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The frequency and reliability of cortical activity using a novel strategy to present pressure pain stimulus over the lumbar spine

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1. Introduction

Functional magnetic resonance imaging (fMRI) combined with psychophysical stimuli has been employed to study cortical sensitization in people with chronic low back pain (cLBP). To date, research groups have differed in their positioning of subjects in the scanner, and the location on the body where the psychophysical stimuli is applied. (Giesecke et al., 2004; Kobayashi et al., 2009) Giesecke et al. (2004) psychophysical approach was applying pressure stimuli to the nail bed of a finger while subjects lie supine in the scanner. While, Kobayashi et al. (2009) used pressure stimuli to the lumbar spine while subjects lie prone in the scanner. Considering that the majority of fMRI studies place the subject in the supine position, we decided to test the feasibility to extend the work of Kobayashi et al. (2009) by creating a method to apply pressure stimuli to the lumbar spine while the subject lies supine.

Here we describe a novel approach to presenting pressure stimuli to the lumbar spine. First, we describe the custom built MR-compatible algometer. Second, we assess the frequency of cortical activity within three *a priori* brain regions, bilaterally, at the individual level. In addition we describe the scan to scan reliability of two commonly reported matrixes of brain function, cluster-size and peak-voxel T-score, for each of the six brain regions. We assess the relationship between stimulus presentation and movement related outliers. And finally, we report on the relationship between stimulus presentation and movement artifacts.

2. Materials and Methods

2.1 Study Design

This paper describes a novel MR-compatible device that was used to apply pressure pain stimuli to the lumbar spine of subjects in the supine position. The frequency and reliability estimates of BOLD responses were secondary analyses of pooled data from a pilot-fMRI project. That project examined cortical responses to “moderate” pressure pain stimuli applied to the lumbar spine with the MR-compatible device.

2.2 Participants

Thirteen participants (6 females; average age $\pm$ standard deviation 42.5 ± 10.5 years) were pooled for this study. All participants read and signed an informed consent approved by the University of Rochester institutional review board. All subjects were recruited via flyers posted on the campus of the University of Rochester. The pooled sample included participants with (N=8) and without low back pain (N=5). Participants were eligible if they were between the ages of 30 and 65, greater than 5 feet 2 inches tall and weighed less than 300 lbs. Specific inclusion criteria are listed in table 1 for participants who had and did not have low back pain, respectively. Exclusion criteria included pregnancy, cauda equine syndrome, spinal neoplasia or metastatic disease, destructive joint pathology, progressive neurologic deficits (such as peripheral neuropathy, lumbosacral radiculopathy, myelopathy, or neurogenic claudication), previous lumbar, hip, or pelvis surgery, chronic migraine headache, fibromyalgia, irritable bowel syndrome, chronic pain from other sources (such as thalamic stroke), contraindications to MRI (such as metal implants or claustrophobia), or ongoing legal proceedings (such as workers' compensation).

2.3 Description of MR-compatible pressure algometer device

The MR-compatible device was created using a single stage electro-pneumatic pressure regulator connected to a pneumatic aluminum piston, see Figure 1. The piston was 2.2 inches tall, with a $\frac{1}{2}$ inch stroke length, and fitted with a 1 cm² rubber tip. This system had a maximum inlet pressure of 3,000 PSI that was generated by a 125 ft³ compressed nitrogen tank. The pressure throughout the system was dynamically controlled via custom written Labview software (version 10.0.1; National Instruments; Austin, TX) in conjunction with a National Instruments A/D board (National Instruments Corp. Austin, TX). The pneumatic aluminum piston was housed in a raised 4-inch wooden-platform, with a cutaway section, which allowed the rubber tip to come in contact with the subject. Subjects were placed on the platform so that the L5 spinous process was in contact with the rubber tip.

2.4 Session Procedures

Following a screening session, all subjects returned for a 2-hour session at the Rochester Center for Brain Imaging. After subjects completed MR safety-screening and demographic questionnaires, they completed the following: a 15-minute response training, fitting to the pressure algometer device (5-minutes), pressure pain threshold and tolerance testing (10-minutes), a multiple-random-staircase procedure to identify pain intensity levels of “no

pain”, “mild pain”, “moderate pain” and “intense pain” (45-minutes), and then underwent a 30-minute MRI session. Greater detail is provided below.

