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# Real-Time fMRI Brain Mapping in Animals

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Subject: The Cover Letter for the Revised Manuscript, JoVE61463_R2

Review Editor
JoVE

Dear Editor,

Please find enclosed the revised manuscript, entitled "*Real-time fMRI Brain Mapping in Animals*". We have revised the manuscript based on the comments of the editoral board and reviewers. Also, we provide a point-to-point responses to reviewers.

We hope that the revised manuscript can be accepted by JoVE.

Sincerely,

Dr. Xin Yu

**TITLE:**

# Real-Time fMRI Brain Mapping in Animals

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**KEYWORDS:**

functional magnetic resonance imaging, fMRI, blood-oxygen-level-dependent, BOLD, brain, real-time, animals, analysis of functional neuroimage

**SUMMARY:**

Animal brain functional mapping can benefit from the real-time functional magnetic resonance imaging (fMRI) experimental set-up. Using the latest software implemented in the animal MRI system, we established a real-time monitoring platform for small animal fMRI.

**ABSTRACT:**

Dynamic fMRI responses vary largely according to the physiological conditions of animals either under anesthesia or in awake states. We developed a real-time fMRI platform to guide experimenters to monitor fMRI responses instantaneously during acquisition, which can be used to modify the physiology of animals to achieve desired hemodynamic responses in animal brains. The real-time fMRI set-up is based on a 14.1T preclinical MRI system, enabling the real-time mapping of dynamic fMRI responses in the primary forepaw somatosensory cortex (FP-S1) of anesthetized rats. Instead of a retrospective analysis to investigate confounding sources leading to the variability of fMRI signals, the real-time fMRI platform provides a more effective scheme to identify dynamic fMRI responses using customized macro-functions and a common

neuroimage analysis software in the MRI system. Also, it provides immediate troubleshooting feasibility and a real-time biofeedback stimulation paradigm for brain functional studies in animals.

**INTRODUCTION:**

Functional Magnetic Resonance Imaging (fMRI) is a non-invasive method to measure the hemodynamic responses[1-9], e.g., the blood-oxygen-level-dependent (BOLD), cerebral blood volume and flow signal, associated with neural activity in the brain. In animal studies, hemodynamic signals can be affected by anesthesia[10], the stress level of awake animals[11], as well as the potential non-physiological artifacts, e.g., cardiac pulsation and respiratory motions[12-15]. Although many post-processing methods have been developed to provide a retrospective analysis of the fMRI signal for the task-related and resting-state functional dynamics and connectivity mapping[16-19], there are few techniques to provide a real-time brain function mapping solution and instantaneous readouts in the animal brain[20] (most of which are mainly used for human brain mapping[21-27]). In particular, this kind of real-time fMRI mapping method is lacking in animal studies. It is necessary to set up an fMRI platform to enable the investigation of real-time brain state-dependent physiological stages and to provides real-time biofeedback stimulation paradigm for animal brain functional studies.

In the present work, we illustrate a real-time fMRI experimental set-up with the customized macro-functions of the MRI console software, demonstrating real-time monitoring of the evoked BOLD-fMRI responses in the primary forepaw somatosensory cortex (FP-S1) of the anesthetized rats. This real-time set-up allows for the visualization of the ongoing brain activation in functional maps, as well as individual time courses in a voxel-wise manner, using the existing neuroimage analysis software, Analysis of Functional NeuroImages (AFNI)[28]. The preparation of the real-time fMRI experimental set-up for the animal study is described in the protocol. Besides the animal set-up, we provide detailed procedures to set up the visualization and analysis of the real-time fMRI signals using the latest console software in parallel with the image processing scripts. In summary, the proposed real-time fMRI set-up for animal studies is a powerful tool for monitoring the dynamic fMRI signals in the animal brain using the MRI console system.

**PROTOCOL:**

This study was performed in accordance with the German Animal Welfare Act (TierSchG) and Animal Welfare Laboratory Animal Ordinance (TierSchVersV). The experimental protocol described here was reviewed by the ethics commission (§15 TierSchG) and approved by the state authority (Regierungspräsidium, Tübingen, Baden-Württemberg, Germany).

## 1. Preparing the BOLD-fMRI experimental set-up for small animal study

1.1 Turn on the console software to control imaging parameters and acquire MRI data.

NOTE: The proposed real-time fMRI set-up is implemented utilizing macro-functions of the console software (version 6) in parallel with the image processing functions of AFNI.

1.2 Find MR sequences (i.e., Position, Localizer, Rapid Acquisition with Relaxation Enhancement (RARE), and 3D echo-planar imaging (EPI) with the workspace explorer, and then drag and append them in the scan list.

NOTE: Position and Localizer sequences are used to identify a region of interest (ROI) in a brain. A RARE sequence is used for an anatomy scan. A 3D EPI sequence is used to measure dynamic BOLD responses.

1.3 Place the predefined macro scripts, "Setup_rt3DEPI" and "Feed2AFNI_rt3DEPI" in the macro script path (e.g., "/opt/(PV version)/prog/curdir/(user name)/ParaVision/macros"). Activate the 3D EPI reconstruction options, "Pre Image Series Activities" and "Execute Macro" in the "Data Reconstruction" user interface menu, and then link the predefined macro script, "Setup_rt3DEPI", before clicking the "**Scan**" button.

NOTE: The macro scripts are included in the **Supplementary files**.

1.4 Install the AFNI software for the real-time BOLD-fMRI analysis and visualization.

## 2. Catheterization and ventilation surgery

2.1 Set up a ventilator and physiological status monitoring systems such as thermometer, blood pressure and respiration recording as shown in **Figure 1**. Set a constant frequency of 60 ± 1 breath/min with the ventilator and a temperature of 37 °C using an MR-compatible heating pad with a feedback control set.

