Cluster analysis of resting-state fMRI time series

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A B S T R A C T
Functional MRI (fMRI) has become one of the leading methods for brain mapping in neuroscience. Recent advances in fMRI analysis were used to define the default state of brain activity, functional connectivity and basal activity. Basal activity measured with fMRI raised tremendous interest among neuroscientists since synchronized brain activity pattern could be retrieved while the subject rests (resting state fMRI). During recent years, a few signal processing schemes have been suggested to analyze the resting state blood oxygenation level dependent (BOLD) signal from simple correlations to spectral decomposition. In most of these analysis schemes, the question asked was which brain areas “behave” in the time domain similarly to a pre-specified ROI.

In this work we applied short time frequency analysis and clustering to study the spatial signal characteristics of resting state fMRI time series. Such analysis revealed that clusters of similar BOLD fluctuations are found in the cortex but also in the white matter and sub-cortical gray matter regions (thalamus). We found high similarities between the BOLD clusters and the neuro-anatomical appearance of brain regions.

Additional analysis of the BOLD time series revealed a strong correlation between head movements and clustering quality. Experiments performed with T1-weighted time series also provided similar quality of clustering. These observations led us to the conclusion that non-functional contributions to the BOLD time series can also account for symmetric appearance of signal fluctuations. These contributions may include head motions, the underlying microvasculature anatomy and cellular morphology.

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Introduction

Functional MRI (fMRI) revolutionized the field of human brain mapping. Based on the fine equilibrium between blood oxygenation levels, areas of task-specific brain activity could be indirectly defined (Belliveau et al., 1991; Jezzard et al., 2002; Ogawa et al., 1990). Although not proven, the recent hypothesis on the biochemical and cellular mechanisms underlying the blood oxygenation changes following brain activity points to a cascade of events that occur following presynaptic activity (Logothetis et al., 2001; Raichle and Mintun, 2006). The fMRI signal was found to correlate with synchronous neuronal activity (Logothetis et al., 2001; Raichle and Mintun, 2006). Because most fMRI studies rely on comparisons between different tasks and resting periods, few questions were raised in the early days of fMRI about the contribution of the basal activity of the brain; for example, what happens to the fMRI signal when we assume that the brain rests? Based on PET studies, Raichle and colleagues (Raichle et al., 2001; Raichle and Mintun, 2006) defined a default network of brain activity consisting of areas that are metabolically active in the resting state. In BOLD fMRI, these areas demonstrate consistent attenuated BOLD signal concurrent with increased response at other locations (Gusnard et al., 2001; Gusnard and Raichle, 2001; Raichle et al., 2001; Raichle and Mintun, 2006; Vincent et al., 2007).

Resting state functional MRI was recently used to access basal brain activity (Beckmann et al., 2005; Biswal et al., 1995; Cordes et al., 2001; Damoiseaux et al., 2006; De Luca et al., 2006; Fox et al., 2005; Fransson, 2005; Greicius et al., 2003; Lowe et al., 1998; Peltier and Noll, 2002; Raichle and Mintun, 2006). In this type of experiment, the BOLD imaging conventionally used for task-related fMRI is acquired continuously while the subject is asked not to perform any specific task. With resting state fMRI data, it became evident that the brain can be segmented into regions of correlated BOLD signal fluctuations (Beckmann et al., 2005; Biswal et al., 1995; Damoiseaux et al., 2006; De Luca et al., 2006; Fox et al., 2005; Fransson, 2005; Greicius et al., 2003; Lowe et al., 1998; Nir et al., 2006; Peltier and Noll, 2002; Raichle and Mintun, 2006). This signal pattern was suggested to reflect a brain network of neurons working in concert (functional connectivity) (Beckmann et al., 2005; Cordes et al., 2001; Greicius et al., 2003; Lowe
et al., 1998; Nir et al., 2006; Peltier and Noll, 2002), or spontaneous neuronal activity (Biswal et al., 1995; Damoiseaux et al., 2006; De Luca et al., 2006; Fox et al., 2005; Fransson, 2005). The latter was suggested to be related to the default brain network originally defined by PET studies (Fransson, 2005).

