Assessment of Resting State Functional Connectivity in an Animal Model Using Optical Intrinsic Imaging

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Abstract

In this investigation, the effects of mild closed head injury (cHI) on resting state functional connectivity (RSFC) were observed. Resting state changes in oxy- and deoxy hemoglobin (HbO, and HbR, respectively) concentrations were measured with optical intrinsic signaling (OIS). After planned imaging of four mice sham mice, and five injured mice, we were unable to analyze data for the complete population due to hard drive issues. However, data for Mouse 1, which was a sham-injured mouse, was preserved and analyzed. According to pilot data, our findings suggested that mice that are subject to cHI show a decrease in the area of the observed brain region, as well as a decrease in correlation, as reflected by the coefficient. Because of unforeseen issues with anesthetic availability, data storage, and technical concerns, the findings are not conclusive.

Introduction

Traumatic Brain Injury (TBI) is characterized by cognitive dysfunction caused by impact to the head. TBI is prevalent in contact sports, and seen particularly in football; thus, it is an area of key focus in the neuroscience and biomedical engineering realms. The most common form of TBI is concussion, scientifically known as mild traumatic brain injury [10]. In the case of concussion or resultant brain trauma, drastic molecular changes can occur in the brain. As many scientific studies have suggested, structure determines function [10, 11]; in this study, this relationship allows for the understanding of how neural and vascular connections can further impact an organism's mobility, thinking, and metabolic processes. Consequently, we can better understand how these areas communicate. This functional relationship between two different brain regions located spatially, as analyzed by temporal similarities over time is referred to as

resting state functional connectivity (RSFC) [11]. As an extension of neuroscience, the relationship between brain injury and functional connectivity is of importance, especially with regards to how metabolic function of a region of the brain may be altered after impact to the head.

Several studies have demonstrated reduced functional connectivity after TBI. These preliminary studies suggest that there is a need to better understand how functional connectivity in the brain changes post-injury in order to develop diagnostic and/or prognostic behaviors. However, RSFC is often difficult to study the effects of repetitive head impact over predetermined periods of time because of the ethical underpinnings involving human research, in that we cannot intentionally cause injury to a human subject [8, 9]. In addition, it is difficult to obtain a uniform patient cohort in which the injury stimulus and area of impact are the same across the board. An alternative to imaging humans is an animal research model [1], which allows researchers to have a more controlled model for mechanism of injury, timing, and other variables.

To study functional connectivity in the brain after repetitive head injury in mice, we use optical intrinsic signal imaging (OIS) [2,4]. In OIS, the exposed skull is illuminated with visible light, and intensity fluctuations of diffuse reflected light caused by changes in hemoglobin concentration are detected. The modified Beer-Lambert law is used to relate changes in the detected intensity over time and at multiple wavelengths to changes in the oxy (HbO) and deoxy-hemoglobin (HbR) [4]. To quantify resting state functional connectivity, resting fluctuations in measured HbO as a function of time are used to create a pixel-based map of resting-state functional connectivity by looking at the strength of the correlation between the HbO time series of a particular pixel and the time series of all other pixels in the image. MATLAB is used to

transform the raw output of the OIS system (i.e., the images of detected intensity as a function of time) to create resting state functionality maps by looking at specific pixels from a particular brain region in each acquired image. These pixels are related that to how each seed changes by frame over time, as indicated by a Pearson correlation test [11].

Methods and Materials

The methods used in this study support the motive to image mice that were subject to concussion in order to analyze changes in RSFC post-injury. Nine adult mice served as test subjects for our animal model. Using appropriate methodology for the investigation, findings were compiled and analyzed.

Animal Subjects

For the purpose of understanding the evolution of injury after human TBI, an animal model of repetitive mild traumatic brain injury was used. A total of nine mice were selected for administration of cHI, imaging, and data analysis. Five of the mice were experimental (injury), while the remaining four mice were classified as our sham (control) group. The cHI mice were subject to one hit per day over the course of five days [7]. The sham, while placed under anesthesia, did not experience any brain trauma. After the fifth hit/sham injury, RSFC data was acquired for a ten-minute imaging period, and euthanasia followed.