2.2 Anesthetize an adult male Sprague-Dawley rat (300-600 g) in a chamber with 5% isoflurane for induction and deliver 2-2.5% isoflurane for surgery from a vaporizer. Check the depth of anesthesia by pinching the hindpaw and confirming the lack of a withdrawal response.

2.3 Intubate the animal with a 14 G plastic cannula for ventilation (60 ± 1 breath/min with a mixture of 70% air and 30% oxygen). Adjust end-tidal carbon dioxide ($CO_2$) to be in the range of 25 ± 5 mmHg[29].

NOTE: The intubation is critical for maintaining proper $CO_2$ levels through fMRI experiments.

2.4 Place the animal in a supine position on a surgery table and shave a thigh with an electric razor. And then, make an incision on the shaved skin with surgical scissors.

NOTE: The length of the incision is around 1-2 cm in a longitudinal direction.

2.5 Find a femoral artery and vein under the incised region for catheterization and separate the individual femoral artery and vein from the surrounding tissues.

2.6 Fasten one side of the separated femoral artery with a surgical suture and hold the other side with micro bulldog forceps. Then, make a small incision between the tied regions on the femoral artery.

2.7 Insert a catheter into the femoral artery through the small incision and tie the catheter and the artery together with surgical sutures. Monitor the arterial blood pressure constantly with the physiological monitoring system to be in the range of 80-120 mmHg and measure the arterial blood gas regularly to maintain $pO_2$ of minimum 90 mmHg and $pCO_2$ of 30-45 mmHg during scanning.

NOTE: This catheterization is critical for monitoring the arterial blood pressure during fMRI experiments.

2.8 Fasten both ends of the femoral vein with silk braided surgical sutures. Then, make a small incision between the tied regions on the femoral vein. Use forceps to perform the suturing.

NOTE: The size of the suture is around 1-2 cm.

2.9 Insert a catheter into the femoral vein. Tie the catheter and the vein together with surgical sutures.

NOTE: This catheterization is critical for administrating alpha-chloralose through the vein and adjusting the anesthetic levels during fMRI experiments. If the animal is not well anesthetized, it will start to breathe spontaneously. In this case, more alpha-chloralose must be administrated to avoid respiratory motion artifacts.

2.10 Suture the surgical incision on the shaved skin. Once the surgical procedures are completed, keep the animal anesthetized by infusing a bolus of alpha-chloralose with the dosage of ~80 mg/kg through the catheter connected to the femoral vein and stop isoflurane administration at the same time.

**3. Placing the animal inside the MRI scanner**

3.1 Transfer the anesthetized animal to the MRI scanner as soon as 2.10 step is done and secure it on a custom-made cradle.

3.2 Insert a real-time feedback rectal thermometer on the animal to monitor the animal's temperature. Place a heating pad under the animal's torso to control the temperature. Maintain the body temperature at 37.0 ± 0.5 °C during MRI scans.

3.3 Deliver alpha-chloralose with ~25 mg/kg/h solution in a mixture of pancuronium (~2 mg/kg/h), a muscle relaxer, continuously while keeping the animal anesthetized and reducing motion artifacts in fMRI images. Monitor the blood pressure and respiration by adjusting the

amount of drug and the rate of ventilation according to the physiological status.

3.4 Administer ophthalmic ointment on the eyes of the animal to prevent dryness during fMRI experiments. Fix the animal's head safely with two ear bars to avoid head motion artifacts.

3.5 Fix a transceiver surface coil on the head. Tune and match the coil to the Larmor frequency (e.g., 599 MHz on 14.1 T) on the head before MRI measurements.

NOTE: Here, 22 mm diameter coil is used to cover the whole brain of a rat.

3.6 Insert a pair of needle electrodes into the skin of the forepaw between digits 1 and 4 and fix them with surgical tape. And then, confirm that the stimulation works properly after connecting a stimulation input cable to these electrodes[30].

3.7 Insert the animal into the MRI bore and place it at the iso-center approximately.

## 4. Measuring anatomical MR images

4.1 Click the calibration menu button in the main user interface. Perform the calibrations of the MRI system clicking the following items in the **Adjustment Platform** user interface (see **Help** menu in the console software): Find the basic resonance frequency, Calibrate the RF pulse power, Set the optimal receiver gain, Measure the B0 map in the animal for shimming, Run global linear shims based on non-localized free induction decay (FID) integral.

NOTE: This step takes less than 2 min.

4.2 Run a Position sequence by clicking the "**Scan**" button to find the head location of the animal inside the MRI bore. If the head is not located at the iso-center, adjust the head location while moving the cradle back and forth until the head is located at the iso-center.

4.3 Run a Localizer sequence by clicking the "**Scan**" button to identify an ROI in the head. Select **Map Shim** and define the ROI of the shim volume to cover the whole brain in the localizer image and then, run a high order (e.g., 2$^{nd}$ or 3$^{rd}$ order) shimming using the "**Shim up to**" option to reduce the main magnetic field (B0) inhomogeneities at the ROI.

NOTE: The high order shimming is a critical step to improve the quality of BOLD-fMRI data when EPI sequences are used.

4.4 Run a T2-weighted RARE sequence by clicking the "**Scan**" button to acquire anatomical images covering the whole brain in a coronal view (e.g., the following sequence parameters are used: repetition time (TR) 4000 ms, effective echo time (TE) 36.1 ms, matrix 128 x 128, field of view (FOV) 19.2x19.2 mm$^2$, number of slices 32, slice thickness 0.3 mm, RARE factor 8).

NOTE: In the following real-time fMRI visualization step, the anatomical images are used to

register 3D EPI images as a template.