2.41 Response Rating Training—Subjects were instructed on how to provide ratings of pain intensity using a custom built finger spanning device (FSD). Subjects were told that the thumb and index finger touching each other corresponded to ‘no pain’ and that the maximum distance achievable between the two fingers corresponded to ‘worst imaginable pain’. The FSD was calibrated to each subject. Because the FSD is an unaccustomed activity for most people, subjects practiced using the device with a simple visual attention task. This task consisted of two 5-minute blocks, where subjects were asked to use the FSD to fill a bar graph to a value between 0 and 10. Subjects were presented a random value (between 1 and 10) for 5 seconds followed by a zero for 5 seconds. Subsequently, subjects used the FSD to provide continuous pain ratings during MR scanning procedures.

2.42 Identification of pressure pain threshold and tolerance—Subjects were strategically placed on the MR-compatible pressure algometer so that the rubber tip of the testing apparatus was in contact with the 5th lumbar spinous process. The amount of pressure that was 1) first perceived as painful (pressure pain threshold) and 2) no longer tolerable (pressure pain tolerance) was identified using an ascending method of limits. For pressure pain thresholds, subjects were instructed to press the first button when they “first feel pain” from the pressure. For pressure pain tolerance, subjects were instructed to press the second button “when the pain is too much”. Pressure stimuli were applied to the lumbar spine for a 5-second duration and then increased in increments of 0.5 kg/cm² at a rate of 1.0 kg/cm²/sec until the subjects pressed the 2nd button (ie pain tolerance) or to a maximum of 18 kg/cm². If subjects did not press the 2nd button, 18 kg/cm² was recorded as pain tolerance and the trial discontinued.

2.43 Identification of “no”, “mild”, “moderate” and “intense” pressure pain—The results of the pressure pain threshold and tolerance testing were used to define the operating limits of the multiple-random-staircase method (MRSN). The MRSN was used to identify the amount of stimulus that subjects reliably categorize as being “no pain”, “mild pain”, “moderate pain”, or “intense pain”. During the MRSN, 6 separately defined staircases were presented pressure stimuli randomly. Two staircases (1-2) used to define “mild pain”, two staircases (3-4) used to define “intense pain”, and two staircases (5-6) used to define “moderate pain”. Subjects used a 4-button response box to rate the stimulus as “no pain”, “mild pain”, “moderate pain”, or “intense pain”. Each stimulus was applied for five seconds. Immediately following the stimulus, subjects gave their response.

The pressure stimuli were delivered at 20-second intervals for 36-minutes, which allowed for 108 stimuli (3 stimuli per minute $\times$ 36 minutes or 18 stimuli per staircase). The pressure stimuli were applied at a rate of 1.0 kg/cm²/sec throughout. The stimulus cycle was 5-seconds ON and 15-seconds OFF. The presentation of stimuli was random according to the following scheme, which was created with Labview software. For each stimulus presentation (1 to 108), one of six staircases was randomly selected. For each pain level staircase pair (mild, moderate, or intense), one staircase began 10% below pain tolerance and one began 10% above pain threshold. Within each randomly selected staircase, the

presentation order of stimuli was dependent on response history. The response history determined if the next stimulus in the staircase was of greater or lesser intensity. For example, if delivering a stimulus from either staircase 1 or 2 to determine “mild pain”, a response of no pain increased the next stimulus, while a response of mild, moderate or intense pain decreases the next stimulus. Likewise, if delivering a stimulus from either staircase 3 or 4 [moderate pain], a response of no or mild pain increased the next stimulus, while a response of moderate or intense pain decreased the next stimulus. Finally, when delivering a stimulus from either staircase 5 or 6 [intense pain], a response of no, mild, or moderate pain increased the next stimulus, while a response of intense pain decreased the next stimulus. The step size adjustment between delivered stimuli was dynamically adjusted between 0.5 kg/cm^2 and 1.0 kg/cm^2 based on the number of pain levels away from the expected response. The stimulus level needed to evoke each subjective pain level (mild, moderate and intense) was recorded as the average value of the final 6 recorded pressure stimuli for both staircases associated with that threshold (12 values total).

2.5 Imaging Procedures

2.51 Overview—Scanning during the fMRI visit included one anatomical and three functional MRI scans. The same experimental paradigm was used in each of the three functional scans (Figure 2).