Preparation of mice

Mice were anesthetized during imaging to reduce motion artifact. For anesthesia during imaging, we prepared appropriate amounts of anesthesia according to a Ketamine-Xylazine Concentration table [Appendix] which was created in the lab. A one-time dose of 80-90mg/kg

ketamine and 5-15mg/kg dose of xylazine was administered. Three mL syringes with a 22G X 1 needle were used for storage of the drugs until the mouse was ready for injection. Once the anesthesia had been prepared, IACUC-defined animal procedure was followed to inject intraperitoneally. After the mouse lost consciousness, its scalp was shaved using a trimmer, an incision was made in the scalp using surgical scissors, and the mouse was placed in the stereotaxic device. The mouse was positioned under the imaging system so that the brain was in the camera's field of view, mineral oil was applied to the exposed skull surface, and a cotton swab was used to clear the transparent skull of any hair in order to provide a clear, full view of the brain. At this point, each of the three LEDs were switched on to ensure proper direction of the light rays to facilitate uniform illumination of the brain and a high signal-noise ratio (SNR). A visual representation can be found in the Appendix.

OIS Instrumentation and Data Acquisition

Image acquisition consisted of two parts: setup and data collection. An Andor camera and lens was used throughout both sessions. During setup (animal positioning), the accompanying imaging software was opened, and the "take video" button was used to open a rolling view of the field of view. For the purpose of imaging, three unique wavelengths were used, including a blue lamp of 470 nm, an orange lamp of 590 nm, and a red lamp of 625 nm [11]. As the video was running, the three LEDs and laser diode were adjusted over the exposed skull to evenly illuminate the surface, and the CCD was adjusted to ensure that the brain was in focus. For data collection, a pre-determined exposure time of 3 milliseconds was set, and the total imaging time of ten minutes was entered into the Andor camera program software. Once the data storage location on the hard drive was specified, we selected the trigger cable box as the method for

external input to guide the LED pattern. At this point, we named the file appropriately, took a signal and began the imaging period. Note: the complete RSFC Imaging Protocol can be found in the Appendix.

Data Analysis

Post-acquisition of data, a series of MATLAB codes were used to produce the desired RSFC map. The codes are used to convert changes in intensity to changes in HbO, filter and process raw data, as well as use an atlas to create an RSFC map.

The Andor software described in the previous section produced what are called "spool files" in a ".dat" format. The objective of the first part of the analysis was to be able to load all spool files and convert raw data to a file format that could be readily loaded at a later point during analysis. These original image files were entered into the first (sif2mat_final_72018.m) of three codes in order to be converted to a ".mat" format.

The second code (ois_analysis_112017) took the intensity images and converted them to images of $\Delta HbO(t)$ using the modified Beer-Lambert law, $\Phi(t) = \Phi_0 * \exp(-\Delta\mu_a(t) * L)$, where $\Phi(t)$ is light intensity, Φ_0 is the baseline light intensity, and $\mu_a(t)$ is the absorption coefficient with reference to blood volume [11]. *Figure 1* (top row) shows time series of $\Delta HbO(t)$ for five different pixels within the olfactory, motor, somatosensory, retrosplenial, and visual regions. The mean HbO(t) was calculated, a bandpass filter was applied to each pixel's HbO(t) to filter data between 0.1 to 0.01 Hz, and the time series of each pixel was resampled for the next step of processing. The penultimate series represents the z-scored signals for each brain seed. The last row shows the raw reading for the global signal regression, which is distinctive in that the mean with regard to the image, and not the HbO(t) value, was used.

The third code (pre_rsfcGUI_P2_113017.m) took the processed HbO(t) images and converted them into a resting state functional connectivity map. After loading raw OIS images, this code asked the user to select two pre-defined landmarks on the brain. By using these reference points, the code is able to transform all images to a standard mouse brain atlas (*Figure 3).* Finally, a unique resting state functional connectivity map was created for the sham-injured mouse that was imaged, as shown in *Figure 4*. *Figure 2* shows the steps involved in execution of seed-based functional connectivity. Each seed time trace (*Figure 2a*) is correlated with each brain pixel's time trace in the image (*Figure 2b*). A Pearson correlation test gives an R value for every pixel in the image, which denotes the strength of this correlation.