## 5. Real-Time fMRI software set-up and fMRI response visualization

5.1 Open a terminal window and go to the real-time AFNI plugin path using the following command:

*cd /home/(user name)/rt_afni*

NOTE: The AFNI plugin script, "afni_rt" is included in the **Supplementary files**.

5.2 Execute AFNI software with the real-time plugin using the command and options below.

*afni -rt*
*-yestplugouts*
*-DAFNI_REALTIME_MP_HOST_PORT=localhost:(port number)*
*-DAFNI_REALTIME_Graph=Realtime*
*-DAFNI_FIM_IDEAL=(Paradigm)*

NOTE: In the first case, the code allows external programs to exchange data with AFNI while in the second case the real-time plugin will attempt to open a TCP socket to the user-defined localhost and port. In the third and fourth cases, the codes will plot the time course of fMRI data in real-time and plot the time course of the user-defined paradigm in the fMRI time course respectively when real-time fMRI data are acquired. For further details, check https://afni.nimh.nih.gov/pub/dist/doc/program_help/README.environment.html.

5.3 Monitor upcoming AFNI BRIK files defined by using the command "Dimon" as shown in **Figure 2** with the following options:

*Dimon -tr (TR of EPI) -nt (NRepetitions of EPI)*
*-rt -quit*
*-infile_pattern realtime*.BRIK*
*-file_type AFNI*

NOTE: "Dimon" is a command to monitor the real-time acquisition of AFNI image files using the following options: "-rt" which executes the real-time plugin and "-infile_pattern (data name).BRIK -file_type AFNI" which allows the plugin to read the specific BRIK files and to send them into AFNI for display and formatting. For further details, check https://afni.nimh.nih.gov/pub/dist/doc/program_help/Dimon.html.

5.4 Use "pvcmd" command with the following options:

*pvcmd -a JMacroManager JMMExecuteMacro -category $USER -macro Feed2AFNI_rt3DEPI*

NOTE: This code exists in the macro script, "Setup_rt3DEPI", to run the background macro script, "Feed2AFNI_rt3DEPI", right after clicking "**Scan**" button for EPI acquisition.

5.5 Use "exec pvcmd" command with the following options to get EPI acquisition

parameters.
*exec pvcmd -a ParxServer -r ParamGetValue -psid $ParSpaceId -param (PVM parameters of EPI) -id 10 -args $AcqKey $ParSpaceId $ProcnoPath*

5.6 Use "exec to3d" command with the following options to convert EPI raw data to AFNI files in real-time in the background macro script, "Feed2AFNI_rt3DEPI".
*exec to3d -omri -xFOV $FOV_X -yFOV $FOV_Y -zFOV $FOV_Z -prefix $LastVolName $ImgFormat$Path2dseq*

5.7 Make sure that EPI geometrical information is consistent with the anatomy orientation.

NOTE: The "to3d" AFNI command will run automatically with the geometrical information such as the field of view (FOV) and matrix size to convert the fMRI raw data into one AFNI BRIK data whenever each 3D volume data is stored after every single TR as shown in **Figure 2**. The image orientation can be changed with the geometrical information parameters of "to3d". For further details, check https://afni.nimh.nih.gov/pub/dist/doc/program_help/to3d.html.

5.8 Turn on an electrical stimulus isolator and perform electrical forepaw stimulation for one evoked fMRI study (e.g., 3Hz, 4s pulse width 300us, 2.5mA) using stimulation blocks.

NOTE: Here, the block-design paradigm consists of 10 pre-stimulation scans, 3 stimulation scans and 12 inter-stimulation scans (15 scans per epoch).

5.9 Run a T2*-weighted 3D EPI sequence by clicking the "**Scan**" button for the BOLD-fMRI study (e.g., the following parameters are used: TR/TE 1500/14 ms, matrix 64 x 64 x 32, FOV 19.2 x 19.2 x 9.6 $mm^3$, and resolution 300 x 300 x 300 $\mu m^3$).

Note: As soon as clicking the "**Scan**" button, monitoring, and processing raw data will be done by using the predefined macro scripts in real-time. Once one AFNI BRIK dataset is converted, voxel-wise time course graphs for 3D EPI images are displayed in the AFNI software and automatically updated for every single TR.

5.10 To overlay the EPI images on top of the anatomical RARE images, convert the RARE images to an AFNI BRIK dataset using the command "to3d" as in step 5.6, then register the EPI images to the anatomical images using the "align_epi_anat.py" AFNI script with the following options:
*align_epi_anat.py -anat anatomy_template_al+orig -epi epi.$(epi data number)+orig -epi_base 1 -suffix _volreg -rat_align -cost lpa -epi2anat*

NOTE: For further details, check https://afni.nimh.nih.gov/pub/dist/doc/program_help/align_epi_anat.py.html.

5.11 To process functional maps of the BOLD responses, calculate the deconvolution of 3D+time dataset with a specific stimulus time series using the "3dDeconvolve" command with

the following options:
*3dDeconvolve -input (input file name)+orig. -nfirst 0 -polort 3 -num_stimts 1 -stim_times 1 (stimulation paradigm file name) 'BLOCK(4,1)' -stim_label 1 forepaw -tout -fout -rout*

NOTE: Image processing steps such as spatial smoothing or temporal filtering have been incorporated into a customized AFNI data processing script. For further details, check https://afni.nimh.nih.gov/afni/doc/help/3dDeconvolve.html.

5.12 To visualize functional maps of the BOLD signals, use an interactive clustering in the AFNI software. Open the "Define Overlay" option and use the "Clusters" function from the AFNI user interface menu.

5.13 After the last fMRI scan, take the animal out of the MRI scanner and euthanize it according to the approved protocols.

