Sample Collection Protocol

A good perfusion is important for obtaining clean section staining results and is essential for producing usable whole-mount results. A well-performed perfusion will minimize the amount of vasculature visible in the brain after immunolabeling.

Day 1

1) Anesthetize the mouse with isoflurane.
    a. ***Tip***: Keep the mouse calm in the hour(s) leading up to the perfusion and immediately beforehand.
    b. ***Tip***: Verify that the mouse has reached deep plane of anesthesia by pinching toe and tail (to observe loss of pedal withdrawal response) before proceeding.
2) Pin the mouse in dorsal recumbent position on inclined plane (with anterior end slightly elevated). Spray the fur over ventral surface with 30% ethanol to prevent fur from sticking to surgical tools.
3) Make an incision in the abdomen below the xiphoid process, cutting through both skin and muscle wall. Cut down to dorsal base of diaphragm on either side of mouse.
    a. ***Tip***: Make sure to cut all the way to dorsal side on the mouse's right side, so that blood will flow freely during perfusion and not pool within the abdomen.
4) Pierce diaphragm, cut along edges, then cut through rib cage on either side to expose heart.
    a. ***Tip***: Once cut through diaphragm, work quickly to begin perfusion while heart is still beating.
5) Perform a standard transcardiac perfusion with 10 mL of room temperature (RT) 1x phosphate-buffered saline (PBS) at 9mL/min and perfuse for ~1 minute, until the blood is completely removed from the tissue.
    a. **Optional**: add 10µg/ml heparin to 1xPBS solution to better wash out blood.
    b. ***Tip***: Keep the needle parallel to the heart's septum within the left ventricle, such that the tip of the needle is pointed straight toward the aortic valve.
    c. ***Tip***: Make the cut in the right atrium large, to permit blood to flow rapidly.
    d. ***Tip***: We use a peristaltic pump to obtain consistent perfusion speeds. Be sure to calibrate the pump speed regularly.
6) Switch the perfusate to 60 mL of fixative solution (RT 4% paraformaldehyde (PFA) in 1x PBS) at 9mL/min, and perfuse for ~7 minutes until the mouse's tail has significantly stiffened.
    a. **CAUTION**: PFA is toxic. Avoid contact with skin, eyes, and mucous membrane. Solutions should be made inside a fume hood.
7) Switch the perfusate to 30mL RT 1xPBS and perfuse at 9mL/min for ~3min, to wash out the PFA before dissection.
8) Dissect the brain (or other organ of interest) and trim to appropriate size or region of interest.
    a. ***Tip***: Be careful to avoid making cuts in the sample during dissection. Slits in tissue may accumulate antibodies during whole-mount immunostaining.
    b. ***Tip***: The brain tissue should appear white when dissected. If the tissue is pink or blood is visible, this indicates the perfusion was not optimal. Tissue may still be used for section staining, but will likely have noticeable vasculature background if staining with mouse-derived antibodies.
9) Post-fix all tissue samples in ice-cold 4% PFA, shake at 4°C overnight.

Day 2

10) Wash fixed samples in 1xPBS/0.02%NaN_3: shake at RT, 1h x 3times (suggested: 2h, 4h, o/n).

Day 3

11) Wash fixed samples in B1n to block excess PFA and preserve antigens: shake at RT, 1h x3 times (suggested: 2h, 4h, o/n).
12) If proceeding immediately to delipidation:
    a. Begin “Delipidation Protocol” on Day 4.
13) If putting brains to storage:
    a. On Day 4, wash fixed samples back to 1xPBS/0.02%NaN_3: shake at RT, 1h x4 times (suggested: 1h, 2h, 4h, o/n).
    b. Samples can be kept in 1xPBS/0.02%NaN_3 at 4°C for many weeks. Fill the tube with media to reduce air oxidation.