Optimizing the Histology Protocol to Determine Long-Term Neuro-Inflammatory Effects of Chronic Cranial Implants

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For this research project, the goal was to determine the neuro-inflammatory effects of chronic cranial implants. Leila Ghanbari, a graduate student in the Biosensing and Biorobotics Laboratory, has designed and tested novel cranial implants. These implants are clear windows which replace part of the skull and act as a way to carry out extensive, in vivo imaging with a large field of view. However, it is necessary to determine the inflammatory response of the brain to the windows as well as to the drilling required to successfully implant them. To do this, two forms of cells should be observed- astrocytes and microglia. Astrocytes are fairly large cells that characteristically aggregate at locations where tissue has had chronic or long-term damage. Microglia are small cells that tend to migrate to injured areas more rapidly. Over the course of this semester, I performed histology experiments with the goal of optimizing the protocol in order to obtain microscopic images of astrocytes and microglia in fixed brain tissue. By quantifying cell aggregation at various time points, we can understand the inflammatory effects of the implants.

Carrying out the histology and staining protocol has many crucial steps. After testing out various procedures and shortcuts, an ideal process was found that resulted in consistently successful staining of both astrocytes and microglia. First, the mice are perfused, which is a process that involves flushing the body with formaldehyde, a fixative, in order to preserve the tissue. Then, the brains are dissected from the skull and kept in formaldehyde solution for 48 hours to ensure complete fixation, after which they are transferred to a 30% sucrose solution, which dehydrates the tissue. This ensures that the brain is not damaged by the formation of ice crystals in the subsequent step when the brains are flash frozen for sectioning. This is accomplished by dunking them in isopentane solution cooled with dry ice to -40°C. The flash frozen brain is sliced into 40µm slices using a microtome. Slices are only taken from affected areas of the brain. The slices are kept in P-g, which is a solution of phosphate-buffered saline (PBS) and glycine.

After the slices are created, the staining process begins. This involves washing the brain slices many times in various solutions. They are first soaked in Ptg (PBS, Triton-X, and glycine)

for 30 minutes three times. Triton-X acts as a detergent that permeabilizes the cell membrane and glycine is an amino acid that takes away any formalin remaining from the perfusion. Then, the slices are soaked in Ptb (PBS, Triton-X, and goat serum, a blocking agent) for two hours on a shaker table. The blocking agent is crucial for later steps because it will assure that the primary antibody only bonds to the cells being monitored. Next, the slices are left to soak overnight in the refrigerator in a mixture of primary antibody and Ptb. The primary antibody used to mark microglia is anti-iba1 made in the rabbit and to mark the astrocytes anti-GFAP antibody produced in the mouse is used. After the slices sit overnight, they are washed twice in P-g to remove excess antibody. Then, they are washed four times in Ptb for 30 minutes each on a shaker table. The secondary antibody is diluted to 1:500 with Ptb and added to the slices. For the secondary antibody, Alexa 488 Goat anti-mouse is used for the astrocyte staining and Alexa 555 Goat anti-rabbit is used for the microglia. The slices are left in this solution for two hours on a shaker table. The excess secondary antibody is removed by washing the slices twice in P-g. Then the slices are soaked in Ptg for 30 minutes, three times, again on a shaker table. Finally, to remove the Triton-X, the slices are put in P-g for 30 minutes. At this point, the staining is complete and the slices are ready to be mounted on slides. They are mounted with Vectashield with DAPI and sealed with clear nail polish. This protocol results in the successful staining of both astrocytes and microglia and will allow for further quantitative analysis of the neuro-inflammatory response of the brain to the novel chronic cranial implants. Figure 1 shows the resulting microscopic images obtained from this protocol.

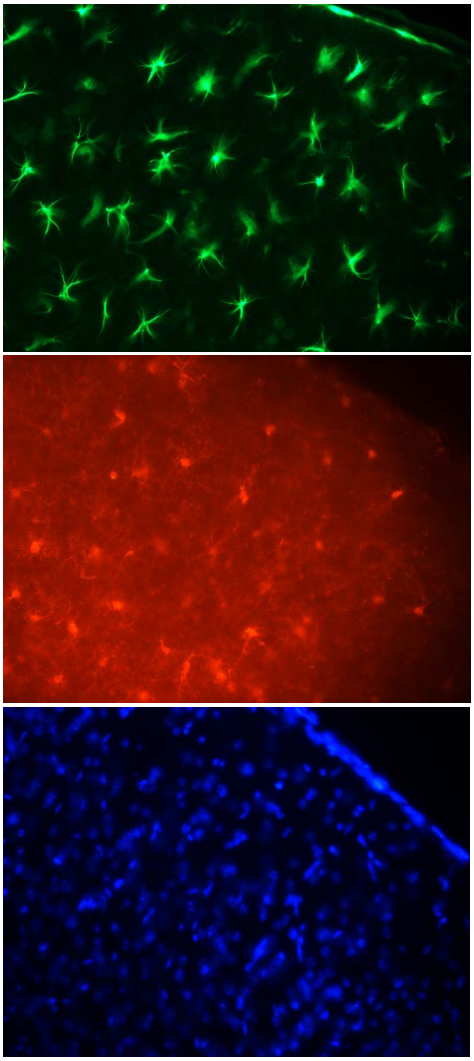

Figure 1: Top: GFAP staining of astrocytes with Alexa 488
Middle: Iba1 staining of microglia with Alexa 555
Bottom: DAPI staining of all cells

There were various challenges faced as this protocol was optimized. Initially, fewer washing steps were completed in an effort to shorten the amount of time

needed to stain each brain slice. This posed a problem because excess antibody was left on the slides and resulted in misleading bright spots on images collected. Also, the microglia staining failed when less washings occurred. This was probably due to a combination of factors but the most apparent issues were that the slices were not soaked in the blocking solution long enough for it be effective and excess antibody remained when it should have been washed off. Because microglia are much smaller than astrocytes, staining them is a more tedious process and it is crucial to follow repetitive washing steps. Another challenge that presented itself was determining the most time effective way to carry out this protocol. Originally one brain at a time was being processed, but because of the time-intensive nature of the experiments, this was found to be too slow. To improve this, a system of batch-processing brains was found to be effective. Three brains at a time are sliced and then refrigerated in P-g until the staining process begins. To carry out the washings with three brains, the timing for each brain is offset slightly. In this way, while one brain is soaking in solution, the other brains are being prepared for the next step. This rotational washing allows for the whole procedure to be done in a much more time effective way. Overall, my goal of optimizing the histology protocol was successfully reached.