NOTE: Image processing functions of AFNI and macro-functions in the latest console software were used to process the real-time fMRI data. Detailed information and descriptions of macro-functions can be found from the help menu in the console software. The AFNI software is a freeware, that can be directly downloaded through the NIMH-AFNI website. The related scripts to build the linkage between AFNI and the console system are attached.

**REPRESENTATIVE RESULTS:**

**Figure 3** and **Figure 4** show a representative real-time voxel-wise BOLD-fMRI time course and functional maps with electrical forepaw stimulation (3 Hz, 4 s, pulse width 300 us, 2.5 mA). The fMRI design paradigm comprises 10 pre-stimulation scans, 3 stimulation scans, and 12 inter-stimulation scans with a total of 8 epochs (130 scans). The total scan time is 3 min 15 sec (195 sec). **Figure 3** shows the voxel-wise time course (black line) of the contralateral FP-S1 corresponding to the block-design paradigm (red line) in the real-time acquisition format. **Figure 4** shows the activated BOLD maps corresponding to the electrical forepaw stimulation. The activated regions are detected and displayed as the colored clusters (red and yellow colors). Experimenters can use the "Clusters" function in the AFNI software to interactively explore clustered volumes and display them as an overlaid color-coded image.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Real-time fMRI experimental set-up for forepaw stimulation.** A simplified schematic of the real-time fMRI set-up and the flow (dashed lines) of the control parameters are shown. One computer (left) is used as a console for pulse sequence execution, stimulus isolator control, and data analysis with AFNI. The other computer (right) is used for monitoring physiological information (e.g., blood pressure, respiration, and chest movement, etc.).

**Figure 2: Diagram of the data processing during fMRI scanning.** A simplified flow chart of data processing with the representative macro and AFNI functions in the real-time fMRI set-up is shown. Before starting fMRI scans, the "Pre Image Series Activities" and "Execute Macro" options are selected among the reconstruction options. The "Setup_rt3DEPI" script is executed

by using those options when clicking the "Scan" button. With the "Dimon" command, the real-time AFNI files are monitored and sent into the AFNI plugin to display dynamic BOLD responses when the background macro script, "Feed2AFNI_rt3DEPI" converts the fMRI raw data to the AFNI files.

**Figure 3: Real-time voxel-wise fMRI responses.** An activated single voxel time course graph (black line) from the primary forepaw somatosensory (FP-S1) cortex is shown during the block-design stimulation paradigm. The repetitive fMRI design paradigm (red line) was defined by the "afni -rt -DAFNI_FIM_IDEAL=(Paradigm)". The graph demonstrates that clear and stable BOLD responses follow electrical stimulation in real-time.

**Figure 4: Functional maps of BOLD responses to electrical stimulation in contralateral FP-S1 regions.** The voxel clusters activated in the FP-S1 regions (yellow and red colors) were identified and significantly synchronized with the repetitive stimulation paradigm, overlaid on the T2-weighted anatomical images.

**DISCUSSION:**

Real-time monitoring of the fMRI signal helps experimenters adjust the physiology of animals to optimize functional mapping. Motion artifacts in awake animals, as well as the anesthetic effect, are major factors that mediate the variability of fMRI signals, confounding the biological interpretation of the signal by itself[31-38]. The real-time fMRI platform offers instantaneous information to assist the optimization of scanning parameters and anesthetic administration schemes. Also, real-time brain hemodynamic responses can be used to provide fMRI-based biofeedback controlling signals for novel stimulation paradigms in multi-modal brain functional studies.

A remaining concern about the proposed real-time fMRI set-up is the technical dependency on the vendor-specific console software. In this protocol, the real-time fMRI analysis scripts implement a series of macro-functions using a console software (see **Table of Materials**) version 6 or higher. The workflow of the MR scan in the previous console software (e.g., PV version 5 or lower) is different from the latest version due to the upgraded user interface and new parameter definition. Using the previous version of the console system (PV version 3), Lu et al. (2008) have shown that the real-time fMRI set-up enabled the monitoring of the drug-induced hemodynamic signal changes in the rat brain to study the cocaine's effect on the central nervous system[20]. However, those set-ups cannot be readily applied to the new console software with state-of-the-art electronic devices. In the latest console software, it is a critical step to run the predefined macro scripts and monitor fMRI raw data right after starting to scan by selecting the "Pre Image Series Activities" and "Execute Macro" options of the "Data Reconstruction".

For further image processing, customized AFNI functions can be readily incorporated into the real-time image processing scripts. In particular, it will be valuable to provide real-time analysis using motion-related traces, e.g., electromyography (EMG) signal for awake animal fMRI[38], and incorporate multi-modal dynamic brain signal, e.g., GCaMP-mediated $Ca^{2+}$, to specify whole-

brain hemodynamic correlation[37]. Furthermore, this real-time fMRI set-up can be extended to animal neurofeedback studies to investigate self-regulating brain and behavior similar to previous human studies[27].

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**DISCLOSURES:**

Sascha Köhler is an employee at Bruker BioSpin MRI GmbH.