2.52 Acquisition—MRI scanning took place on a Siemens 3T Trim Trio research-dedicated system, fitted with a 32-channel head coil. High resolution structural data were collected using a T1-weighted MP-RAGE protocol with the following parameters, 160 1mm sagittal slices, matrix (mm) = $256 \times 256 \times 160$, repetition time (TR) = 2350 ms, echo time (TE) = 3.4 ms, field of view (FOV) = 256 mm, flip angle (FA) = 7° , parallel imaging parameters = GRAPPA, acceleration factor = 2, voxel size = 1 mm^3 . Functional MRI data were collected using a gradient-echo (GRE) echo-planar imaging (EPI) protocol with the following parameters: 40 contiguous 4.0 mm trans-axial slices, matrix (mm) = $256 \times 256 \times 160$, TR/TE = 3000/30 ms, FOV = 256 mm, order of acquisition of slices = interleaved, FA = 90° , voxel size = 4 mm^3 . Four dummy volumes were discarded at the beginning of each fMRI run to reduce saturation effects due to the B_0 field inhomogeneity. Each run lasted five minutes (276-seconds), and all three runs were conducted consecutively. The FSD was used to provide continuous pain intensity ratings throughout each run.

2.53 Preprocessing—Image preprocessing was conducted using SPM12b (Wellcome Trust Centre for Neuroimaging, London, UK) with MATLAB 2011b (MathWorks, Sherbon, MA, USA). Functional MRI preprocessing procedures consisted of slice-time correction, 3D motion correction with realignment to the middle volume of each sequence, coregistration to the structural MRI followed by normalization to a standardized MNI template, and spatial smoothing [6mm isotropic Gaussian kernel (FWHM)]. Data were spike-corrected to reduce the impact of artifacts using the post-processing Artifact Detection Tool (ART) toolbox for fMRI data (http://www.nitrc.org/projects/artifact_detect). Spike-correction consisted of identifying time points that were removed during the individual-level general linear model (GLM). Points where the mean global signal changed by above 3 standard deviations,

translation movement exceeded 0.5mm or rotational movement exceeded 0.01° were considered a spike (ie outlier).

2.6 Functional MRI Analyses

2.61 Overview—The present study utilized a within-subjects design to assess the frequency and consistency of pressure pain-related brain activity in three *a priori* brain regions across three fMRI runs. The *a priori* brain regions were the anterior cingulate cortex (ACC), insular cortex (INS) and primary somatosensory cortex (SI) bilaterally. These brain regions were chosen because they are included in the “pain processing network” (Tracey, 2008). Although these regions are not specific to processing painful stimuli they have been consistently activated at the group level in prior fMRI studies (Brooks and Tracey, 2005; Tracey, 2005; Treede et al., 1999). Brain activity significantly associated with “moderate” pressure pain stimuli at the individual- and group-levels were identified using a random-effects general linear model (RFX-GLM).

2.62 Individual-level—For individual-level contrasts, the task regressor (i.e., “moderate” pressure pain stimulation periods) was deconvolved on the canonical HRF, and temporal/dispersion derivatives were modeled to remove confounds associated with differences in peak response latency and peak response duration, respectively. Additional regressors of no-interest included: the 6 motion parameters and any outlier data points that were identified during pre-processing. To extract values for the *a priori* ROIs (i.e., bilateral ACC, INS, and SI), we conducted one-sample t-tests within inclusive anatomical masks (i.e., search spaces). The anatomical masks were created with the WFU PickAtlas (WFU PickAtlas, v2.4). We used anatomical search spaces rather than individual- or group-generated BOLD volumes of interest to emulate methods that would be potentially feasible and standardized in a clinical setting. Images from individual participants for each run were thresholded [$p < .05$, cluster minimum (k) = 5 voxels], and cluster size and peak -voxel T-score values were extracted from each anatomical mask to calculate subsequent internal consistency coefficients (ICCs).

2.62 Group-level—At the group-level, an RFX-GLM (pain vs. no pain) was conducted on fMRI data to examine whether ROI activity was similar to the individual-level results. Data were thresholded using a false-discovery rate corrected p-value ($p_{FDR} < 0.001$) and a minimum cluster size of (k) = 5 and the same *a priori* inclusive anatomical-masks were applied.