Conventional fMRI analysis extracts task-related BOLD signal changes by using a statistical comparisons between the time series and the task timings (Beliveau et al., 1991; Jezeard et al., 2002). In contrast, resting state fMRI analyzes the BOLD signal with no information about the timing of neuronal events, but rather uses only the similarity of fluctuations in different voxels. Thus, it can not separate blood oxygenation contributions (T2* effect) from any other signal sources. Noise contribution to the BOLD signal has been thoroughly studied (Bodurka et al., 2007; Kruger and Glover, 2001). It has been demonstrated that a typical BOLD signal embraces several noise sources; non-physiological noises that result from the MRI scanner and physiological noises originated from the subjects. The physiological noises are divided into two main sources: functional and non-functional. The physiological noises were also shown to be weighted by the measured absolute signal (Kruger and Glover, 2001) implying that its fluctuations might be tissue specific.

A number of signal processing schemes have been suggested for analyzing the resting state BOLD signal, ranging from simple correlations to spectral decomposition of frequency analysis (Beckmann et al., 2005; Biswal et al., 1995; Damoiseaux et al., 2006; De Luca et al., 2006; Fox et al., 2005; Fransson, 2005; Greicius et al., 2003; Lowe et al., 1998; Peltier and Noll, 2002). Interestingly, frequency analysis revealed low-frequency fluctuations of the data (below 0.1 Hz) that are correlated between functionally related regions of the brain (Biswal et al., 1995; Raichle and Mintun, 2006). In this work we wish to characterize these low frequency BOLD signals by ‘k-means’ clustering analysis of the fluctuated signal and its spectral decomposition. Such analysis was performed on the cortex, sub-cortical regions (e.g. thalamus) and white matter. As input we used the original time series rest-fMRI signal as well as a short-time Fourier transform (spectrogram) that provides information not only on the frequencies of the fluctuating signal, but also on their amplitude changes over time. The functional relevance of the rest fMRI signal fluctuations was investigated by comparing the obtained clusters with anatomical and cyto-architectonic atlases (Talairach (Talairach and P., 1988) and Brodmann (Garey, 1999)). To define to what extent the rest fMRI signal fluctuations reflect tissue functionality, we repeated such analysis on non-BOLD MRI time series measurement and on BOLD white matter signal. We also investigated the effect of non-functional sources of physiological noise (i.e. head motion) to the observed rest fMRI clusters.

Materials and methods

**Human experiments**

**Data acquisition**

Eleven healthy subjects aged 22–42 years underwent MRI in a 3 T scanner (GE, Milwaukee, USA) using an 8-channel head coil. The institutional review board (IRB) committee approved the research protocol and each subject signed an informed consent. The MRI protocol included high resolution anatomical images: T2-weighted images using a fast spin-echo sequence (TR=7000/85 ms, echo train length of 16), fluid-attenuated inversion recovery (FLAIR) images (TR=7000/102/2100 ms) and 3D spoiled gradient echo (SPGR) images (TR=50/3 ms, Flip angle=10°). T2 weighted and FLAIR sequences were acquired in axial position with field of view (FOV) of 24 cm, slice thickness of 5 mm and whole brain coverage. SPGR images were acquired with the abovementioned FOV and resolution of 0.93×0.93×1.5 mm³. In addition to the high resolution sequences, a series of gradient-echo planar-imaging (GE-EPI) images were acquired with the following parameters: TR/TE=600/45 ms, matrix of 80×80, FOV of 24 cm, 7 axial slices with slice thickness of 5 mm and no gap localized at the level of the thalamus. The GE-EPI protocol was repeated 800 times to produce a pixel-by-pixel time series of the BOLD signal for 8 min. During the GE-EPI protocol, the subject was asked to lie still with their eyes closed and not to perform any special task.