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Figure 1



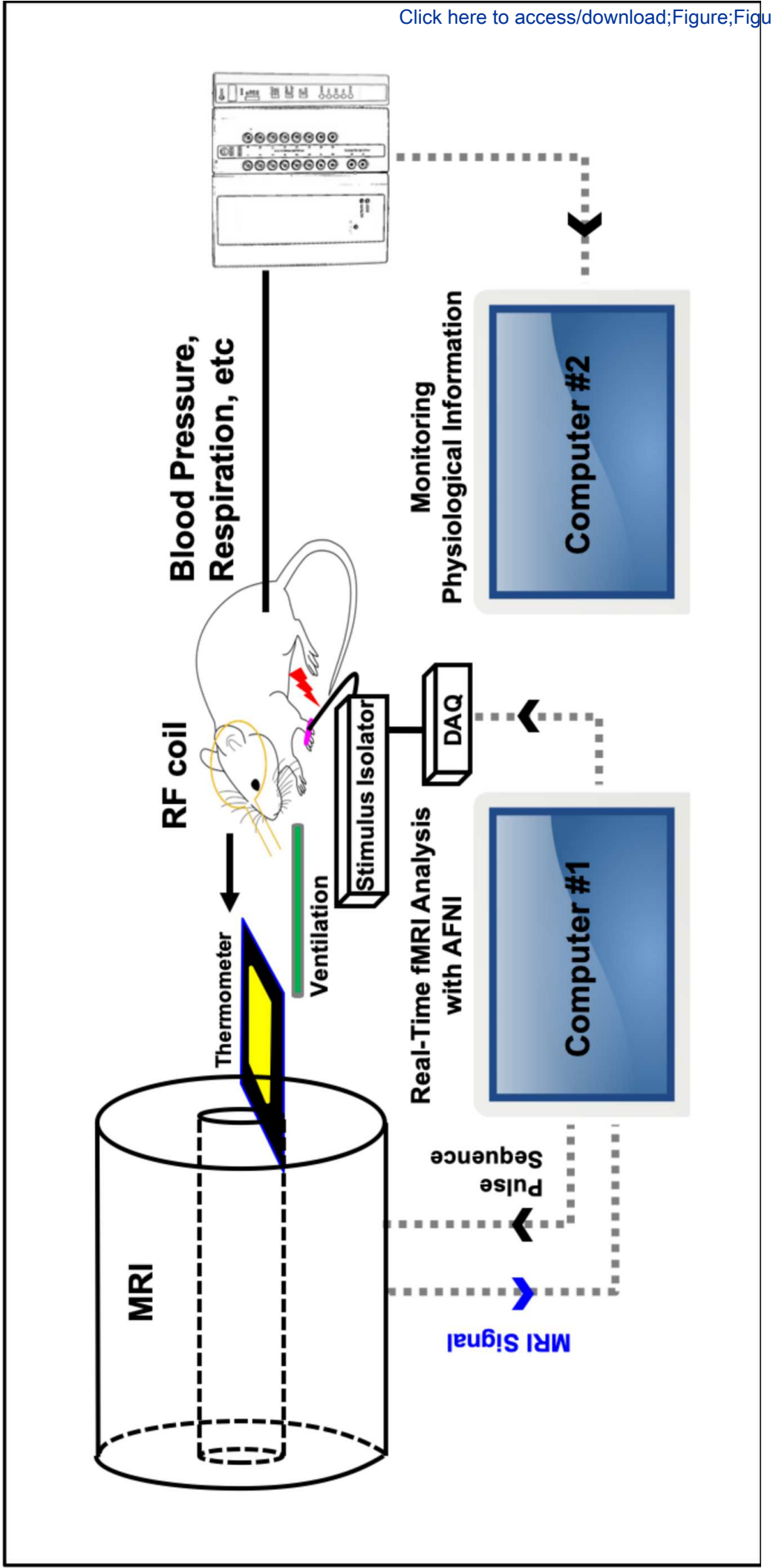

Figure 2