2.7 Statistics

2.71 Frequency—Frequency of brain activity was assessed at the individual-level for each brain region of interest (ROI). We created a single contrast (pain vs no pain) that averaged the three fMRI scans. Each subject was assessed for activity within each *a priori* ROI, using a threshold of $p < 0.05$ and minimum cluster size (k) of 5 voxels. Frequency was reported as the percentage of the 13 participant sample who demonstrated activity. Statistics were conducted using SPSS v21.0 (SPSS Inc., Chicago, IL, USA).

2.72 Consistency of Brain Activity—Consistency of brain activity was assessed with internal consistency coefficients (ICCs) of absolute agreement. This statistic provides a

measure of reliability through a ratio of between-subject variance to total variance, (Caceres et al., 2009) and is commonly used to examine reliability of fMRI summary statistics. (Brandt et al., 2013; Caceres et al., 2009) ICCs were conducted on ROI cluster sizes and peak T-scores, for the following time points: run 1 vs. run 2, run 2 vs. run 3, run 1 vs. run 3, all 3 runs. ICC coefficients range from 0 to 1 and classification of reliability has been suggested as the following: Less than 0.4 = 'poor', between 0.4-0.6 = 'fair', between 0.61-0.8 = 'good', and greater than 0.8 = 'excellent'. Results are described in terms of these criteria. Statistics were conducted using SPSS v21.0 (SPSS Inc., Chicago, IL, USA).

2.73 Task-Related Artifacts—Task related motion artifacts (i.e. spikes) are problematic in fMRI studies. To assess for task related artifacts we looked at the percentage of data points that were identified during the task and compared that to the percentage of time subjects were doing the task. The task paradigm was 5 blocks of 25 seconds with an inter-stimulus of 25 seconds. Each scan produced 92 data points (3 second TR), for a total of 276 data points per subject. If movement occurred at random we would expect the percentage of points removed to be equal to the relative percentage of time during task, which would be approximately 45.5%.

3. Results

3.1 Frequency of Individual-Level ROI Activation

Table 2 shows the frequency of individuals demonstrating cortical activity within the 6 *a priori* brain regions, the mean peak voxel T-score and the mean cluster size around the peak voxel. Frequency of individuals showing activity ranged from 84.6% to 100%. The left primary somatosensory cortex activity was found in all 13 subjects, while both the left anterior cingulate and insular cortices had 11 of the 13 subjects. Cortical activity within all of the brain regions was found in 11 or the 13 subjects (84%).

3.2 Reliability of Peak-Voxel T-Score (Test-Retest)

Table 3 shows the ICCs for peak-voxel T-scores within all six ROIs. Coefficients for the scan to scan consistency ranged from 0.59 to 0.90, suggesting fair to excellent reliability. Coefficients for all three runs ranged from good to excellent (ICC = 0.78 to 0.89) reliability.

3.3 Reliability of Cluster Size (Test-Retest)

Table 4 shows the ICCs on cluster size within all six ROIs. Coefficients for the scan to scan consistency ranged from 0.09 to 0.90, suggesting poor to excellent reliability. Coefficients for all three runs ranged from poor to good (ICC = 0.43 to 0.76) reliability.

3.4 Group-Level Region of Interest Activity

At the group-level, bilateral SI and right INS were all significantly activated during pressure pain stimuli compared to no pain [L-SI: $t_{12}=7.01$, $p < .001$, $k = 50$ voxels; R-SI: $t_{12}=6.43$ $p < .001$, $k = 5$ voxels; R-INS: $t_{12}=5.07$, $p < .001$, $k = 10$ voxels).

3.5 Task-Related Artifacts

Each fMRI scan had 92 time points and each subject had 3 scans, which resulted in 276 time points per subject. Table 5 presents the total number of outliers detected per person, as well as the number of outliers that were during a stimulus block on a per run basis. The entire data set consisted of 3,588 total time points, of which 233 were removed during the first level GLM. Of the 233 time points that were removed, 59.7% occurred during a stimulus block period. The pressure stimulus was only being presented 45.5 % of time.

4. Discussion

4.1 Summary of Results

The present study describes a psychophysical test procedure using pressure stimuli to the lumbar spine while the subject is supine within the MR environment. Using this approach, we found a high frequency of individuals demonstrating activity within the ACC, INS, and SI bilaterally. At the group-level we found activity within the bilateral SI and right INS. The consistency of cluster size activity and peak voxel t-scores across three consecutive fMRI runs were “fair” reliability was found for the cluster size around the peak-voxel and “good to excellent” for peak-voxel T-score.