Additional experiments included: 1. Scanning 5 subjects with the abovementioned protocol but with different TE’s (30 and 60 ms). 2. Scanning 2 subjects with the abovementioned parameters but with TR of 2000 ms (24 slices, 5 mm thickness, 300 repetitions). 3. Scanning 2 subjects with a T1 weighted 2D fast SPGR (TR/TE=30 ms/2 ms, 5.3 s per image acquisition, Flip angle=5°, 6 slices, 5 mm thickness, 300 repetitions). 4. The abovementioned GE-EPI protocol was also acquired for a sphere filled with doped water that served as a homogenous phantom.

**Pre-processing**

Pre-processing of the time series data included the following steps:

1. Correction of head motions using a least squares algorithm and 6 parameter (rigid body) transformations; 2) Normalization to the Talairach coordinate system; 3) Segmentation into gray matter, white matter and sub-cortical gray matter. These procedures were done using the SPM software (version 2, UCL, London, UK). Realignment was done with a 4th degree b-spline interpolation with no wrap and no mask. Normalization was done by applying first a 12-parameter affine transformation followed by an estimation of non-linear deformations using discrete cosines transform basis function. Segmentation was done using clustering of the image and analysis of the likelihood of a pixel to be assigned as either of the tissue types. For all pre-processing we used the default SPM settings.

**Data processing**

The following data processing was done in Matlab (© Mathworks, USA).

**Time series.** The first 50 points of each time series were discarded to assure that the GE-EPI signal reached a steady state. The analysis was continued on the 750 time series data points which were normalized both to fluctuate around zero and to have the same standard deviation in time (for all voxels). The MRI signal drifts were corrected using open source Matlab functions (Pastushenko, 2004).

**Spectrum analysis.** For each voxel, the time series was transformed to the frequency domain using the FFT Matlab function. Cluster analysis (see below) was done on the resulting spectrum following low-pass filtering of frequencies below 0.2 Hz. This was done to minimize the contribution of heart beats and respiratory cycles to the spectrum.

**Windowed Fourier transform analysis.** This analysis provided a 2D matrix for each voxel depicting the frequency changes along time. Here the data was first linearly interpolated to an apparent sampling rate of 60 ms (factor of 10) using the interp matlab function. Next the windowed Fourier transform was obtained using the specgram matlab function with a window size of 440 time points and an overlap of 200 time points. Cluster analysis (see below) was done following low pass filtering (<0.2 Hz) of the spectrogram matrix for the abovementioned reasons. As input for the cluster analysis, the spectrogram matrix was reshaped to a vector.

**Clustering**

The data vectors (time series, spectrum and spectrogram) for all pixels were used as input for cluster analysis. In this analysis we used a modified k-means clustering algorithm. The k-mean algorithm was run 10 times for each number of clusters (k). The clustering analysis was repeated for different k from k=3 to k=45 (in steps of 1). The
clusters were evaluated using a squared Euclidean distance index between the values of the data vectors of each pixel (i.e. the time series or spectrum or spectrogram data). It should be noted that the pixel’s spatial information was not used in the clustering as additional input thus it does not impose geometrical constrains on the resulted clusters. For each k-means clustering result, a repeated measure ANOVA test was used to test for difference between the clusters. Each pixel in each cluster was regarded as an individual observation, which resulted in 300–750 individual observations in each cluster (depend on the data types). The data was then tested for statistical significance using a repeated measure ANOVA, with the clusters as the independent factor and the data vector (time series, spectrum or spectrogram) as the repeated variable. A p-value of 0.001 was considered the significance cut off value.

For the cortex the input data vectors included data from one slice (slice level – z=0 in the normalized brain space), the thalamus it included data from 13 slices (the entire thalamus volume) and the white matter included data from 12 slices. The number of clusters (k) varied according to the tissue type. For the cortex Similarity index analysis (see below) k was 12 since 12 regions were defined in the Brodmann map of the same slice (z=0). For the thalamus k was 6 as the number of the large nuclei masses (pulvinar, medio-dorsal, ventral lateral, ventral posterior lateral, ventral anterior and anterior). For the white matter k was set to 8 which were the optimal number based on trial and error (at larger number of k, the clustering pattern appeared noisier with reduced symmetry between the hemispheres).