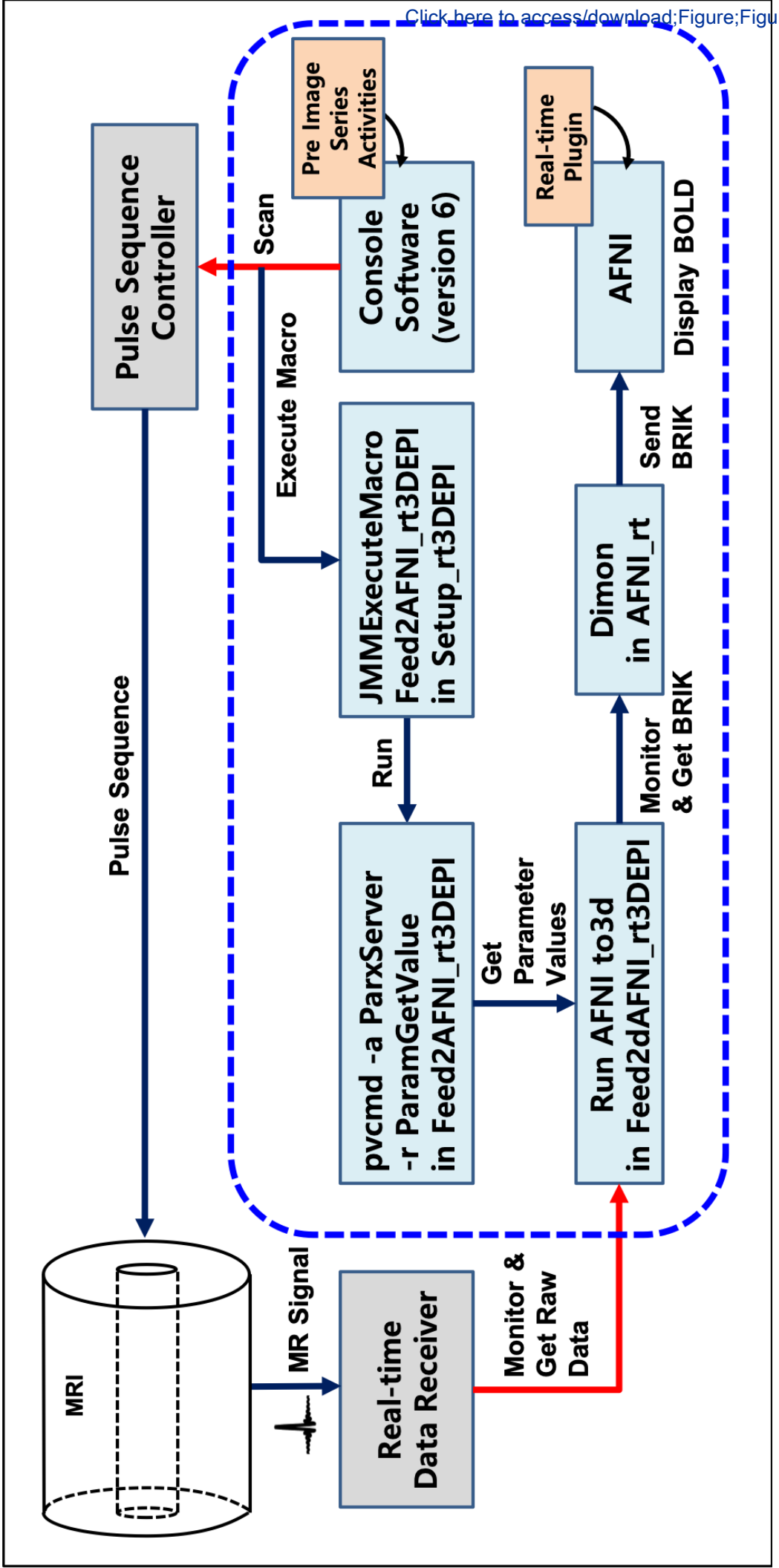

Figure 3

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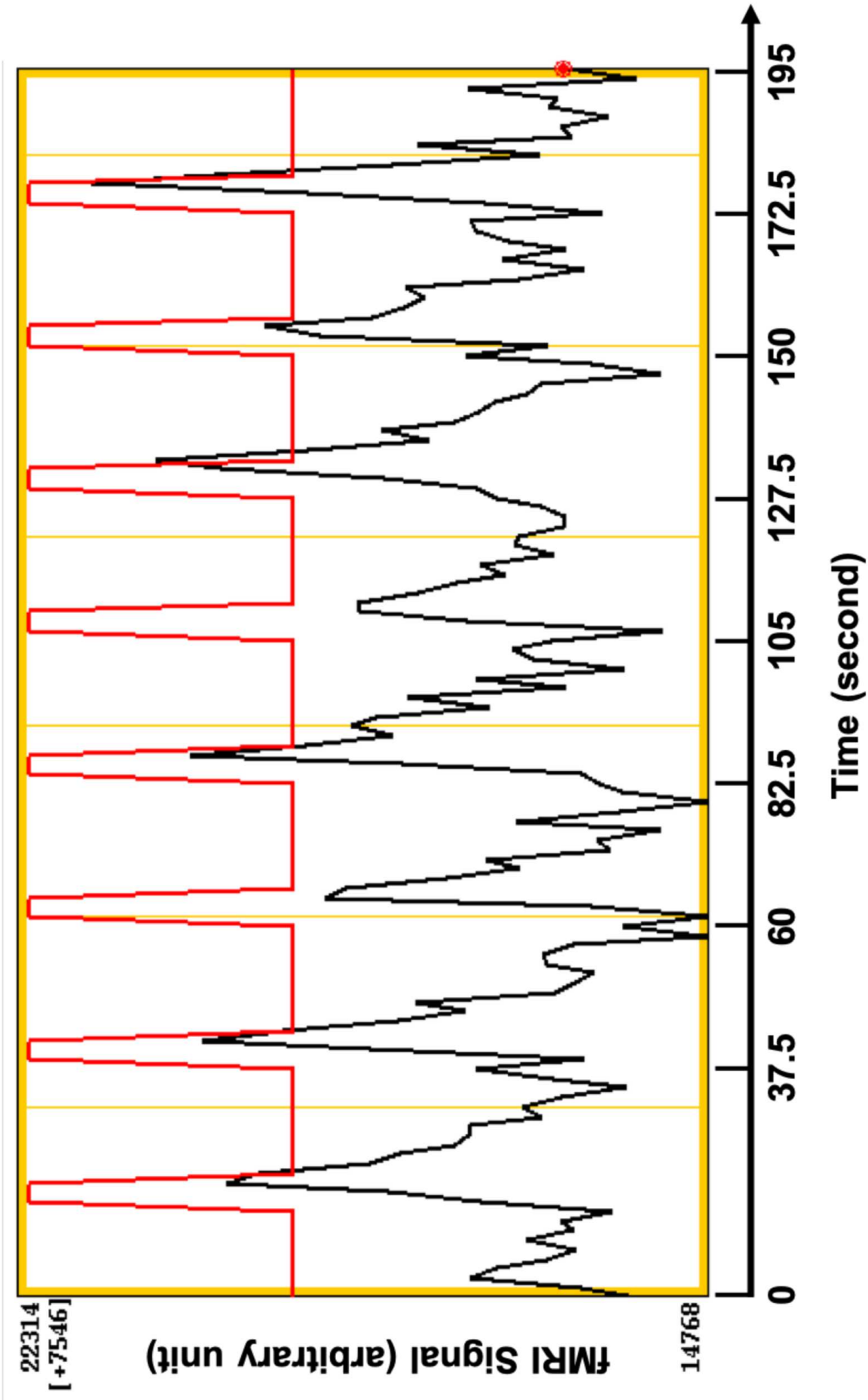

