint and the files of the second session might be reduced compared to other files of the first session. You can also choose a file that seems more pertinent that the first or the last of the session, but you have to select it before to select any other file.

Several more options have to be chosen among the Estimation options and the Reslice options. Let’s first consider the Estimation options:

- **Quality**: the default value is 0.9. I usually keep this value. The value has to be between 0 and 1, 1 being the highest quality. The higher is the quality, the slower will be the estimation speed.
- **Separation**: The default value given is 4mm, it is adapted for human brains. As we are doing for rodents and the resolution of our EPI images is 0.5mm, I’m using 0.5mm as a value here. In general, I would use the same value as for the resolution of the images.
- **Smoothing (FWHM)**: Here I’m choosing a FWHM of 0.5mm for the same reasons as above. This is high enough to clean images from the noise.
- **Num passes**: Here I usually use “Register to first”. Note that if you choose to “Register to mean”, the software will first register to the first image before to make a second estimation and register to the mean image.
- **Interpolation**: I keep here the default “2nd degree B-Spline”.
- **Wrapping**: I have kept “No wrap” until now. However it might be wise to choose to wrap along the phase encode direction (X or Y depending on the orientation of the image) in case you have some folding artifacts in the image.
- **Weighting**: I don’t add any weighting file as there are no particular extra-brain motions at resting-state.

About the Reslice options:

- **Resliced Images**: Here I choose “All Images + Mean Image” as we are using the Mean Image in the next steps.
- **Interpolation**: Here I choose “4th Degree B-Spline” as it gives a good result in a reasonable time.
- Wrapping: The same as in the Estimation options.
- Masking: I choose “Don’t mask images” here.
- Filename Prefix: Up to you to choose the prefix that will appear before the name of the resliced images, personally I kept the default “r”.

Once you have chosen all the values, I would advise you to save the batch so that you keep track of all what has been done. Also it can help if you make the same for many subjects, so you don’t have to do it all over again (See alternative path).

You now just need to click on “Run” (the green arrow) to execute the program.

Alternative path:

To make the process faster you can load the .mat file used in the previous dataset analysis in case the conditions of acquisitions are the same (for example within a same study). In this case, after have loaded the .mat file, you only need to remove the dataset selected there and replace by the data to be processed.

Using “spmbatch.m”: 
In case you got many subjects with the same conditions and you don’t want to setup all the preprocessing steps using SPM one by one, there is a program called “spmbatch” that you can find on the website of the SPM with the other toolbox to be added. This program allows in the case your files are organised all in the same way for all the subjects to create and execute all the batches following a template batch that you created with one of the subjects.

After you created the batch file for realigning following the procedure given just before, you can run the function spmbatch.m by typing “spmbatch” in the main MATLAB window as for any function. A window will appear, choose the .mat file you want to reproduce and click “done”. A new window appears, you have now to choose the folder of the subject corresponding to the template and click “Done”, A last window will appear, here you must choose the folders of all the subjects for which you want to reproduce the batch you created before and click on “Done” once this done. The program will then create all the batches and run the program for all the subjects. In case you have some little differences for some subjects, you can type “spmbatch ('run', 'no')” instead of just “spmbatch”, this will only create the batches and not execute them. You can then modify and execute by loading them with the batch editor from SPM (button “Batch” in the window Menu of SPM).

12.1.3.2 Coregistration
Choose in the Menu window drop down menu Coregister: Estimate and Reslice. A batch window appears (cf. fig 2).

You have to select a few files:

- Reference Image: The image that will be the reference to which to align the data. In our case it is the skull-striped bias-corrected FSEMS data.
- Source image: The image that will be used to calculate the transform, in our case, it is the mean image given by the Realign step and that was skull-striped.
- Other images: The images on which will be applied the transform calculated. It is in our case the data given by the Realign step.