The above clustering procedure was also used to analyze the homogenous phantom time series and on a simulated white noise time series as well as on the T1 weighted time series.

**Similarity index analysis**

To test the similarity between the obtained clusters and known anatomical structures we devised a similarity index (SI) (Eq. 1) which describes the weighted fit between cluster and anatomical regions. The similarity index has two components: the first weights for the percentage of voxels of a specific cluster that resides within a specific anatomical region; the second weights for percentage of a voxel of a specific anatomical region that resides within a specific cluster. The similarity index is defined as the multiplication between the two components and it receives values between zero and one where zero means that none of the cluster voxels resides within the anatomical region and vice versa. A value of one, means that all cluster voxels reside within the anatomical region and vice versa. The formula for the SI was defined as follows:

$$SI_i = \left( \frac{|\alpha_i \cap F_j|}{|\alpha_i|} \right)^2$$

\(\alpha_i\) — a cluster; \(i=1\ldots n\); \(F_j\) — Atlas brain region; \(j=1\ldots m\) where \(SI_i\) is the probability of a cluster \(\alpha_i\) to belong to an atlas region \(F_j\), \(n\) is the number of cluster in the analysis and \(m\) is the number of regions in the atlas. For each cluster \(\alpha_i\) we define the region that has the highest \(SI_i\) and note it as \(SI_i\).

At the end of this process a 2D matrix was obtained where one axis represent the cluster and the other the anatomical regions. This matrix was used to automatically assign a cluster to an anatomical region (based on the highest similarity criterion). If more than one cluster is assigned to a specific region, these clusters were combined in their figure representation. The cluster assignment is done after the calculation of the SI and therefore, this procedure does not bias the SI results. This procedure was done for visualization purpose in order to reduce ambiguity in the cluster assignment where one anatomical region could be presented by few clusters. Following assignment, the similarity index for all regions (\(SI_i\)) was averaged to obtain an estimate for subject’s clusters similarity (SI) with the atlas. The cortex data was compared with the Brodmann cortical classification and the thalamus data was compared with Talairach segmentation.

**Head movement analysis**

The head movement coordinates for each point in the time series (as extracted from the SPM2 motion correction procedure) was used to define the distance (\(Dis_{xyzt}\)) that each voxel moved in time.

$$Dis_{xyzt} = \sqrt{(x_t-x_{t-1})^2 + (y_t-y_{t-1})^2 + (z_t-z_{t-1})^2}$$

(2)

The minimum of each head-motion function (\(Dis_{xyzt}\)) was defined as the center of head movement in time (\(CM_t\)).

$$CM_t = \text{Min}(Dis_{xyzt})$$

(3)

To account for the head motion effects on the SI (Eq. 1), for each subject, we correlated between the SI and the mean head movement in time (MCM) for each subject, where MCM is defined as:

$$MCM = \text{CM}_t$$

(4)

MCM is a single parameter that represents the amount of head motion during the measurements. Please note that our subjects head motion doesn’t consist of a pure rotation (or any other pure movement direction) that might have resulted in a wrong estimation of the MCM.

Additional estimate of the effect of head motion was done by performing a multiple regression of the rigid-transformation parameters and the BOLD signal. The residuals of this regression were used as a time-series for the clustering analysis. Theoretically the “realignment-residuals” time series should contain less head-motion information.

**Porcine experiments**

T2* time series on excised fixed porcine brain were acquired on a 7 T scanner (Bruker, Karlsruhe, Germany). Porcine brain samples were obtained from Lahav Institute of Animal Research (Kibbutz Lahav, Israel). The brain was placed in a plastic tube filled with Fluorinert (FC-77, 3 M) to avoid susceptibility effects. The experimental protocol included a time series of gradient-echo EPI (800 repetitions) with the following parameters (TR=650 ms, TE=19 ms, 16 coronal slices of 3 mm thickness and in-plane resolution of 1×1 mm² (matrix of 64×64). The experiment was repeated twice and on the second...
repetition minor physical vibrations were applied on the tube causing slight un-controlled motion of the sample. The data underwent the same pre and post-processing (without normalization) as mentioned above.