Figure 4

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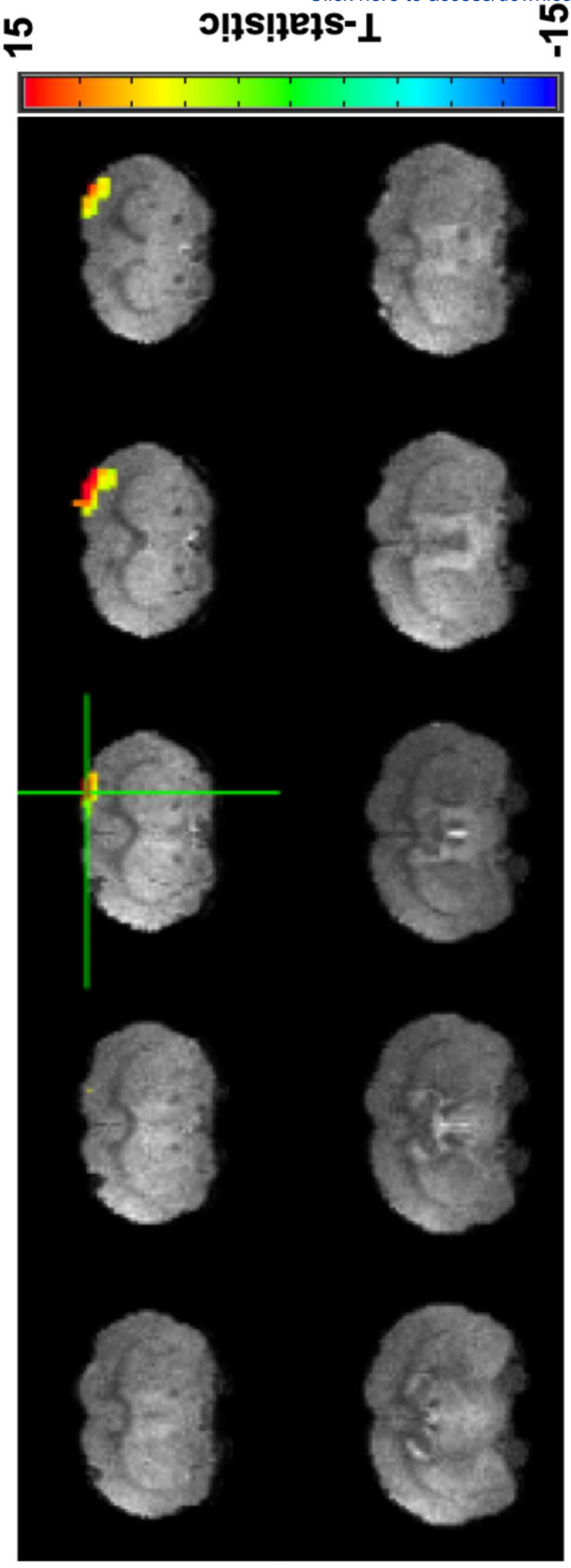

Table of Materials



| Name of Material/Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 14.1T Bruker MRI system | Bruker BioSpin MRI GmbH | N/A | |
| A365 Stimulus Isolator | World Precision Instruments | N/A | |
| AcqKnowledge Software | Biopac | RRID:SCR_014279, http://www.biopac.com/product/acqknowledge-software/ | |
| AFNI | Cox, 1996 | RRID:SCR_005927, http://afni.nimh.nih.gov | |
| CO2SMO (ETCO2/SpO2 Monitor), Model 7100 | Novametrix Medical Systems Inc | N/A | |
| Isoflurane | CP-Pharma | Cat# 1214 | |
| Master-9 | A.M.P.I | N/A | |
| Nanoliter Injector | World Precision Instruments | Cat# NANOFIL | |
| Pancuronium Bromide | Inresa Arzneimittel | Cat# 34409.00.00 | |
| ParaVision 6 | Bruker BioSpin MRI GmbH | RRID:SCR_001964 | |
| Phosphate Buffered Saline (PBS) | Gibco | Cat# 10010-023 | |
| Rat: Sprague Dawley rat | Charles River Laboratories | Crl:CD(SD) | |
| SAR-830/AP Ventilator | CWE | N/A | |
| α-chloralose | Sigma-Aldrich | Cat# C0128-25G;RRID | |

**Editorial comments:**

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

➔ *We have revised the manuscript using the attached version.*

2. Please address all the specific comments marked in the manuscript.

➔ *We have addressed all the specific comments marked in the manuscript as follows:*

TITLE:

2.1 Please ensure that the title is focused on the presented protocol.
Real Time fMRI Brain Mapping in Animals using …..
The manuscript needs a thorough proofreading.

➔ *Following the suggestion above, we have revised the title and thoroughly proofread the manuscript.*

AUTHORS & AFFILIATIONS:

2.2 Please include email addresses of all authors as shown above for corresponding author.

➔ *We included the email addresses of all the authors.*

SUMMARY:

2.3 Please tone down the language to focus on the protocol instead.

➔ *We replaced the word with a neutral word to tone down the language.*

INTRODUCTION:

2.4 Please expand. Is this open access? If not, please use generic term instead.
Please do not include the link to the lab in the table of materials.