Several more options can be chosen:

- Objective Function: I would choose Normalized Cross Correlation as we are making a within modality coregistration.
- Separation: Here I chose [2 1] that corresponds to making an estimation first with considering having a 2mm separation between the reference image and the source image objects and then with 1mm afterwards. This is done by entering simply the values 2 and 1 with a space in between.
- Tolerances: Here I have set the following values [0.02 0.02 0.02 0.001 0.001 0.001 0.01 0.01 0.01 0.001 0.001 0.001]. This means that iterations stop when the difference between successive estimates is less than these values. You can try other combinations but this one worked in my case. In any case a 1 by 12 array must be entered (12 values separated by a space between each).
- Histogram Smoothing: I kept the default value [7 7] for this as the size of the histogram is independent from the dimension of the objects to be coregistered.
- Interpolation: I chose here 2nd-Degree B-Spline and it seems to give a good result. Any higher order interpolation would probably give a good result as well but would take more time.
- Wrapping: I chose “No wrap” as for the Realignment step.
- Masking: I chose “Don’t mask images” as for the Realignment step.
- Filename prefix: The default value is “r” but I used “co” instead as r is also used for the Realignment step.

You can now save your batch and run it if you want to. You can also use spmbatch as explained before in the Realignment chapter.

![Coregister batch window](image)

**fig 2: Coregister batch window**

### 12.1.3.3 Normalisation

Choose in the Menu window drop down menu Normalise: Estimate and Reslice. A batch window appears (cf. fig 3).

Here we can add some Subject with few files to select for each:
- Source Image: the image used to calculate the transform matrix, in our case, it is the skull-striped bias-corrected fsems used as reference in the coregistration step.
- Source weighting image: I keep it empty as I’m using skull-striped brain image as source image.
- Images to write: the images resulting from the coregistration step.

We have also to choose some other options:

- Template image: In our case it is a skull-striped fsems dataset. This file is called “rot brain ref 64” and is in the folder “rsfmri tools/analyze ref”
- Template weighting image: I keep it empty as I’m using skull-striped brain image as source image.
- Source image smoothing: I set it at 0.5, this is done to match the smoothing of the template image
- Template image smoothing: I set it at 0.5, same as above. The both images are smoothed so that noise would not affect the normalisation step.
- Affine Regularisation: Here I choose “Average sized template” as the template image chosen is about the same size as the brains from all the subjects.
- Nonlinear Frequency Cutoff: I kept the default value (25)
- Nonlinear iterations: I kept the default value (16)
- Nonlinear regularisation: I kept the default value that is 1
- Preserve: I chose “Preserve Concentrations”
- Bounding Box: As the dataset is such as there are 11 1mm thick slices with a field of view of 32x32mm, I’m using [-15.75 -15.75 -5.25, 15.75 15.75 5.25]
- Voxel sizes: I use [0.5 0.5 1] as the resolution from the images used to make the transform.
- Interpolation: I chose “2nd Degree B-Spline” as for the coregistration step.
- Wrapping: I chose “No wrap” as before
- Filename Prefix: I kept the default prefix “w”

You can now save your batch and run it if you want to. You can also use spmbatch as explained before in the Realignment chapter.
12.1.3.4 Smooth

Choose in the Menu window the button Smooth.

A batch window appears (cf. fig 4).

For smoothing a few options have to be set:

- **Images to Smooth**: Those are the images to be smoothed, in our case, those are the images resulting from the Normalisation step.
- **FWHM**: This is the size of the Gaussian kernel to apply on the images. I have chosen 0.6, 0.6 and 1 mm for respectively x, y and z as the resolution of our images is 0.5 0.5 and 1 mm.
- **Data Type**: I chose to keep the default SAME, meaning that the images are registered in the same format as they were before smoothing.
- **Implicit Masking**: I kept the default value “No” as I don’t see any reason to apply any masking here.
- **Filename Prefix**: I kept the default value “s”
12.1.4 Time filtering

For making the time filtering step you need to add the files ‘datatimefilter5.m’ and ‘writeanalyze.m’.

For doing this, in the main window of MATLAB you must type:

```
addpath '/home/peter/Desktop/rsfMRI tools/pack preprocessing' (if you’re on Appendix), or addpath 'path where are situated these functions' otherwise.
```

You need also gift to make it work, so type `addpath 'home/peter/Desktop/GroupICATv2.0e/icatb'` (if you’re on Appendix) or `addpath 'path of gift'` otherwise. And then launch gift by typing `gift`.