Results

In this work we used a spectrogram (short-time Fourier transform) analysis in order to characterize the time dependency of BOLD time series frequency variations. This analysis procedure was compared with conventional analysis on the time series. Fig. 1 shows typical spectrogram of one cortical voxel of one representative subject. We found that the amplitude of certain frequencies of the resting-state fMRI changes significantly with time (a 2–3 fold increase or decrease was frequently observed) in a non-repeated manner (Fig. 1). This time dependent frequency variations were used as input to the clustering algorithm (see methods).

The cortex clusters

Cluster analysis of the resting data of the 5 slices (from the 7 available following realignment and cortex segmentation) revealed a symmetric pattern of clusters between the two hemispheres for any cluster number (k, from k = 3 to k = 45). The spatial appearance of the clusters resembled the atlas segmentation of the brain for any given k (see Fig. 2 with k = 12 following normalization to MNI). To quantify this similarity, we first normalized the images to MNI coordinates and repeated the clustering analysis. For comparison with the MNI normalized Brodmann map we devised a similarity index that compares the clusters with the Brodmann cortical segmentation (see methods). We found that the initial number of clusters (k) did not have a clear effect on the similarity between the atlas and the rest fMRI clusters and therefore we set the number of clusters to 12 (the number of Brodmann brain regions in this slice) when comparing

![Fig. 2. BOLD Signal Spectrogram Based Segmentation. (A) Brodmann mask of one slice of a representative subject depicting 11 different cortical areas. (B) BOLD signal clustering identifying 9 distinct regions (the cluster analysis consist 12 regions but 3 of them were merged in the image as they fall within the same atlas regions). The BOLD clustering was done following spectrogram analysis incorporating both time variation and frequency information (time window of 18 s with 12 s overlap, 0.16 Hz threshold low pass filter). (C) Talairach mask of the thalamus for one slice of a representative subject and in 3D showing that large nuclei mass including the pulvinar (PUL), ventral–posterior lateral (VPL), ventral–lateral (VL), medio-dorsal (MD) and mid-thalamic (MT) nuclei groups. (D) BOLD signal clustering identifying 6 distinct sub-regions within the thalamus (shown on a representative slice and in 3D) with high resemblance to the Talairach segmentation.](image-url)
between the atlas and the clusters maps. With the spectrogram analysis, 11–12 statistically different clusters \( (p < 0.001, \text{ANOVA test}) \) were found for all subjects. Similar results were observed when the BOLD time series data was used for clustering but there the clusters were not significantly different from each other according to the ANOVA test. The similarity index (SI) of all clusters, for all subjects, was between 0.075–0.196, considerably above chance level (0.04). In general some brain regions were better defined than others with preference for the bigger regions. In particular, Brodmann region 17 was identified in some subjects with very high similarity with a mean index of 0.3.

It should be noted that the SI between the clusters and the atlas was made to relate the cluster to the anatomical features. We did not test the SI to other brain atlases as this was beyond the scope of this work.

In addition, cluster analysis of a time-series produced by the residuals of a regression between the BOLD time series and the realignment (movement correction) parameters resulted in similar cluster pattern and SI (data not shown).

The thalamus clusters

Working on smaller scale tissues such as the sub-cortical gray matter nuclei masses (e.g. the thalamus) yielded similar results. Using the clustering approach, the thalamus could be segmented into 6 distinct sub-regions, symmetric between hemispheres (Fig. 2). The clusters highly resembled the morphological division of the thalamus to its sub-nuclei groups. In general, the thalamus segmentation had a much higher similarity index (running between 0.17 and 0.4) to the Talairach brain atlas than the cortex. Some sub-regions were better defined than others; for example, the pulvinar nucleus in the thalamus was selectively well defined in all subjects (mean index of 0.45).