The results of this study were partially consistent with previous publications. Prior research has shown processing a painful stimulus is associated with cortical activity within the *a priori* brain regions we choose. As such the high frequency of individuals demonstrating activity within these regions is consistent with these prior studies. The good to excellent scan-to-scan consistency of peak voxel t-score is consistent with a prior study using a similar reliability methodology and thermal stimuli. The scan-to scan consistency of the cluster size around the peak voxel was lower in our study, “fair”, where prior reports using thermal stimuli report slightly higher reliability, “good ”

We suggest this feasibility study demonstrates our method can be successfully applied in larger cohorts of individuals. The advantage of using this approach is the supine patient position. The supine position allows pressure stimuli to be applied to both locations, locally over the lumbar spine and in remote locations such as the foot and thumbnail. Further, the supine position is the common position in arterial- spin labeling and resting-state fMRI as well as others. The unified position would allow for a more multi-modal imaging approach to be used during a scanning session.

Applying our fMRI approach with additional anatomical locations of pressure stimuli can be used to assess the spatial extent of central sensitization. Central sensitization manifests in the clinical setting as hyperalgesia and allodynia(Woolf, 2011). In cohorts with chronic low back pain, pressure hyperalgesia and allodynia measured locally and over remote locations discriminate healthy from clinical populations (Neziri et al., 2012). Longitudinal changes in the central representations of pressure hyperalgesia and allodynia can be used to investigate central sensitization normalization. min

4.2 Strengths and Limitations

Although there has been an explosion of fMRI studies over the past decade, few laboratories have published reliability measures associated with their particular stimulus presentation. The strength of this paper is provision of those measures along with the description of the testing apparatus and experimental design. However, caution is needed when trying to translate our estimates of reliability to different scanning environments, protocols or presentation strategies.

4.3 Implications for Future Research

Our findings suggest that using a priori anatomical search regions will demonstrate a high frequency of activity using our pressure pain stimulus. The within session scan-to-scan reliability of peak-voxel t-score appears to be better candidate than cluster-size around the peak voxel. A logical next step is to test the reliability across multiple scanning sessions. This step is needed as longitudinal imaging studies are being employed to assess central mechanisms of pain-relieving treatments.

5. Conclusion

Our results show a promising methodology with some limitations. There was a high frequency of individuals demonstrating cortical activity within the bilateral primary somatosensory cortex, left insular cortex and bilateral anterior cingulate cortex. Further, we found that there was good to excellent within session, scan-to-scan consistency of peak-voxel T-scores for each of the brain regions. The reliability of the cluster-size around the peak-voxel was poor to good, and not as consistent as the peak-voxel T-score. We did find a higher proportion of motion artifact during stimulus presentation than rest, suggesting our stimulus presentation strategy may induce some movement and would need to be accounted for in the future.

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Figure 1. MR-Compatible Pressure Algometer

Figure 2.

Three fMRI runs were collected. The paradigm for each run included five 25-second blocks of “moderate” pressure pain stimuli with a 25-second inter-stimulus interval. The amount of pressure used for each participant was individually tailored based on the multiple-random-staircase procedure conducted prior to scanning.

Table 1
Inclusion Criteria separated by group

Asymptomatic Subjects	Symptomatic Subjects
No LBP in the last 3 months	LBP > 3 months in duration from L1-SI joint
No pain elicited upon deep palpation or mechanical maneuvers of lumbar spine	Pain elicited upon deep palpation or mechanical maneuvers of the lumbar spine
mODI score of < 10%	mODI score of $\geq 20\%$
Baseline NPRS < 3	Baseline NPRS ≥ 3
No pain on pressure algometry $\leq 5 \text{ kg/cm}^2$	Pain on pressure algometry $\geq 5 \text{ kg/cm}^2$

LBP = Low Back Pain; mODI = modified Oswestry Disability Index; NPRS = Numerical Pain Rating Scale;