Click then on ‘Setup ICA analysis’ and add the datasets to timefilter as for doing an ICA analysis (cf. next chapter). Once the data added and the prefix set (I usually choose ‘ft’ for this) just click on ‘ok’ without setting anything else.

Then load the content of ‘yourprefixSubject.mat’ file (for example ‘ftSubject.mat’ in my case). Save in a variable called ‘fileN’ the ‘files.name’ data. For doing so, type in the main window of MATLAB: `fileN=files.name`. 
And then launch ‘datetimefilter5’ by typing `datetimefilter5folder` or `datetimefilter5` depending if you want the results to be saved in the same folder as the data to be used (`datetimefilter5`) or in the parent folder (`datetimefilter5folder`).

The program will ask you the repetition time of the acquisition of the data (Tr). Type the value in seconds.

Then it will ask the frequency range to keep, type `[min_freq high_freq]` with min_freq and high_freq in Hertz.

You’ll get one file with the prefix ‘ft’ at the end.

Open this file and save it with ImageJ so that gift analysis works with it.

### 12.2 Using GIFT

Open GIFT by typing in MATLAB `addpath 'path to the GIFT toolbox folder'`. Then type `gift`. A window appears (cf. fig 5).

![fig 5: Main menu of GIFT](image)

Click on the button *Setup ICA Analysis*.

A window appears (cf. fig 6) for selecting the folder in which you want to save the outcomes from the analysis.
Once you chose it, a new window appears (cf. fig 7), This is the main window for the setup of the ICA Analysis.
Several options must be chosen there:

- Enter Name(Prefix) Of Output Files: Here you must indicate the prefix you want the outcomes of the analysis to have.
- Have You Selected the fMRI Data Files: Click on ‘Select’.

A new window appears asking if the data are stored in one folder. It is asks in fact if all your data from all the subject are organised under one folder with one folder per subject and the different sessions in different folders under each subject. If it’s the case, choose ‘Yes’, otherwise choose ‘No’.

If you choose Yes, a new window appears asking to select the folder in which is stored all the data (cf. fig 8).
Once selected, another window appears with few options (cf. fig 9):

- **Select file pattern for reading data**: you must enter the beginning of the name under which are stored the data, for example if you followed the preprocessing procedure given above, your file should be prefixed ‘ft_swcor’ so you would have to enter ‘ft_swcor*.img; *.nii’
- **Are session folders inside subject folders?** if it’s the case choose ‘Yes’, otherwise choose ‘No’
- **Enter file numbers to include**: I usually leave it empty as I usually select all. But if you want to include only a part of the timepoints, enter it using for example 50:250 if you want to consider only the timepoints between 50 and 250, 50:2:250 if you want only every second file between 50 and 250 or as 1 3 4 7 8 9 if you want to pick the timepoints or files 1, 3, 4 etc only.
- **Click OK** once you have chosen all these options, you will see the considered folders in the MATLAB main window listed.

If you choose No, a window appears asking to precise the number of Subjects and the number of sessions per subject (cf. fig 10). Fill both cases and press ‘ok’.
fig 10: Fill in the number of Subjects and Sessions per Subject

A window (cf. fig 11) appears with a list (‘Subject1Session1’, ‘Subject1Session2’, etc). Click on each a window will appear where you can select the data for the corresponding session. Do it for all the sessions.
If you make a mistake, you can click on the corresponding session and click the button ‘change’ and the selection window will appear. You can also check if your selection is right by clicking on ‘View’. Once all the data are chosen, click ‘OK’. An error message will appear if you forgot one or more sessions.

There are still more options to choose in the Setup ICA window (cf. fig 7):

- Do you want to estimate the number of independent components?: I don’t do it as it takes a lot of time if you have several subjects and the results from the estimation doesn’t seem correct when used after the time filtering function I wrote (it gives always 54 as a result when combined with my time filtering function as the number of frequencies retained is 54).
- Number of IC: I usually use 30 as given by Jonckers article (ref.) for rats but other number can also be tried.
- Do you want to autofill data reduction values: I usually choose ‘Yes’. It is asked here if you want the program to fill the number of principal components you want for the data reduction steps (PCA)
- Which ICA Algorithm Do You Want To Use?: I usually choose the ‘Infomax’ algorithm, it seems to work well and be the most adapted for the resting-state fMRI on rodents.
- Which Group ICA Analysis You Want To Use?: Choose ‘Regular’, ‘ICASSO’ is used to test the algorithmic reliability. This is not what we are searching to do here.