Inspecting the frequency decomposition of each of the clusters (following assignment to atlas region) across subjects revealed a unique region-specific pattern for each subject, precluding their comparison. This was not surprising since the frequency power changes over time (see Fig. 1), as indicated by the short-time Fourier analysis, revealed that the low frequency fluctuations represent either neuronal, physiological or artifactual changes that are chanced in time (Triantafyllou et al., 2005).

The white matter cluster analysis

It is generally accepted that because the blood supply to the white matter is significantly lower than that to the cortex (less than one fourth), the BOLD contribution to the white matter signal is low. Thus, symmetric, functionally related BOLD signal fluctuations are expected to be in much smaller magnitude if any than the  

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*Fig. 3. BOLD Signal Clustering of the White Matter. (A) Two axial slices of one representative subject depicting the BOLD signal clustering superimposed on an anatomical MRI image in gray scale. Note the delineation of symmetrical white matter structures. Clustering was done as in Fig. 1 following spectrogram analysis and low-pass filtering of the data. (B) The same as in (A) but given in 3D for a portion of the scanned volume depicting the white matter structures, of which the corpus callosum (CC), brainstem (BS) and part of the optic radiation (OR) could be identified.*
cortical ones. Nevertheless, using the above analysis routine, the white matter could be clustered into regions of highly correlated BOLD signals. Interestingly, these clusters resemble known white matter fascicles tracts (e.g. the corpus callosum, the inferior longitudinal fascicules) (Fig. 3). In addition, the amplitude of the frequency decomposition of the white matter did not differ much from that of the gray matter regions. This result was reproduced across subjects.

Non-BOLD time series cluster analysis

BOLD acquisition through T2* weighted MRI enhances the contribution of blood oxygenation to the MRI signal, increasing its sensitivity to functionally induced blood volume and flow changes. Other contrast mechanisms (e.g. T2 and T1) are known to provide only marginal correlation with brain activity as the blood oxygenation level contribution to the contrast is low. In the following experiment we acquired a time series of conventional T1 weighted images (repeated 300 times). We found that the cluster analysis of this series yielded clusters that presented good similarity to the atlas appearance of brain regions (Fig. 4).

Cluster source validation

The T1 and the white matter anatomical clustering encouraged us to search for a non-functional explanation for the synchronous fluctuations signal that might affect the cluster analysis.

Phantom data

To rule out the possible mechanical effect on the clustering we repeated the cluster analysis for a simulated white noise time series and for a BOLD signal that was acquired on a homogenous spherical phantom. Both validations didn’t show any symmetric anatomical clusters; therefore, we conclude that white noise or scanner-related noise were not the cluster source.

Respiration and heart beats

Additional sources for non-functional contribution to the low-frequency signal fluctuation could be due to respiration, heart beats and head movement. Although we can’t rule the first two effects (respiration and heart beats) we tried to narrow it by selecting a short TR of 600 ms which minimizes the frequency aliasing of these processes. This allowed removing their main effect in the analysis routine although marginal effect may still be present.

Head motion

Rigid body transformation applied on the time series data allows the correction of most of the head motion effect. The range of motion correction for the different subjects was found to vary between 0.1 and 1.0 mm from the initial location in all three axes (X,Y,Z) and between 0.1 and 1° in the pitch and yaw angles and between 0.05 and 15° at the roll angle.

Nevertheless, residual noise caused by head motion is still present in the data following these corrections. We found that the frequency characteristics of this head motion function are similar to those of the BOLD fluctuations. Using the head-motion function we defined the shift in the center of movement and calculated its mean (MCM) as an indicator of the global magnitude of head-motion. We found that the head-motion correction effect on the pixels signal value gradually grows in accordance with the distance from the center of movement (where the movement is minimal). Therefore any effect which originated from the movement or from the movement correction will be stronger at larger distance from the MCM. In addition, this effect was distributed roughly symmetrically between the two hemispheres. Fig. 5 shows the correlation between the MCM and the entire BOLD SI. Strong correlation was found in any of the classified clusters; for example that clusters that correspond to Brodmann’s area 17 (which also had the highest SI) show strong and significant correlation with the MCM (Fig. 5B).