➔ *We expanded the word. As described in Code availability, 'AFNI' is open access software. We did not include any link to the lab in the Table of Materials.*

PROTOCOL:

2.5 Please remove the redundancy from the protocol and make the steps crisps especially for the last section.

➔ *We removed the redundancy from the protocol and have revised the steps of the last section to make them luculent.*

2.6 Please ensure that the highlighted section is no more than 2.75 pages including headings and spacings.

➔ *We highlighted the sections which is no more than 2.75 pages including headings and spacings.*

2.7 How is this done? (step 1.2)

➔ *We have revised the step 1.2 to explain how to be done. The step is clear for anyone who has ever used MR sequences.*

2.7 Please move this note where image processing is being done. (step 1.4)

➔ *We moved this note to step 5.11 where image processing is being done.*

2.8 How is this done? (step 2.1)
➔ *We added the information about how to be done.*

2.9 Size of the suture? Material? (step 2.8)
➔ *We added the information about the suture.*

2.10 Do you let the animal recover after this? Please describe the post operative steps. (step 2.10)
➔ *We have revised this step to make the meaning obvious. As described in this step, we administrate alpha-chloralose, which is an anesthetic, meanwhile stopping isoflurane administration. Therefore, we keep the animal anesthetized after this step. If we administrate both alpha-chloralose and isoflurane at the same time, the animal would die. No post-operative steps required.*

2.11 This part can be divided into two sections: Animal preparation and measurement. (step 3)
➔ *Based on the comment, we divided step 3 into two sections*

2.12 Also if steps are highlighted please highlight the headings as well. (step 3)
➔ *We highlighted the headings if steps are highlighted (e.g., step 4).*

2.13 Is there a specific reason to do so? (step 3 NOTE)
➔ *We removed the NOTE because we included the information in the Table of Material.*

2.14 Time frame after surgery? Do you anesthetize the animal before placing on the MRI scanner? (step 3.1)
➔ *We included the information about time frame in step 3.1. Yes, we keep the animal anesthetized since the surgery procedure started.*

2.15 Reasons for doing this? (step 3.3)
➔*Alpha-chloralose is an anesthetic. The whole protocol is conducted with an anesthetized animal as described in the abstract and introduction. Therefore, we deliver alpha-choralose to keep the animal anesthetized.*

2.16 In the software? Clicking or checking these items? (step 3.8)
➔ *It is performed in the software (Adjustment Platform) by clicking the items. We have revised this step to make the meaning clear.*

2.17 Please include how much time is taken to perform the calibration. (step 4.1)
➔ *We added the information in step 4.1 NOTE.*

2.18 How is this done? (step 4.2)
➔ *We added the information about how to be done.*

2.19 How is this done? (step 4.3)
➔ *We added the information about how to be done.*

2.20 Please expand the abbreviation. (step 4.3)
➔ *We have already expanded the abbreviation in the first place (step 1.2) before used it in step 4.3.*

2.21 From here on please describe the action to show what is being done and in the following lines write the code/command being used to show how the action is being performed. Please refer to the example manuscripts attached along with this email. (step 5)
➔ *We have revised this section in step 5 referring to the examples attached.*

2.22 Please ensure that the codes/commands are written exactly how it is to be used in case of performing your experiment. Please write the codes/command in a new line. (step 5)
➔ *We have revised this section in step 5 adding the codes/command in a new line.*

2.23 Please ensure that the steps are written in the order. (step 5)
➔ *We have written the steps in the order.*

2.24 Presently there is a missing link between step 3.12 and 4.1. Please bring out continuity. (step 4.4 and 5.1)
➔ *We have added a NOTE between step 4.4 and 5.1 to bring out continuity. Step 4.4 is conducted for anatomical images and then, we acquire 3D EPI images in step 5 to overlay the EPI images on top of the anatomical images after setting up real-time fMRI software.*

2.25 Open the tcp/IP socket or connect the TCP/IP socket? (step 5.2)
➔*In this step, we open the TCP/IP socket to the corresponding host and port. Then, based on the socket communication, the TCP/IP socket is connected when a request comes from the client.*

2.26 Please make this as a numbered action step. When is this done? (step 5.2)
➔ *We have revised step 5.2. This step is to execute AFNI software with the real-time plugin using the command and options. It cannot be divided into numbered action steps.*

2.27 How is this used? (step 5.3)
➔ *We added the command and options in new lines referring to the example attached.*

2.28 Please ensure that these macro scripts are present as supplementary files. (step 5.4)
➔ *We included the macro scripts in the supplementary files.*

2.29 Please make substeps. Please include how each step is performed. (step 5.5 and 5.6)
➔ *We made substeps and added the command and options in new lines.*

2.30 Is this included with the submission? (step 4.8)
➔ *We included the "align_epi_anat.py" file in the supplementary file.*

DISCUSSION:
2.31 Please ensure the discussion contains all of the following with citations.
a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
➔ *We have included the above contents in the Discussion.*

3. The manuscript needs thorough proofreading.
➔ *We have thoroughly proofread the manuscript.*

4. For the last section of the protocol please see attached example manuscript and format accordingly.
➔ *As described above, we have revised the last section.*

5. Once done please ensure that the highlighted section is no more than 2.75 pages including headings and spacings.
➔ *As mentioned above, we highlighted the sections.*

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**Reviewers' comments:**
**Reviewer #1:**
Manuscript Summary:
The authors describe a protocol for real-time functional magnetic resonance brain mapping in rats, which uses a particular software (Paravision version 6 or higher) available from one vendor (Bruker Biospin) in combination with the AFNI software package.
The protocol is relevant and of interest for a number of researchers performing fMRI in rodents. Overall, the protocol describes the procedures in a comprehensive manner.
The revision has substantially improved the manuscript. All procedures are now clear. Errors have been corrected/removed and missing details and descriptions have been added.

Major Concerns:
I have no major concerns that speak against publication.
➔ *Thank you for the comment.*

Minor Concerns:
There are still some grammatical and linguistic errors. Especially the use of direct articles is sometimes awkward.
➔ *We carefully corrected all the grammatical and linguistic errors following the comments.*

**Reviewer #2:**
In addressing the reviewers' concerns the authors have provided an additional figure (Fig. 2) and supplementary codes (Suppl. codes 1-3), revised figure (Fig. 1), and revised text.
The manuscript is much better now after the revision and I consider the manuscript suitable for publication.
➔ *Thank you for the comment.*

real-time set-up macro script

Click here to access/download

**Supplemental Coding Files**

Feed2AFNI_rt3DEPI