Figures

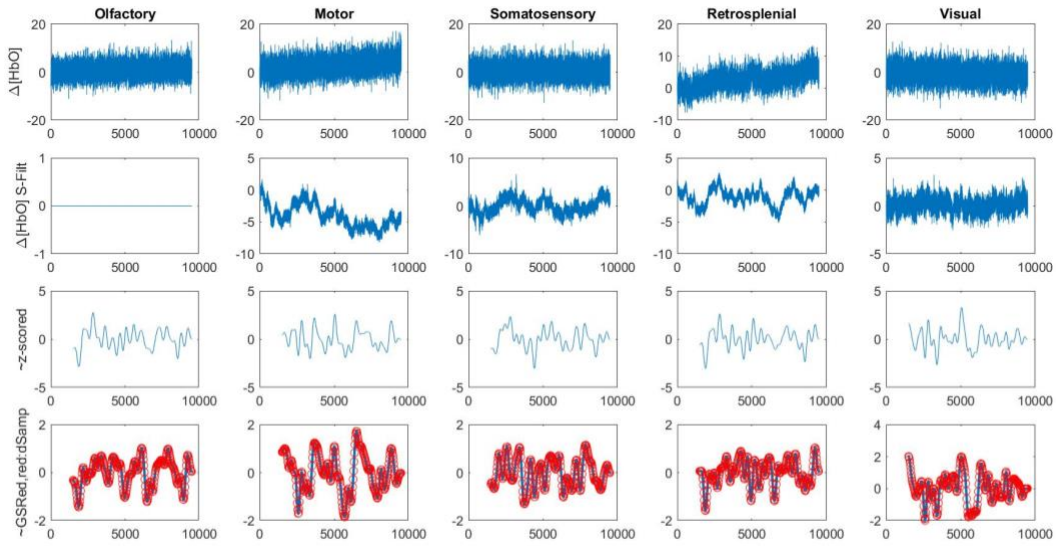

Figure 1. *Processed Time Series for Mouse 1. The processing time series across five brain seeds are shown, with specific views of the change in hemoglobin oxygen concentration for a raw sample, after application of a bandpass filter, z-scored dataset, and global signal regression.*

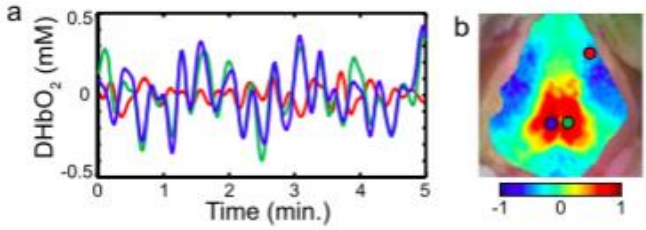

Figure 2. *Execution of seed-based resting state functional connectivity with Optical Intrinsic Imaging. RSFC map made by comparing each seed-based time trace with each corresponding brain pixel. Correlation and functional relationship are directly related, both high in this case, while a value of zero indicates no correlation [10].*

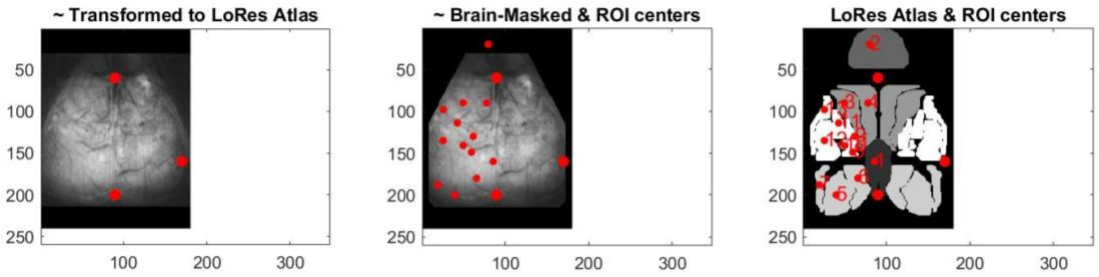

Figure 3. *Transformation of raw data to mouse brain atlas using anatomical landmarks. Region of Interest (ROI) centers on middle panel are transformed to the LoRes Atlas, shown by the left panel. The right panel shows transformed atlas with the ROI centers, which is used to make RSFC maps, presented in Figure 4.*

