Immunocytochemistry (ICC) Protocol for Intestinal Organoids.

Protocol

1. Passage organoids into 50 µL domes made of 50% Matrigel (1:1 mixture of Matrigel and DMEM/F-12) on glass coverslips in a 24-well culture plate. Grow in Intesticult™ Organoid Growth Medium (Mouse) supplemented with 50µg/mL gentamicin at 37°C and 5% CO_2.

2. Remove organoids from culture conditions at desired stage of development.

3. **WASH STEP:** Gently aspirate media from wells to be stained. Be careful not to disturb the Matrigel® domes. Add 600 µL of room temperature PBS and incubate at room temperature for 5 minutes.

4. Remove PBS and add 600µL of cold 4% PFA (paraformaldehyde), leave at room temperature for 30 minutes.

5. Repeat step 3 (wash step) twice.

6. Add 600µL of Triton-X 100 (0.2% in PBS). Incubate at room temperature for 30 minutes.

7. Repeat step 3 (wash step) twice.

8. Add 600µL of room temperature NH_4Cl (50 mM in PBS) and incubate at room temperature for 30 minutes **Optional step – see notes**

9. Repeat step 3 (wash step) twice

10. Add 600µL of 5% BSA in PBS and leave at room temperature for 60 minutes

11. Repeat step 2 (wash step) twice

12. Add 600µL of primary antibody (made up in PBS + 0.1% BSA) and leave overnight at 4°C. ***** Expect to use a higher concentration of antibodies than in 2D culture ICC; see notes *****

13. Repeat step 2 (wash step) five times

14. Add secondary antibody (made up in PBS) and leave for 2 hours at room temperature or overnight at 4°C. ****expect to use a higher concentration of antibodies than in 2D culture ICC; see notes****

15. Repeat step 2 (wash step) five times

16. Remove the coverslip from the well with forceps. Add 1 drop of PermaFluor™ containing 1.0 µg/mL DAPI counter stain onto a glass slide. Carefully place the coverslip organoid side down onto the PermaFluor™ droplet. Incubate at room temperature for at least 10 minutes before imaging. For long term storage, store at 4°C.

Notes

- Addition of 4% PFA fixes the organoids to the bottom of tissue culture treated dishes quite effectively. Thus, the user does not have worry as much about potentially disturbing the organoids as they would if they were still embedded in the Matrigel dome. Upon removal of 4% PFA, the Matrigel dome often collapses and the organoids are fixed onto the glass coverslip.
- Until the organoids are fixed with 4% PFA all solutions applied to the Matrigel dome must be room temperature to prevent the domes from dissolving.
- NH_4Cl is added to quench the auto-fluorescence from shed cells in the lumen when using an excitation wavelength of 488nm. Do not use if there are fluorescently tagged proteins in the lumen that are excited at the same wavelength. If the 488 excitation wavelength is not being used, this step can be skipped.
- Organoid ICC requires a greater concentration of antibodies than 2D culture systems due to greater density offered by the 3D architecture and therefore decreased permeability. For primary antibodies expect a range of 1:50- 1:500 as a starting point, and for secondary antibodies we recommend starting with a 1:200 dilution.
- With regard to the triton-X concentration, some sources suggest it be increased to 0.5% to increase permeability, improving signal-to-noise ratio. In our experience increasing the triton-X concentration on its own does not have a significant effect on signal-to-noise ratio, but when modified with antibody concentration can lead to better results.
- Depending on the given combination of organoids and antibodies it is possible to extend or shorten the antibody incubation times. Typically, longer incubations are done at 4°C overnight and shorter times are done at room temperature (2-4 hours).

Materials

- Corning Growth Factor Reduced and Phenol Red Free Matrigel
- 16% PFA (Alfa Aesar, 43368)
- Triton-X 100 (Sigma, X100)
- NH_4Cl (Sigma, A4514)
- BSA (Stemcell Technologies, 09300)
- Permafluor Mountant solution (Fisher Scientific TA-030-FM), DAPI must be added separately

Acknowledgements
This protocol was optimized for organoid culture with Intesticult from the following source:

Mahé, Maxime M et al. “Establishment of Gastrointestinal Epithelial Organoids.” *Current protocols in mouse biology* 3 (2013): 217–240. *PMC*. Web. 12 June 2015.