Once all these options filled, click on Setup Defaults on the top left of the window. A new window appears with more options (cf. fig 12):
- Select Type of Data Pre-processing: I usually choose ‘Remove mean per voxel’, also ‘Intensity Normalization’ can be chosen. There are no reasons to choose ‘Remove mean per timepoint’ and Variance Normalization does not seem to give good results in our case.
- What Mask Do You Want To Use? Choose ‘Select Mask’ and use the mask called ‘mask ref rot 64’ in case you have used ‘brain ref rot 64’ as a template for the normalisation previously. You can use another mask more adapted in order to exclude other tissues than brain in the analysis. Choose ‘Default Mask’ if all you data are skull striped.
- Select Type Of PCA: I keep ‘Standard’ here.
- Select Type Of Group PCA: Here I keep ‘Subject Specific’
- Select The Backreconstruction Type: I keep ‘GICA’
- Do You Want To Scale The Results?: Here I choose ‘Z-scores’ when I use ‘Remove mean per voxel’ and ‘No Scaling’ if I use ‘Intensity Normalization’ as the data are already scaled by the preprocessing step then.
- How Many Data Reduction(PCA) Steps Do You Want To Run?: Here I keep the default value given by the program (1 in case you have only one subject and 2 in case you have several subjects).
- Number of PC(Step 1, Step 2 and Step 3): I usually keep the default values. But you can increase the number of PC on the Step 1, this would allow more data to get through the data reduction, possibly giving larger areas for the components, however I did not make any serious study on the matter. To be investigated.

Once all this is set click on Ok.

Click then on OK on the main setup window.

A final window related to the ICA algorithm options is opening, here I keep the default values and click directly on OK.

You can now run the ICA analysis by clicking on ‘Run Analysis’ in the main menu of GIFT. A window open where you have to select the parameter file of your analysis, this one should have the prefix you gave it during the setup of the analysis and should be stored in the folder you chose for the output files.

Once this done, another window opens asking what step you want to run, you can run it step by step or run all at once. I suggest this last option as we are not interested in the intermediary steps. Choose what memory you want to allocate to the Group PCA step at the bottom of the analysis and click ‘Done’. Then the process will start and a progression bar will appear.

This will take a while.

Once the analysis is done a GUI window for watching at the results appears. On the top left of the window, there is the button ‘Display Defaults’, if you click on it, a window will appear to choose the number of components you want to see per figure, if you want to see the positive values or positive and negative or absolute values, or the threshold for the values. At the bottom left there is a button to load the anatomical images on which to display the components, you should typically use the reference image you used to normalize all your brains for example.
You can also choose what you want to display, ‘components’ for the maps of the components for one of the subject (choose the subject in the menu on the left), ‘subject’ to see one particular component for all the subjects (menu on the right for choosing the component), but also ‘orthogonal’ (to see a component for a particular subject in orthogonal view) and ‘composite’ (to see several components in the same time on the same map).
Appendix B: Script for time filtering:

datatimefilter5.m

% This script has for goal to read the .img files we want to analyse and
% filter the intensities for each voxel along the time.
% We first open the data using the script icatb_read_data, so you need prior
% to launch this program to add the path of gift in MATLAB and to create a
% subject.mat file using 'Setup ICA' within GIFT and loading the data to be
% filtered.
% This program allows to filter the data for all the subjects in the same
% time.
% The data once opened are Fourier transformed, then multiplied by a vector
% value per value by one within the frequency range \([w_1, w_2]\) and by zero
% outside
% The data is then inverse Fourier transformed and saved into an Analyze
% file prefixed 'ft_' and situated in parent folder of the data treated.