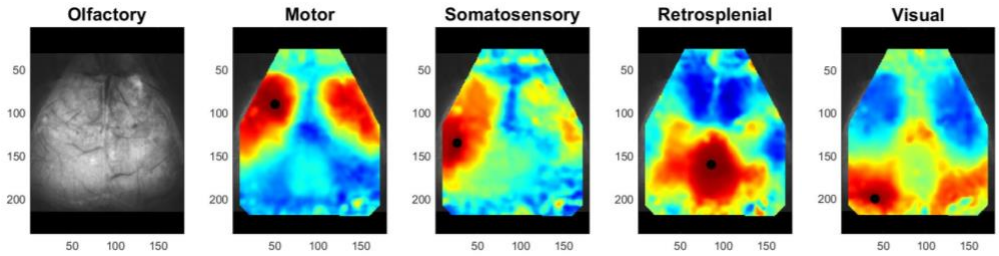

Figure 4. *Resting state functional connectivity maps. The findings for five brain seeds, olfactory, motor, somatosensory, retrosplenial, and visual, are pictured for sham-injured Mouse 1. The olfactory panel is an anatomical view due to the lack of transformation of the olfactory ROI center onto the atlas (Figure 3).*

Results

Based on the RSFC map generation from *Figure 4,* each seed-specific region can be analyzed to suggest possible findings. The olfactory seed shows an anatomical view of the brain as a comparison alongside the RSFC maps generated for the motor, somatosensory, retrosplenial, and visual seeds of interest. According to trends suggested by White and colleagues [11], the maps should be reflective of

bilateral functional connectivity, as seen in their RSFC maps [Appendix] for specific seeds. For sham-injured Mouse 1, the motor seed appears to be similar to the expected finding, as reflected by strong correlation between the right and left hemispheres. While less observably bilateral, the retrosplenial seed also shares positive correlation between the hemispheres. The somatosensory seed appears to have the most lateralized effect, with an observably red region reflecting strong correlation on the left side, but not on the right side of the brain. Finally, the visual seed follows a trend similar to the motor map, in which a positive correlation is observed. Correlation and functional relationship are directly related, while a value of zero, which would appear green in color on an RSFC map, would indicate no correlation [11].

Discussion

The findings of this study can serve as useful measures for the application of brain function to cases of brain trauma. While only one of the mice were able to provide us with analyzable data, the figures acquired from the experiment can be beneficial in recreating aspects of the investigation, as well as understanding possible implications of closed head impact in long term exposure settings.

Because of unexpected circumstances throughout the study, the validity of the investigation is not entirely robust. Issues with storage space after the second mouse was partially imaged made securing complete data files very difficult. In response to the issues that arose, many preventative measures were implemented, such as the creation of a RSFC troubleshooting protocol, and modifications of the pre-existing experimental protocol, and replacement of equipment including an Andor lens. Should the experiment be repeated, a more precise protocol is expected to yield a much more transient workflow from data acquisition to analysis of work.

Our expected outcome should reflect a decrease in brain area and a decrease in Pearson correlation with increased brain trauma in the form of closed head injury. However, because of the insufficient sample size which led to data for only a single sham-injured mouse, fluctuations in the measured variables cannot be used as conclusive evidence. The probable data with consideration of oxygen supply across brain seeds supports the idea that if one region were to be affected, a direct relationship to another pixel within that seed was likely presumed.

With regards to previous work, this investigation aligns precisely with that of a group of researchers at Washington University, St. Louis. This provides us with a general understanding of the impact of closed head injury on neuronal function, with specific analysis of oxygen saturation and cerebral blood flow, as imaged by the primary technique of interest, optical intrinsic spectroscopy.