Table 2
Frequency of Individual-Level Region of Interest (ROI) Activity

ROI	# of Subject (%)	Mean peak Voxel T-score (SD)	Cluster size around peak voxel (SD)
Left ACC	11 (84.6%)	5.39 (3.4)	52.3 (43.1)
Right ACC	11 (84.5%)	4.84 (2.9)	46.2 (43.4)
Left INS	11 (84.6%)	6.5 (3.5)	107.8 (72.1)
Right INS	12 (92.3%)	6.6 (3.1)	106.9 (72.5)
Left SI	13 (100%)	6.9 (1.8)	229.3 (115.1)
Right SI	12 (92.3%)	7.4 (1.9)	163.5 (102.7)

Region of Interest (ROI), Number (#), Standard Deviation (SD), Anterior Cingulate Cortex (ACC), Insula (INS), Primary Somatosensory Cortex (SI)

Table 3
Intraclass correlation coefficients for cluster size test-retest reliability among fMRI runs

ROI	Run 1 vs. Run 2	Run 2 vs. Run 3	Run 1 vs. Run 3	All Runs
Left ACC	0.168	0.088	0.901 ^{***}	0.555 [*]
Right ACC	0.150	0.414	0.935 ^{***}	0.597 [*]
Left INS	0.486	0.606	0.645 [*]	0.680 ^{**}
Right INS	0.615 [*]	0.702 [*]	0.699 [*]	0.763 ^{***}
Left SI	0.531	0.149	0.566	0.529
Right SI	0.221	0.090	0.593 [*]	0.433

* significant at $p < .05$;

** significant at $p < .01$;

*** significant at $p < .001$

Region of Interest (ROI), Anterior Cingulate Cortex (ACC), Insula (INS), Primary Somatosensory Cortex (SI)

Table 4
Intraclass correlation coefficients for ROI peak T-score test-retest reliability among fMRI runs

ROI	Run 1 vs. Run 2	Run 2 vs. Run 3	Run 1 vs. Run 3	All Runs
Left ACC	0.671 *	0.722 *	0.786 **	.806 ***
Right ACC	0.660 *	0.588	0.810 **	0.784 **
Left INS	0.627	0.664 *	0.867 **	0.809 ***
Right INS	0.744 *	0.647 *	0.901 ***	0.852 ***
Left SI	0.644	0.877 ***	0.760 *	0.841 ***
Right SI	0.828 **	0.869 ***	0.854 ***	0.895 ***

* significant at $p < .05$;

** significant at $p < .01$;

*** significant at $p < .001$

Region of Interest (ROI), Anterior Cingulate Cortex (ACC), Insula (INS), Primary Somatosensory Cortex (SI)

Table 5
Number of outliers detected per subject and during data and correlation to stimulus paradigm

Number of outlier data points (Percentage of Outlier data points within Stimulus Paradigm)				
Subject	PP Block Run 1	PP Block Run 2	PP Block Run 3	Total # All Runs
Subject 001	4 (7.4%)	4 (7.4%)	4 (7.4%)	19 (6.9%)
Subject 002	4 (7.4%)	0 (0.0%)	5 (9.3%)	11 (4.0%)
Subject 003	11 (20.4%)	8 (14.8%)	0 (0.0%)	22 (8.0%)
Subject 004	9 (16.7%)	0 (0.0%)	3 (5.6%)	16 (5.8%)
Subject 005	4 (7.4%)	2 (3.7%)	4 (7.4%)	16 (5.8%)
Subject 006	3 (5.6%)	2 (3.7%)	2 (3.7%)	13 (4.7%)
Subject 007	4 (7.4%)	0 (0.0%)	0 (0.0%)	15 (5.4%)
Subject 008	2 (3.7%)	8 (14.8%)	0 (0.0%)	12 (4.3%)
Subject 009	1 (1.9%)	0 (0.0%)	1 (1.9%)	7 (2.5%)
Subject 010	11 (20.4%)	5 (9.3%)	8 (14.8%)	47 (17.0%)
Subject 011	4 (7.4%)	6 (11.1%)	4 (7.4%)	19 (6.9%)
Subject 012	5 (9.3%)	6 (11.1%)	2 (3.7%)	32 (11.6%)
Subject 013	0 (0.0%)	1 (1.9%)	2 (3.7%)	4 (1.4%)
Total	62 (8.8%)	42 (6.0%)	35 (5.0%)	233 (6.5%)

Each subject was scanned three times. Each scan was 276 seconds long and provided 92 time points. Each subject had a total of 276 time points.