The Fixation Protocol Alters Brain Morphology in Ex-vivo MRI Mouse Phenotyping

A. Elizabeth de Guzman[1,2], Michael D. Wong[1,2], Jacqueline A. Gleave[1], and Brian J. Nieman[1,2]

[1]*Mouse Imaging Centre, Hospital for Sick Children, Toronto, Ontario, Canada,* [2]*Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada*

Target Audience: Our target audience is scientists who regularly perform *ex-vivo* animal brain imaging and are interested in the effects that changes in fixation protocols have on morphometric and volumetric analysis.

Introduction: *Ex-vivo* MRI has become a popular method for morphology based mouse brain phenotyping, owing to its high SNR and resolution capabilities when compared to in-vivo MRI [1]. In order for these studies to be possible, chemical fixation is performed to preserve macromolecular structure and prevent breakdown of tissue over long term storage [2]. Despite its common use, the possible confounding effects of tissue fixation for morphological MRI analysis are only partly characterized, and a wide variety of fixation protocols are in use. The purpose of this study was to characterize the effect that increased fixation by chemical cross-linking has on brain structure volume detectable with MRI.

Methods: Perfusion: Eight C57BL/6J mice underwent transcardiac perfusion through the left ventricle, as previously described [3]. A first perfusate solution included 30mL phosphate-buffered saline (PBS), 1µL/mL of heparin and 2mM of ProHance (gadoteridol, Bracco Diagnostics Inc., Princeton, NJ) and was administered at a rate of 1mL/min. This was followed by a second solution containing 30mL of 4% paraformaldehyde (PFA) in PBS and 2mM of ProHance. The head was decapitated and the brain, left inside the skull, was soaked in 4% PFA and 2mM ProHance for 24 hours. It was then transferred to a solution of PBS, 2mM ProHance and 0.02% sodium azide for 6 days before being imaged. After a first MR scan, samples were placed back in 4% PFA ProHance solution for 48 hours followed by a second MR scan. A third and final image was taken after another 48 hours of fixation. After each period of fixation, samples were washed in a PBS ProHance solution for at least 5 hours before imaging in order to wash all free fixative solution. **MRI:** *Ex-vivo* images were acquired on a 7T scanner (Agilent Technologies Inc.) using a T2-weighted 3D fast spin echo sequence; 56µm isotropic resolution, 6 echoes at 14ms echo spacing, TR/TE=2000/42ms, 450x252x250 matrix, and ~11.5 hour scan time. **Image Analysis:** An automated registration process was used to align all images to a consensus average. 62 automatically-segmented brain structures were aligned with the consensus average and the Jacobian determinant of individual deformation fields were used to calculate the volume of each structure. Statistical calculations were performed using R (www.r-project.org) and p-values were corrected for multiple comparisons through the false discovery rate (FDR) method.

Results and Discussion: The volume of each brain structure was fit with a linear mixed effects model allowing structure volume to vary linearly with post-perfusion fixation time (Figure 1) and including a random intercept for each subject to account for population variability. Shown in Figure 2 are coronal slices of the brain identifying structures that increased (red) or decreased (blue) in volume with increased fixation time. The percent change from the initial volume (24H in 4%PFA) per day in fixative solution is indicated by the colour scale. Only structures whose slope was statistically different from zero (5%FDR) are presented. Many of the structures that have decreased in volume with increasing fixation time are those in close proximity to large ventricles and ducts. Most likely the expansion of nearby ventricles occurs in order to compensate for volume decreases in adjacent tissue. Because the pattern of change is non-uniform, morphological analyses may yield quantitatively different results dependent on the exact fixation protocol. Consequently, inconsistent fixation protocols may produce results that masquerade as phenotypic differences.

Conclusion: This study has demonstrated that differences in volume due to varying fixation time are detectable with MRI. Specifically, the rate at which structure volume changes with fixation time varies depending on the location in the brain. Previous data has also shown that fixation affects water permeability in tissue [4]. Further investigation will be performed to determine if long term storage of these samples in PBS may affect structure volume further. These results provide a cautionary note to studies of mouse brain morphology, highlighting the potential role that the sample preparation protocol may play.

References: [1] JP Lerch et al. (2012) Front Neuroinform **[2]** JD Bancroft et al. (2008) Theory and Practice of Histological Techniques **[3]** LCahill et al. (2012) Neuro Image **[4]** TM Shepherd et al. (2009) Magn Reson Med

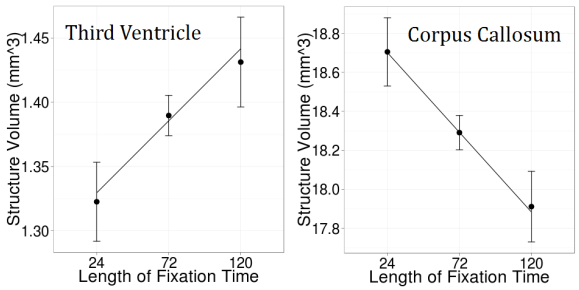

Figure 1. Average structure volume plotted with respect to length of post perfusion fixation time. The confidence intervals have been plotted after the removal of between subject variability.

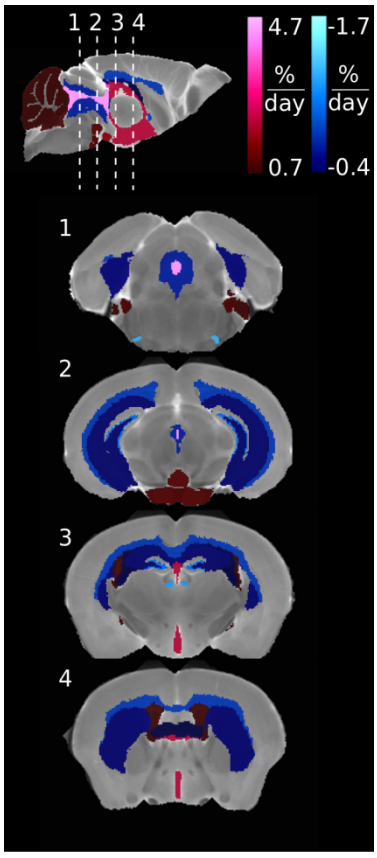

Figure 2. Coronal slices of the average brain. A colour map has been overlaid indicating the percent change in volume per day in fixative of statistically significant structures (5%FDR)