Future Work

Using experiences from this investigation, several modifications can be made within the lab. The LSCI component can be refined by further manipulating the exposure time for each speckle to obtain a better spatial resolution. In addition, the main issue, hardware and storage, should be taken note of prior to the start of the experiment. In order to analyze data, it must be stored in a larger hard drive. Movement and dismantling of the OIS and LSCI systems should be minimized, if done at all, as damage to equipment may cause complications in other projects that may use the same equipment. Prior to imaging mice, if any technical difficulties arise, each aspect of the equipment should be checked to ensure whether there is an issue with coding and analysis, or a physical replacement is needed.

In the future, several applications of this work may be applied to other potential studies. It is possible that imaging through the skull of the mouse may provide a means to image awake animals without the confounding influence of anesthesia. Cranial windows may facilitate longitudinal imaging in the same animal as repetitive resection and suturing may not be necessary. Thus, if a mechanism in which an optical window could be installed on the surface of the skull, the methodology for the experiment may be improved, and the procedure would not be terminal for each imaging session. In this way, the change in neural connections over time could be better studied. The research could also be taken one step further by investigating how variances in head impact location can affect neuronal connections, as measured by oxygen concentration and cerebral blood flow [3], in other regions of the brain.

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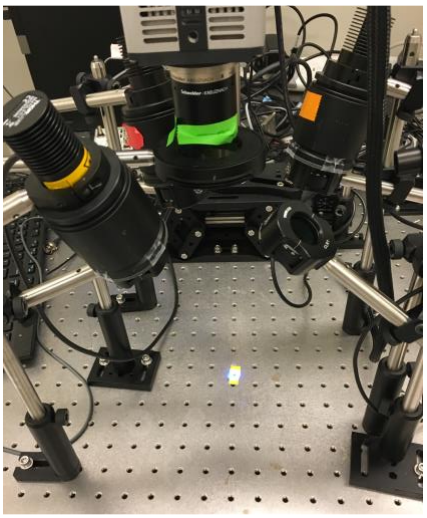
Appendix

A. Ketamine-Xylazine Concentrations Reference Table for a 25g Mouse

Ketamine/Xylazine Concentrations for Mice	
Refer to calculations from 8/12/15 in brown power of team lab notebook	
Weight of Mouse (g)	25
Ketamine Concentration	
Ketamine from bottle (mL)	0.1
Concentration of ketamine in bottle (mg/mL)	100
Saline to dilute (mL)	0.9
Ketamine dose (mg/kg)	85
Volume of ketamine-saline dilution to inject in animal (mL)	0.21
Xylazine Concentration	
Xylazine from bottle in mL	0.1
Concentration of xylazine in bottle (mg/mL)	100
Saline to dilute (mL)	2.9
Xylazine dose (mg/kg)	13
Volume of xyl/saline dilution to inject in animal (mL)	0.10
Extra Infusion	
Extra ketamine/saline dilution for infusion (mL/hr) - 40% initial dose	0.085
Bupivicaine	
Bupivicaine Dose (mg/kg)	2
Concentration in bottle (mg/mL)	5
Bupivicaine from bottle (mL)	0.1
Saline to dilute (ml)	1.9
Volume of bupivicaine dilution to inject (mL)	0.2

B. Setup of OIS and LSCI systems. For OIS, the head is illuminated with collimated LED light of three distinct wavelengths (one of which is labeled orange, and another red in the image below; third is not pictured) a CCD camera (labeled green) is used to image diffuse reflected light, and cross-polarizers (one of which is positioned below the orange LED) to reduce specular reflections.



C. **OIS and laser speckle contrast imaging system experimental protocol**
(last update 06/18/2018)

Animal preparation

- Locate the mice at ECC and bring them to the HSRB E197.
    - Make sure the mice stay at least 24 hours after delivery
    - Be sure to cover the cage during mice transport.
- Prepare anesthesia.
    - Calculate the necessary volume of ketamine and xylazine. The Excel spreadsheet is a great resource.
        i) Dose (IP injection): ketamine = 85 mg/kg, xylazine = 15 mg/kg
        ii) When using solution in vials from the Stein lab - ketamine: 100mg/ml, xylazine = 20 mg/ml), if the mouse weight = x g,
            (1) Ketamine (ml) = 85 * (x/1000)/100
            (2) Xylazine (ml) = 15 * (x/1000) /20
        iii) e.g.) if x = 30 g, ketamine = 25.5 ul & xylazine = 22.6 ul
    - Mix together in the same syringe for 1 total injection. Be sure to use sterile saline. The total injection volume should be < 0.5 ml.
    - After IP injection, make sure the mouse is unconscious by pinching its toe.