N=size(files);

Tr=input('please precise the Tr value for this session');
Wn=input('please indicate the cutoff frequencies of the filter such as Wn
=[w1 w2]');

for i=1:N(2)

    fileN=files(i).name;

    % Load data
    data = icatb_read_data(fileN);
    % data is a 4D matrix \((x, y, z, t)\)

    sizedata=size(data);

    Nvol=sizedata(4);
    step=1/Nvol;
    width=1/Tr;

    freq=-width/2:step:width/2-step;

    filt=zeros(1,Nvol);
    y=abs(freq)>Wn(1)&abs(freq)<Wn(2);
    filt(y)=1;

    wdata=zeros(1,Nvol);
    %filter the data voxel-by-voxel
    for x=1:sizedata(1)
        for y=1:sizedata(2)
            for z=1:sizedata(3)
                wdata(1,:)=data(x,y,z,:);
                fftdata=fftshift(fft(wdata));
                data(x,y,z,:)=ifft(fftshift(fftdata.*filt));
            end
        end
    end
t_max = size(fileN);

vox_size = [32/65 32/65 1];
isize = size(data);

filenam = fileN(1,:);
ind = findstr(filenam, ', ', ');
if ~isempty(ind)
    filenam = filenam(1:ind-1);
end
ind = findstr(filenam, '\ ');
if ~isempty(ind)
    siz = size(ind);
    root = filenam(1:ind(siz(2)-1));
    filenam = filenam(ind(siz(2))+1:end);
end
filename = [root, 'ft_', filenam];
writeanalyze(data, isize, filename, vox_size);
end
Appendix C: Magnetic Resonance Imaging

Magnetic Resonance Imaging or MRI is a medical imaging technique that is based on a physics phenomenon called Nuclear Magnetic Resonance.

The Nuclear Magnetic Resonance property is obtained by submitting the subject to a strong magnetic field to align the magnetization of the nuclei of hydrogen atoms in the body and images are acquired by first altering systematically the alignment of the magnetization using a Radio Frequency (RF) pulse forcing the nuclei to produce a rotating magnetic field that can be measured by the scanner. The image is produced thanks to the use of a gradient of magnetic field that is generated and makes the nuclei in the different area of the excited volume to precess with a different frequency. The image is then rebuilt using a Fourier transform.

Different contrasts can be produced depending on how the sequence of the RF pulse and the gradients as well as the timing of the measurement are chosen. This allows observing and distinguishing different types of tissues and pathologies.

MRI provides good contrast between different soft tissues of the body, which makes it especially suitable for imaging the brain.
Appendix D: Software used in this project

MATLAB is a software developed by MathWorks. MATLAB is a numeric computing environment with its own programming language; it allows manipulating matrices and the implementation of all types of algorithms. In this project this software was mainly used as the environment that allows not only using the diverse toolboxes but also applying some basic programs created during the project.

During the project such toolboxes as SPM, GIFT and FNC were used in addition to MATLAB:

SPM means Statistical Parametric Mapping, it is a statistical technique created for examining differences in the brain activity recorded during functional neuroimaging experiments. It is also the name of the toolbox developed by the Wellcome Department of Imaging Neuroscience that applies this technique within the MATLAB environment (22). This toolbox is freely available on their website (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) (22). This program was used during the project mainly for preprocessing the datasets and preparing them to be analyzed by another toolbox called GIFT. I used it also for implementing statistical tests on the results of the analysis. I used for those purposes mainly SPM8 but I used also the previous version, SPM5, with its toolbox spmmouse (without much success).

GIFT is a toolbox for MATLAB developed by Dr Vincent Calhoun and Dr. Tulay Adali of the MIALAB (http://mialab.mrn.org/software/gift/index.html) (23). This software implements multiple algorithms for independent component analysis and blind source separation of group functional MRI data. This toolbox has been used to apply the main step of the processing of the resting-state fMRI data.

FNC is a toolbox developed by the MIALAB (http://mialab.mrn.org/software/fnc/index.html) for finding and displaying temporal relations between the components resulting from GIFT (24). I tried to use it during the project for determining the causal relations between the components and try to rebuild the functional networks.

ImageJ is a Java-based software for image processing developed by the National Institute of Health (NIH). ImageJ has an open architecture and allows creating and implementing plugins and macros through its built-in editor. This program has been used during the project for skull-stripping the datasets as well as for checking results from the different steps of the analysis.

Some other softwares, FSL and spmmouse, have been used or investigated during the project but did not give any added value to the project, this is the reason why they are not mentioned in this report.