Post-mortem analysis

In this section we used an excised and fixed porcine brain to explore the abovementioned noise contribution to the BOLD clusters. Regular acquisition of a BOLD time series resulted in a noisy clustering pattern (Fig. 6). To estimate the contribution for head-motion we...
sightly shook the excised brain container. This manipulation resulted in non-controlled movement of the brain with much lower magnitude that typical human brain head motions (data not shown). Nevertheless, analysis of this time series resulted in well defined clusters although not symmetrical. The non-symmetry might be due to the low magnitude of the movement and its center that resides outside of the brain.

Discussion

The first, most prominent finding of this work is that the clustering analysis of multi-dimensional acquisition of BOLD and non-BOLD MRI signals allows segmentation of the brain into its neuroanatomical regions in both gray matter and white matter regions.

In this work we used clustering analysis to classify brain regions according to their fluctuated BOLD signal. Since we used the Euclidean distance as the factor of the cluster analysis, this procedure is similar, in principle, to the conventional correlation analysis for the time series data. With conventional correlation, analysis specific voxel signal fluctuations are correlated with all other voxels. This analysis can be repeated for other seed voxel to reveal additional correlated networks. In the cluster approach, these networks are obtained simultaneously without the need to define a seed voxel of interest.

Applying cluster analysis to the resting state fMRI data points out a strong link between tissue architecture and the fluctuating BOLD signal, as the clusters resembles known cyto-architectonic atlas segmentations. Yet, the functional organization of the brain is known to be correlated with its anatomical segmentation. Therefore, to more precisely characterize the origins of the fluctuating BOLD signal and its clustering pattern, we performed the analysis on two different non-functional MRI time series signals (analysis on white matter BOLD signal and T1 time series).

It should be noted that the T1-weighted images could still be influenced by inflow effects, and therefore be sensitive to brain activation fluctuations over time. However, these inflow effects are likely to be much smaller than the BOLD signal changes. The T1 and the white matter clustering suggest a hypothesis that fluctuations in repeated measures of MRI signal (T2⁎ or T1) includes significant non-functional information. However, recent observations do find links between non-fMRI physiological measures of tissue function and the resting state fMRI signal, implying that there is functional content to this signal as well (Peltier et al., 2005; Triantafyllou et al., 2005). These observations and our findings led us to speculate that the resting-state BOLD signal probably embraces contributions from both physiological (e.g. functional) and artificial (noise) factors that can not be easily separated.

The different noise sources to the BOLD signal have been thoroughly studied and modeled in several recent works (Bodurka et al., 2007; Kruger and Glover, 2001; Logothetis and Wandell, 2004). It has been demonstrated that typical BOLD signal embraces several noise sources; non-physiological noises that result from the measurement instruments and physiological noises originating from the subject. The physiological noises are divided into two main sources: the functional and non-functional effects.

Tissue microstructure effect

Grugur and Clover (Kruger and Glover, 2001) show that the functional contribution to the signal is the strongest source in gray matter fMRI signals. Accordingly the BOLD rest fMRI mainly refers to the basal activity of a region in which sporadic episodes of sporadic activity may lead to the observed signal fluctuations. Importantly, in that work it has been also demonstrated that physiological noise factors are signal-dependent (Kruger and Glover, 2001). Consequently, the specific tissue proton density will be the prime factor to influence the magnitude of the measured signal. Therefore, the signal fluctuations are correlated with the underlying proton density. Thus, it is possible that fine microstructure differences between brain regions (i.e. cell density, synaptic density) lead to different MRI signal fluctuations. These proton density differences are not observed in a regular single MR image, but when repeated enough times (i.e. multi-dimensional acquisition), cluster analysis of the signal fluctuations might extract such subtle differences. Nevertheless, one should bear in mind that the magnitude of the measured signal is dependent not only on the proton density, but also on the T1, T2, and T2⁎.