Mounting in the stereotaxic frame (~ 5 minutes)

- Before mounting the mouse, put a heating pad under where the mouse body will lie.
- Carefully insert a rectal probe, and switch on the temperature controller. The SV should read about 37.0.
- Load the mouse's front teeth over the bite bar. The tongue should hang below the bite bar along with the lower jaw.
- While holding the mouse's neck with your thumb and forefinger, move the right horizontal ear canal onto the right ear bar.
- While holding the right side of the head steadily, slide the left ear bar into the mouse's left horizontal ear canal.
- Screw down the nose bar. Be EXTREMELY gentle.

Resection of the scalp (~ 5 minutes)

- Inject 0.5% bupivacaine at the scalp injection site where you will be making an incision.
- Again, use the spreadsheet for an accurate calculation of the estimated proportion of drugs.
    1. 0.5 % = 5 mg/ml. Max. dose is 2mg/kg. Thus, 0.4 ml/kg.
    2. volume (ml) = 0.4 * (x/1000). If x = 30g, volume = 12 ul

Imaging system setup (~ 5 ~ 10 minutes)

- Setup the mouse head position
- Turn on the camera and open the 'Solis' software.
    - (For troubleshooting, restart Windows with camera off, then turn on the camera, and open the Andor Solis application). The basic setting parameters are below:
    - Array size: 512 * 512
    - Binning: 1*1
    - Exposure time: 0.003 s (3 ms)
    - Shutter: Rolling
    - Resolution: 16 bit
    - Zoom lens magnification: 0.77 (Currently, the enlarging lens is set up)
    - The Mode should be Internal.0-

1. Set 'Single Scan' for the acquisition mode and Run 'Take Video'.
2. While watching the video, move the stereotaxic device (x – y direction) slightly such that the mouse head will be positioned at the center of the image.
3. Once the head is located at the center, adjust the focus by rotating a knob of the z-axis mount.

a. If necessary, adjust the zoom such that the mouse head fills almost the entire imaging area.

4. Adjust the position/angles of the LEDs and laser diode (LD) to ensure that each illumination covers the whole imaging area. During this set-up, switch to "continuous mode" in the LED controller.
5. Adjust the intensities of the LED and LDs such that the max. count number per each channel has similar values.
6. By rotating each polarizer without displacing the 3D printed donut, minimize specular reflection by rotating each polarizer.
7. After adjusting the intensities, switch back to "trigger mode (center)" on the LED controller.
8. Dial the LD controller knob to '40 mA'. The LD will be switching between 40 mA (off) and 80 mA (on) during the procedure.

Image acquisition (25 ~ 35 minutes)

- 1st round: 10-minute resting-state functional connectivity (RSFC) with fcOIS with three wavelengths (Blue, Orange and Red - 470, 590 & 625 nm) and laser speckle contrast imaging.

  1. Switch to "trigger mode" in the LED controller
  2. Select "Kinetic series" in the acquisition mode.
  3. Set 'Exposure Time' : 0.003 s (3 ms)
  4. Set 'Trigger Mode' : External
  5. Insert "# = 125 x 60 x experiment time in minutes" in the 'Kinetic Series Length'. The current trigger pulses are set to acquire 1000/8 = 125 images per second.
  6. You MUST CHECK on the "Enable Spooling to Disk" in the "Data Spooling" tab and specify the saving folder (RSFC operation - data – analysis -> data-> experiment). Go back to the folder to ensure that the files are being saved in at the indicated location.
  7. Open the MATLAB script "Trigger_050117.m". Set the "exp_time" to the desired imaging duration in minutes.
  8. Press the "Take Signal" button in Solis. Now it's waiting for the external trigger. Run the "Trigger_050117.m" code.

- 2nd round: 6-minute functional activation with OIS with three wavelengths (470, 590 & 625 nm) and laser speckle contrast imaging.

  # of series : 125 * 360 = 45000

D. Sample Resting State Functional Connectivity Maps (White et. al 2010)