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Intestinal 3D immunostaining

0. Perfusion (Optional) – If you will be looking at the vasculature or associated structures, perfusing the animal will greatly reduce the amount of autofluorescence and increase the shelf life of the sample. It also helps to do a live perfusion, but you will need to be trained before you can attempt that procedure.

    a) Perfuse the mouse with PBS, I find ~30mls is a sufficient volume.
    b) Switch to your fixative and perfuse another 10-20 mls of the solution.

1. Before linearizing, extract the intestine and flush it with PBS from both ends to remove all fecal matter.
2. Flush the intestine with the fixative you will be using and allow it to sit for 30 minutes on ice.
    a. This step will make it easier to keep the intestine flat when you pin it later. Leaving it longer than this will cause it to curl upwards, and less time (or not pre-fixing it at all) will cause the edges of the tissue to curl under.
3. Linearize the intestine, then pin it to a silicone plate with the pins ~1cm apart.
4. Submerse the tissue in your fixative and leave at 4° on an orbital shaker overnight
5. Remove the fixation buffer and replace it with ice-cold 1× PBS.
6. Wash the samples on an orbital shaker at 4 °C for 5 min. Repeat this step two more times.
7. After the last wash with 1× PBS, remove 1× PBS and replace it with 10% (vol/vol) sucrose solution.
8. Incubate the samples on an orbital shaker for 3 h at 4 °C.
9. Remove 10% (vol/vol) sucrose solution and replace it with 20% (vol/vol) sucrose + 10% glycerol solution.
10. Incubate the samples on an orbital shaker O/N at 4 °C.
11. Remove 20% (vol/vol) sucrose + 10% glycerol solution, rinse the samples once with ice-cold 1× PBS and submerge the intestine pieces in PBS + NaN3.
    a. PAUSE POINT. Samples can be stored at 4 °C for up to several months.
12. Cut a piece of necessary size from the sample for further staining and transfer it to a 12 well plate lined with silicone and pin it down.
    a. I usually do $1cm^2$, but you can adjust it to suit your needs
13. Add blocking buffer to the samples; use 1 ml per well for a 12-well plate or 2 ml per well for a 6-well plate. Incubate the samples on an orbital shaker for 1–2 h at 4 °C.
14. Prepare an appropriate primary antibody mix in blocking buffer.
15. Replace the blocking buffer with the primary antibody staining buffer and incubate the samples on an orbital shaker O/N at 4 °C.
    a. Primary antibody mix can be left on for several days if necessary or if working with a large piece of tissue

16. Remove the primary antibody mix and add ice-cold PBS + 0.3% (vol/vol) Triton X-100. Wash the samples on an orbital shaker at 4 °C for 1 h. Repeat this step four more times.
17. Prepare an appropriate secondary antibody mix in blocking buffer
18. Replace PBS + 0.3% (vol/vol) Triton X-100 with secondary antibody mix. Incubate the samples on an orbital shaker O/N at 4 °C.
    a. CRITICAL! Although primary antibodies can be left on the samples for several days, secondary antibodies cannot be left. Allow the secondary antibodies to incubate for a maximum of 16 h at 4 °C.
19. Remove the secondary antibody mix and add ice-cold PBS + 0.3% (vol/vol) Triton X-100. Wash the samples on an orbital shaker at 4 °C ten times for 30 min each. At this point, samples can be analyzed using a fluorescence stereomicroscope to determine that primary and secondary antibody immunostaining has worked.
20. Replace PBS + 0.3% (vol/vol) Triton X-100 with ice-cold 1× PBS and wash the samples twice for 10 min each at 4 °C on an orbital shaker.
21. Replace 1× PBS with ice-cold 4% (wt/vol) PFA. Incubate the samples on an orbital shaker for 2 d at 4 °C.
    a. 1 day is generally enough, but 2d will make it much easier to work with
22. Remove 4% (wt/vol) PFA and add ice-cold PBS. Wash the samples on an orbital shaker at 4 °C three times for 30 min each.

    At this point I diverge from the published protocol because I prefer to clear and mount with TDE

23. Cut the tissue into strips that are 1-2 villi thick and place them in PBS with 10% TDE
24. After all strips have been cut, equilibrate the pieces in a stepwise fashion using 10%, 25%, 50%, 97% PBS/TDE. Incubate for 2 hours at 4° at each concentration, and overnight for the final 97% stage.
25. Transfer the strips to a microscope slide with the adhesive spacer, attach coverslip and image

(References)

1. This protocol is adapted from High-resolution 3D analysis of mouse small-intestinal stroma by Jeremiah Bernier-Latmani & Tatiana V Petrova. You can find the recipes for all of the buffers there.