The tissue unique blood vessel distribution (Duvernay et al., 1981) is also a potential noise source in any rest fMRI analysis. Indeed it was shown that blood capillary distribution correlates with homodynamic-based functional imaging in the cerebral cortex (Harrison et al., 2002). More than that, the veins symmetric distribution in primary visual brain areas was shown to affect the BOLD EPI signal with high variances in time (Olman et al., 2007). In this work the time series were normalized for the variance in time and therefore this is probably not the main cause for the difference in the clusters. A model presented by Logothetis and Wandell (2004) summarizes the blood vessel distribution regional effects on the BOLD signal. This model led to the conclusion that direct comparison between BOLD amplitudes of two cortical regions should only be made when the homodynamic response functional is equal or at least known.

Head movement effect

An alternative explanation for the synchronous signal fluctuations may originate from a non-functional source, either physiological or
mechanical (scanner instability). The fact that white matter pixels in our work behave in a similar manner to gray matter pixels supports the non-functional explanation for the signal fluctuations. The question is why these fluctuations bear within them anatomical information? We ruled out the possible mechanical effect on the cluster analysis with simulated white noise time series and for signal acquired on phantom. Another possible noise source is non-functional physiological source that embraces several factors like respiration (Birn et al., 2008; Thomason et al., 2007) heartbeats (Shmueli et al., 2007) and head-movement.

In our experimental protocol we choose a TR of 600 ms and TE of 45 ms in order to have fast acquisition (minimizing heart beat sampling) and optimal T2* BOLD contributions, respectively. Even so, TR of 600 ms is not short enough to sample the cardiac-induced fluctuations; yet, we selected this TR in order to have a reasonable brain volume acquisition with minimizing the effect of cardiac induced fluctuations. To verify that these conditions did not bias our clustering results we performed the same analysis on data acquired with longer TR (2000 ms, and, on different subjects, with different TE (30 ms and 60 ms), data not shown). None of these experimental conditions affected the quality of clustering analysis.

The signal fluctuation caused by head-movement bears that same low-frequency pattern of the entire BOLD signal. This was observed in all subjects although other subjects may have head movements that result in higher frequency fluctuations. Although head-movements are corrected by rigid body transformation signal variation caused by movement induced partial volume effects (most probably at the CSF–gray matter border) still resides within the signal time series. We suggest that part of the neuro-anatomical segmentation of the BOLD signal could result from partial volume effects (i.e. a region can be characterized by the neighboring tissues signal). Such effect depends on the amount and direction of the movement as well as on the number of samples. This finding might have a significant impact on the field of resting state fMRI as it suggest that head movement are a partial source of the observed results. Nevertheless, additional, more targeted, studies should be aimed to explore this issue in depth. We found a correlation between the BOLD signal clusters SI (the correlation to the atlas index) and the MCM movement function. The MCM probably contains several movement sources like breathing and heartbeats, as well as other directed and undirected ones. This was exemplified in the results of the excised porcine brain where subtle movement in the tissue caused the appearance of correlated signal fluctuations. One should also take into account the fact that we scanned only 7 axial slices which may lead to not optimal movement estimation therefore might result in additional motion-related noise in the MR time series that cannot be corrected.

Conclusions

Based on the anatomical appearance of the resting-state clusters and the abovementioned validation steps, we argue that the tissue microstructure and non-functionally related noise contributors influence correlated BOLD signal fluctuations. Without imposing functional paradigms on the BOLD time series, separating the functional contribution to the signal fluctuation from the anatomical/noise contributions is challenging. In view of the above results, the resting-state fMRI and functional connectivity analysis of it should be re-examined. Is the resting-state fMRI a true measure of brain connectivity? Our results can not tell whether the morphology (micro or macro) of the tissue is the sole factor in such measurements. However, its weighting might have a substantial influence and should be taken into account when estimating basal brain activity and connectivity based on these measures.

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