Immunofluorescence Optimization and Size Determination of Intracerebral Hemorrhage Sites in Mice Brains

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Background

Intracerebral hemorrhage (ICH) occurs when penetrating arteries in the brain parenchyma burst (1). ICH has a mortality rate of 50% after 30 days of onset with the main treatment being blood pressure (BP) management and supportive care (1,2).

The inflammatory milieu of ICH includes microglia, macrophages, T cells, and neutrophils (3,4). Local microglia and macrophages switch to a pro-inflammatory state (2). This transition can be quantified by upregulation of cell surface receptor CD68 with immunofluorescence (IF) antibody test. The Iba1 receptor can be used to enumerate total number of microglia and macrophages (2). Neutrophils are recruited to an ICH site from the vasculature (4). Their quantification depicts the acute immune response and be quantified by the LY6G receptor (5).

Numbers of neurons and astrocytes are impacted by the sterile activity of immune cells (4). Neuron numbers can be quantified by antibodies to neural-specific nuclear protein (NeuN) (6). Astrocytes upregulate glial fibrillary acidic protein (GFAP) due to the oxygen and glucose deprived ICH region (7).

BP medications are mainstay treatment in acute ICH with the goal of lowering blood pressure to normal ranges. However different BP drug classes can impact the immune response and prognosis of ICH, but these effects currently are unknown (2). Elucidating these effects are the goals of the future extensions of this project.

This capstone project optimizes IF antibody concentrations for future staining and begins to analyze inflammatory responses after ICH in mice treated with different BP medications (8).

Methods

Cryostat Sectioning and ICH Diameter Measurement

Mice brains were removed from -80°C storage for sectioning into 7-micron width slices onto Fisherbrand Tissue Path Superfrost Plus Gold Slides by blinded user. Multiple images of ICH were captured with metric ruler in view of Samsung Galaxy S20 camera for qualitative size analysis for inclusion of mouse in experimental cohort. Prepared slides were then stored in 4°C.

IF Staining

See Table 1. Hoechst/DAPI was used to stain nuclei. Sections stained with only secondary antibodies were used as negative controls for nonspecific staining. Stained slides were stored at 4°C in a dark box until imaging.

Table 1: IF staining matrix. Each secondary antibody is prepared at 1:500 dilution.

Staining Condition	*Primary Antibody Marker*	*Concentration*	*Host*	*Secondary Antibody Fluorophore*	*Host*	*Target*
1	Ly6G	1:300	Rat	AF488	Goat	Anti-rat
1	Iba1	1:400	Rabbit	AF594	Goat	Anti-rabbit
Counterstain	Hoechst	1:10,000				
2	CD68	1:500	Rat	AF488	Goat	Anti-rat
2	Iba1	1:400	Rabbit	AF594	Goat	Anti-rabbit
Counterstain	Hoechst	1:10,000				
3	GFAP	1:500	Chicken	AF647	Goat	Anti-chicken
3	Neun	1:200	Rabbit	AF488	Goat	Anti-rabbit
Counterstain	Hoechst	1:10,000				

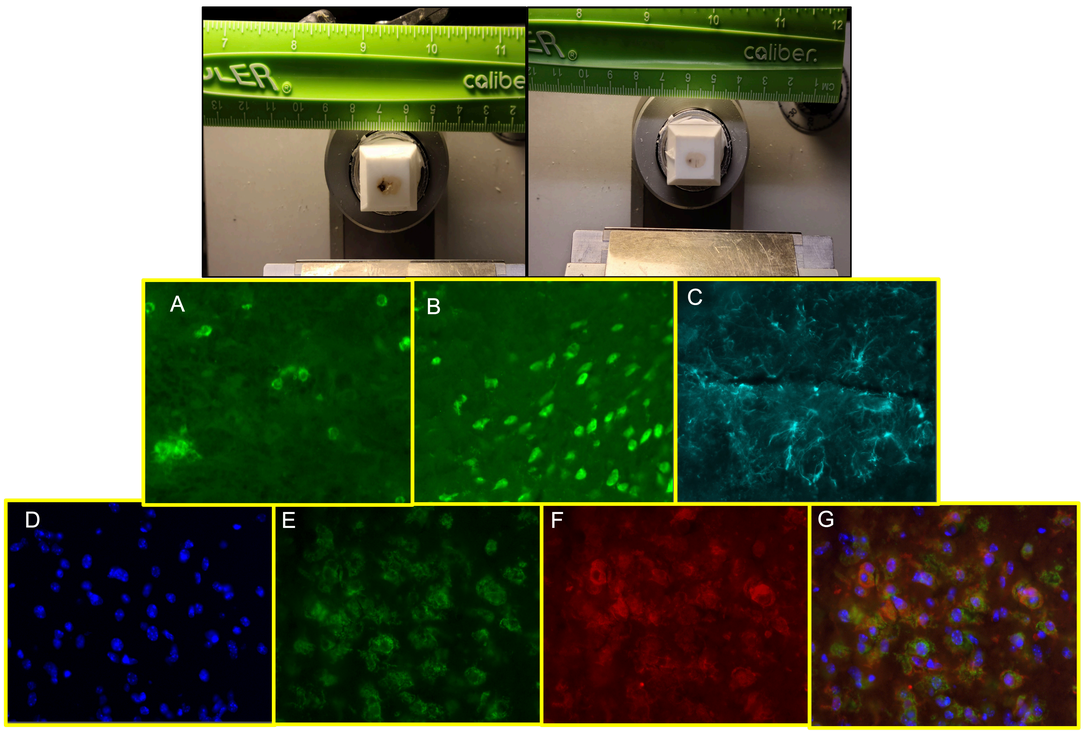

Figure 1: (Top row) ICH size characterization. Successful ICH in left image and unsuccessful one in right image. **(Bottom 2 rows)** Exemplar IF images at 40x magnification. A=Ly6G, B=NeuN, C= GFAP, D= DAPI, E= CD68, F=Iba1, G=Overlay of CD68, Iba1, and DAPI.

Results and Conclusions

As can be seen in **Figure 1**, this study provided a qualitative ICH sizing framework to measure ICH sizes at different points in sectioning. To further refine the precision and accuracy of this method, the relative distances of the ruler and cutting stage should be noted as well as the angles of the setup and camera. IF staining was attained visually with the concentrations listed in **Table 1**. Further staining can be done with this provided experimental layout alongside further analysis such as mean fluorescence intensity and cell counting to quantify the inflammation and cell numbers in the ICH site. This study therefore has provided a numerical system to classify ICH size for final cohort inclusion and determined the optimal antibody strengths for future IF staining. Quantification across the BP groups of mice is currently in progress.

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